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# Arterial and venous plasma concentrations of adenosine during haemorrhage

Yong Zhang & [1]W. Wayne Lautt

Department of Pharmacology & Therapeutics, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0W3

A haemorrhage model was used to impose severe metabolic stress in anaesthetized cats by removing blood (15.3 ml min$^{-1}$) to attain an arterial pressure of ca. 50 mmHg for a 2 h period. Adenosine levels in central venous blood rose by 5 min, reached a peak of about 3.5 times control levels by 15 min and then returned to the basal level (1 $\mu$M) by 60 min. However, the adenosine concentration in arterial blood remained unchanged for the entire 2 h period of hypotension. These data demonstrate that haemorrhage results in rapid adenosine release, but the released adenosine is not able to serve a role as a systemic circulating vasodilator even in this severe model.

**Keywords:** Adenosine; haemorrhage; anaesthetized cats

**Introduction** Extreme metabolic stresses, such as ischaemia and haemorrhage, evoke endogenous adenosine release. The released adenosine dilates vascular smooth muscles and restores tissue blood flow to exert protective effects including limitation of vascular injury (Babbitt *et al.*, 1989), improvement of myocardial recovery (Bolling *et al.*, 1990) and prevention of neuronal damage (Dux *et al.*, 1990). Adenosine receptor antagonists, caffeine in rats (Conlay *et al.*, 1988) and 8-phenyltheophylline in cats (Lautt & Légaré, 1985), result in less decrease of blood pressure for a given volume of blood loss. It is unclear, however, if this evidence of a hypotensive role of adenosine is entirely secondary to actions locally at the site of the adenosine release or whether adenosine can recirculate to serve a generalized systemic hypotensive effect. It has been suggested that adenosine release, which is reported to be maximal at the time of haemorrhagic decompensation, may account for the decline in plasma catecholamines that is seen at that time (Tung *et al.*, 1987).

We have examined the relation of endogenous adenosine to haemorrhage using an improved high performance liquid chromatography (h.p.l.c.) fluorometric method for measurement of adenosine in plasma (Zhang *et al.*, 1991). The objective was to determine if adenosine is released into venous blood and, second, if locally released adenosine could attain levels in arterial blood that could lead to generalized systemic effects. The data show that adenosine is released into venous blood during haemorrhage but is eliminated from plasma before reaching the arterial side of the circulation, suggesting that adenosine is not able to serve a role as a systemic circulating vasodilator even under conditions of extreme metabolic stress.

**Methods** Experiments were performed on mongrel cats (2.5–5.0 kg) of either sex. The animals were fasted for 18 h and anaesthetized with sodium pentobarbitone (32.5 mg kg$^{-1}$, i.p.); supplementary doses (6 mg, i.v.) were given as required to maintain adequate anaesthesia. Body temperature was maintained at 37.5°C by use of a rectal probe and a thermal-controlled unit (Yellow Springs Instruments, model 72) operating heating rods in the surgical table. A cannula was placed in the right brachial vein for administration of fluids and maintenance doses of anaesthetic. The left femoral artery was cannulated for measurement of systemic arterial pressure, and the aorta was cannulated via the right femoral artery for the withdrawal or infusion of blood. the left carotid artery was cannulated for arterial sampling. The inferior vena cava was cannulated by two catheters via the right and left femoral vein, respectively. The right catheter, with the tip located below the junction of the renal vein, was used for measurement of central venous pressure and the left one, with the tip located 2 cm below the heart, was used for venous sampling.

Plasma adenosine was measured by h.p.l.c.-fluorometric detection as previously described (Zhang *et al.*, 1991). Briefly, each blood sample (360 $\mu$l) was rapidly collected into a 1 ml syringe containing 40 $\mu$l of a stopping solution consisting of dilazep (1 mM) to inhibit adenosine uptake into and release from erythrocytes; erythro-9-(2-hydroxy-3-nonyl)adenine (10 $\mu$M) to block adenosine deaminase activity and indomethacin (2 $\mu$g ml$^{-1}$) to inhibit nucleotide release from platelets (final concentrations). Blood samples were immediately centrifuged, deproteinated with 50% trichloroacetic acid, and neutralized with 3.3 N potassium hydroxide. Adenosine in blood samples was converted by use of chloroacetaldehyde into $N^6$-ethenoadenosine for h.p.l.c. analysis.

After the surgery, 4000 units of heparin were administered intravenously via the right brachial vein and each animal was permitted a 1 h stabilization period. Then, four control samples at 0, 15, 30, 60 min before starting haemorrhage were taken for analysis of adenosine and blood gases. The haemorrhage model (Lautt *et al.*, 1980) utilizes a rapid arterial blood withdrawal (15.3 ml min$^{-1}$) with the rate held steady by a Harvard withdrawal-infusion pump. The animal was bled into a heparinized 60 ml syringe until blood pressure reached 50 mmHg (1 mmHg = 133.322 Pa), which was designated as time 0. At that time haemorrhage ceased and the blood pressure was held at ca. 50 mmHg by withdrawal or infusion of blood for 120 min. Samples of blood were drawn for analysis at 5 min and then at the end of each 15 min period.

Data are expressed as mean ± s.e.mean. Statistical evaluation was performed by blocked ANOVA. Differences were considered significant at $P < 0.05$.

**Results** Systematic arterial blood pressure was 121.3 ± 3.4 mmHg and during withdrawal of blood the mean pressure decreased to 51.1 ± 0.7 mmHg where it was held for 2 h by withdrawal or return of blood. Central venous pressure declined from 4.3 ± 0.1 mmHg to 3.2 ± 0.2 mmHg by 5 min and remained at a similar level thereafter. The basal levels of arterial and venous plasma adenosine were similar (ca. 1 $\mu$M) and stable within 1 h after surgical preparation (Figure 1). The concentrations of plasma adenosine in central venous efflux were significantly increased from 5 min to 45 min after haemorrhage and then declined back to the basal levels ($P < 0.05$, Figure 1a). In contrast, plasma adenosine concentrations in arterial blood were not elevated during the whole haemorrhage procedure (Figure 1b). Figure 2 represents blood volume removed to cause blood pressure to decline to ca.

[1] Author for correspondence.

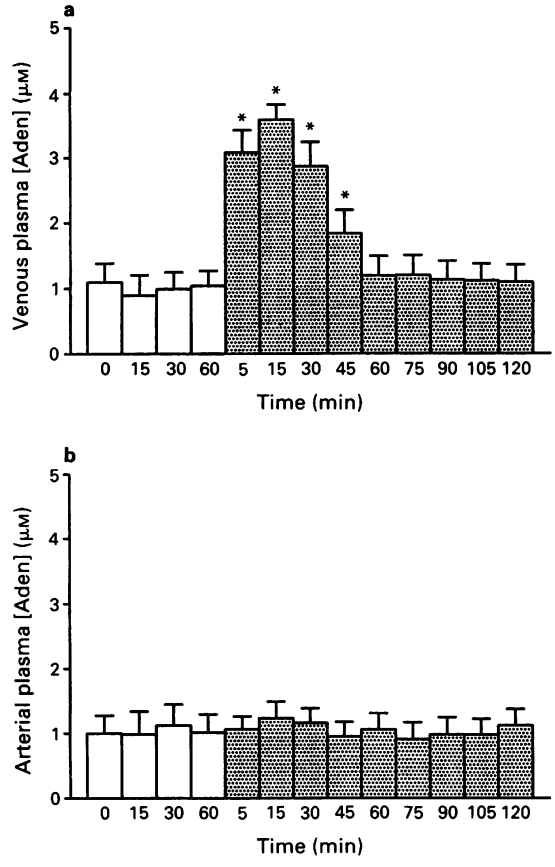


**Figure 1** Central venous (a) and arterial (b) plasma adenosine concentrations [Aden] before (open columns) and after (stippled columns) haemorrhage. * denotes that the mean of the post-haemorrhage data is significantly different from the mean of the pooled prehaemorrhage data ($P < 0.05$, blocked ANOVA).

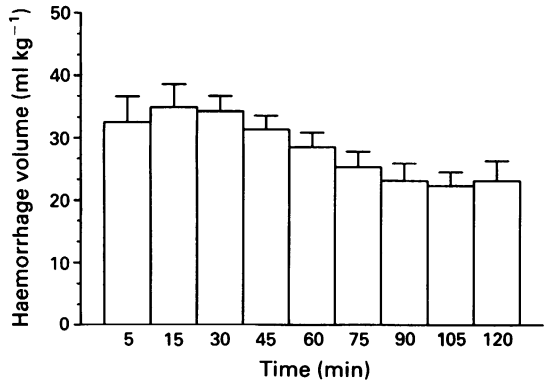


**Figure 2** Blood volume removed to maintain blood pressure at the haemorrhage set point of ca. 50 mmHg. The initial rate of bleeding was 15.3 ml min$^{-1}$ until pressure reached the set point whereupon additional blood was withdrawn or replaced as required. All times are measured from time 0, i.e., the time at which blood pressure reached 50 mmHg.

50 mmHg and be maintained at that pressure. The initial rate of blood removal was rapid (15.3 ml min$^{-1}$) and was maintained until pressure reached 50 mmHg (6.4 ± 1.1 min). The times referred to throughout are taken from $t = 0$ at the time the blood pressure reached the set point. The volume of blood withdrawn reached a peak of 35.3 ± 4.3 ml kg$^{-1}$ at 15 min.

**Discussion** Adenosine is produced and released from a variety of tissues in response to metabolic stress. A wide range of effects on blood vessels, metabolism, nerve functions and hormonal secretion occur in response to local adenosine. However, it is not clear if adenosine is able to serve a hormonal role by attaining sufficiently high circulating levels to cause systemic effects at sites distant from the sites of production. It has been suggested that elevated adenosine levels may suppress catecholamine release (Tung *et al.*, 1987) and lead to decompensation after prolonged haemorrhage in rabbits. Our results do not support this conclusion. First, the pattern of release of adenosine in our results is contradictory to that of Tung *et al.* (1987). Tung *et al.* (1987) found no elevation of venous adenosine 30 min after the onset of haemorrhage and after 1 h the levels were less than 2 μM (control levels 0.7 μM); at the point where 60% of the blood had to be reinfused to maintain arterial pressure at the set-point (41 mmHg) the venous adenosine levels were further elevated to 2.4 μM. In contrast, our results showed that the peak time of adenosine release correlated well with the peak time of blood volume removed and there was no correlation between adenosine release and decompensation as reported by Tung *et al.* (1987).

Regardless of the differences in release pattern and venous plasma concentration, the possibility of adenosine serving a role as a circulating vasodilator or autonomic nervous modulator is unlikely. Despite the sharp rise in venous levels, the adenosine was eliminated from the blood prior to reaching the arterial side of the circulation. Adenosine is, therefore, not capable of serving a hormonal-type function of producing effects at sites removed from the tissues of production. The possible exceptions would be that even though it was not clear where the adenosine carried in venous blood came from, adenosine may modulate functions downstream in the heart or lungs or in the tissues downstream of the portal circulations of the pituitary, kidney, and liver.

Adenosine appears to contribute to the hypotension seen during haemorrhage since caffeine in rats (Conlay *et al.*, 1988) and 8-phenyltheophylline in cats (Lautt & Légaré, 1985) decreased the hypotensive response. However, the effects are probably restricted entirely to those tissues that generate the adenosine since recirculation of adenosine does not occur. Since such effects are not seen during a major and generalized stimulus like haemorrhage, it seems unlikely that any other localized stimuli would lead to elevations in arterial adenosine levels.

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**SPECIAL REPORT**



# Identification of inhibitors of nitric oxide synthase that do not interact with the endothelial cell L-arginine transporter

Richard G. Bogle, *Salvador Moncada, Jeremy D. Pearson & [1]Giovanni E. Mann

Vascular Biology Research Centre, Biomedical Sciences Division, King's College, Campden Hill Road, London W8 7AH and *Wellcome Research Laboratories, Langley Court, Kent BR3 3BS

The effects of inhibitors of nitric oxide (NO) synthase and other cationic amino acids on unidirectional L-arginine transport were studied in porcine aortic endothelial cells cultured in microwell plates or perfused in microcarrier columns. L-Homoarginine, L-lysine and L-ornithine inhibited transport of L-arginine. The NO synthase inhibitors $N^G$-monomethyl-L-arginine and $N^G$-iminoethyl-L-ornithine also reduced L-arginine uptake, whereas $N^G$-nitro-L-arginine and its methyl-ester had no inhibitory effect. The ability to modulate selectively endothelial cell L-arginine transport or NO synthase activity will allow further characterization of the arginine transporter and its role in regulating NO biosynthesis.

**Keywords:** Endothelial cells; nitric oxide; L-arginine transport; nitric oxide synthesis inhibitors; system $y^+$; L-homoarginine; $N^G$-monomethyl-L-arginine (L-NMMA); $N^G$-nitro-L-arginine methylester (L-NAME); $N^G$-iminoethyl-L-ornithine (L-NIO); $N^G$-nitro-L-arginine (L-NOARG)

**Introduction** Synthesis of nitric oxide (NO) from L-arginine has been identified as a widespread mechanism involved in the regulation of cellular function and communication. Vascular endothelial cells generate NO from L-arginine via a $Ca^{2+}$/calmodulin-dependent constitutive enzyme and a cytokine-inducible $Ca^{2+}$-independent NO synthase not normally present in endothelial cells (see Moncada *et al.*, 1991). Analogues of L-arginine such as $N^G$-monomethyl-L-arginine (L-NMMA), $N^G$-iminoethyl-L-ornithine (L-NIO), $N^G$-nitro-L-arginine (L-NOARG) and $N^G$-nitro-L-arginine methylester (L-NAME) are potent inhibitors of the release of NO from vascular endothelial cells (Moore *et al.*, 1989; Rees *et al.*, 1990), but the time course of maximal inhibition by these analogues and their reversibility by L-arginine appear to differ.

Endothelial cells take up L-arginine by a saturable transport system $y^+$ which is inhibited by L-NMMA and other cationic amino acids (Mann *et al.*, 1991) and stimulated by bradykinin (Bogle *et al.*, 1991). In the present study we have examined the inhibitory potency of other arginine analogues on L-arginine uptake by porcine aortic endothelial cells.

**Methods** Porcine aortic endothelial cells were isolated and cultured in Dulbecco's modified Eagle's medium supplemented with penicillin 100 units $ml^{-1}$, streptomycin 100 $\mu g\ ml^{-1}$, 10% foetal and 10% new born calf serum and L-glutamine 4 mM. When confluent, endothelial cells were either plated into 96-well plates or transferred onto Biosilon microcarriers (Nunc, Denmark).

Confluent monolayers in 96-well plates ($2 \times 10^4$ cells per well) were rinsed with HEPES-buffered Krebs solution (see Bogle *et al.*, 1991) and L-arginine uptake was measured following incubation of monolayers with 50 $\mu$M L-[$^3$H]-arginine and D-[$^{14}$C]-mannitol (an extracellular tracer). Transport was terminated by washing monolayers 3 times with ice-cold phosphate-buffered saline and protein was determined with the BioRad reagent. Monolayers were digested with formic acid and samples taken for scintillation counting. Recovery of D-[$^{14}$C]-mannitol was always $<0.1\%$. Microcarrier cultures ($\sim 5 \times 10^6$ cells per column) were perfused at 0.5 ml $min^{-1}$ with HEPES-buffered Krebs solution and cells were exposed briefly (150 $\mu$l in 30 s) to 0.5 $\mu$M L-[$^3$H]-arginine and D-[$^{14}$C]-mannitol. Fractions of the column effluent were collected sequentially to determine uptake of L-arginine relative to D-mannitol (Mann *et al.*, 1991). Unlabelled L-arginine analogues (0.05–5 mM) were added individually to the incubation media or microcarrier column perfusate.

L-[2,3-$^3$H]-arginine and D-[1-$^{14}$C]-mannitol were obtained from NEN (Germany), unlabelled amino acids, L-NAME and L-NOARG from Sigma and L-NMMA, D-NMMA and L-NIO from Wellcome, Beckenham, U.K.

**Results** Uptake of L-arginine by endothelial cell monolayers was linear for up to 20 min, hence, all measurements were made after a 15 min incubation period. L-Arginine uptake at 50 $\mu$M was $153 \pm 27$ nmol $mg^{-1}$ protein $h^{-1}$ (mean ± s.e.; $n = 4$). Transport was saturable over the concentration range of 1–100 $\mu$M, although at higher concentrations a non-saturable component became apparent (data not shown). Uptake of L-arginine (50 $\mu$M) was inhibited by L-homoarginine (1 mM; $52 \pm 4\%$; $n = 3$) but not by D-arginine (1 mM) or analogues selective for amino acid transport systems L (β-2-amino-bicyclo-[2,2.1]-heptane-2-carboxylic acid), N (6-diazo-5-oxo-L-norleucine) or A (2-methylaminoisobutyric acid). L-Arginine uptake was inhibited in a concentration-dependent manner by L-NMMA or L-NIO (Figure 1a) but was not inhibited by L-NOARG or L-NAME (Figure 1b). Although L-NOARG and L-NAME significantly ($P < 0.05$; unpaired $t$ test) elevated the uptake of L-arginine, this effect did not appear to be dose-dependent and may reflect *trans*-stimulation of arginine entry (Mann *et al.*, 1991).

The effect of these NO synthase inhibitors on L-arginine uptake was investigated further in porcine aortic endothelial cells cultures and perfused on microcarrier beads. Under these conditions rapid (15 s) L-arginine transport was partially saturable (Mann *et al.*, 1991), sodium-independent (data not shown) and inhibited by 1 mM L-lysine, L-ornithine or L-homoarginine (Figure 2). Uptake of L-arginine (0.5 $\mu$M) was also inhibited by L-NMMA (1 mM; $57 \pm 6\%$) or L-NIO (1 mM; $41 \pm 4\%$) whereas L-NOARG (1 mM; $8 \pm 2\%$) and L-NAME (1 mM; $2 \pm 3\%$) were either weak or inactive inhibitors (Figure 2). D-NMMA and D-arginine were not inhibitors of L-arginine uptake (Figure 2).

**Discussion** Our results demonstrate that L-NOARG and L-NAME are poor inhibitors of L-arginine transport whereas

[1] Author for correspondence.

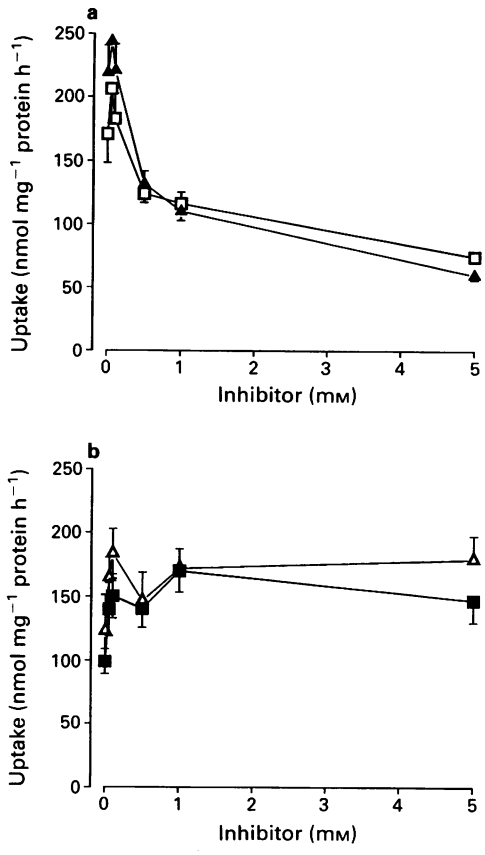


**Figure 1** Effects of NO synthase inhibitors on L-arginine transport in endothelial cell monlayers. Inhibition of 50 μM L-[$^3$H]-arginine uptake by (a) $N^G$-monomethyl-L-arginine (□) or $N^G$-iminoethyl-L-ornithine (▲), (b) $N^G$-nitro-L-arginine (■) or $N^G$-nitro-L-arginine methyl ester (△). Values are mean of 15 replicates derived from three experiments using different batches of endothelial cells; vertical bar show s.e.

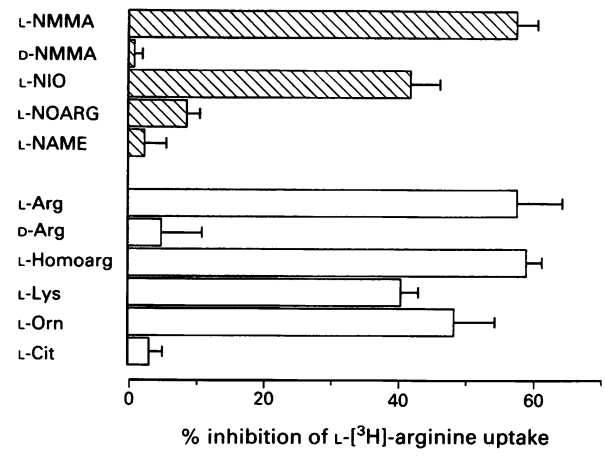


**Figure 2** Effects of NO synthase inhibitors and other arginine analogues on rapid L-arginine transport in perfused endothelial cell microcarrier cultures. Cells were challenged in the absence of ($J_c$) and then the presence ($J_i$) of arginine analogues (1 mM) and the % inhibition of L-[$^3$H]-arginine uptake (0.5 μM) was calculated from $(1 - J_i/J_c) \times 100$. Values are mean of $n = 3-5$ experiments from different batches of endothelial cells, horizontal bars show s.e. For abbreviations, see text.

L-NMMA and L-NIO inhibit transport substantially, thus identifying inhibitors of NO synthase that do not block entry of L-arginine. Other cationic amino acids including L-lysine, L-ornithine and L-homoarginine, which do not inhibit the constitutive form of NO synthase (see Moncada *et al.*, 1991) also reduced L-arginine transport. The fraction of L-arginine transport insensitive to inhibition by arginine analogues may reflect entry via a second carrier with a lower substrate affinity.

Differences between the pharmacological profiles of NO synthase inhibitors have been described previously (Rees *et al.*, 1990), and it is possible that this is due to differences in the mechanisms by which they enter endothelial cells. In neutrophils and J774 cells inhibition of NO synthase has been shown to occur more rapidly with L-NIO than L-NMMA, and the effects of L-NIO were prevented following concomitant incubation with L-arginine (McCall *et al.*, 1991). This latter study also suggested that uptake of different arginine analogues may be mediated by different transport systems. Our findings are consistent with this idea, since the analogues that inhibited L-arginine transport did so reversibly and are most likely to compete with L-arginine for entry via the $y^+$ transporter.

The lack of effect of L-NOARG and L-NAME as inhibitors of endothelial cell L-arginine transport suggests that these compounds do not enter the cell via system $y^+$. Diffusion may account for the uptake of the lipophilic molecule L-NAME, however such a mechanism is unlikely to account for the entry of L-NOARG. The pathways by which these analogues are transported into endothelial cells can only be further elucidated by studies using radiolabelled L-arginine analogues.

It is likely that the major proportion of endotheial NO synthase activity is particulate (Förstermann *et al.*, 1991) and may be localised close to or at the plasma membrane (Boje & Fung, 1990). Close coupling between the L-arginine transporter and NO synthase could account for the regulation of NO release as well as for the ability of circulating L-arginine to reverse the inhibition of NO synthase by L-NMMA. Our results offer the potential to modulate L-arginine transport or NO synthase activity selectively. Recent cloning of NO synthase (Bredt *et al.*, 1991) and system $y^+$ (Kim *et al.*, 1991; Wang *et al.*, 1991) will allow application of immunocytochemical techniques to co-localise NO synthase and system $y^+$ in normal and diseased vascular endothelium.

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**SPECIAL REPORT**



# A specific $B_2$-bradykinin receptor antagonist HOE 140 abolishes the antihypertrophic effect of ramipril

[1]Wolfgang Linz & Bernward A. Schölkens

Department of Pharmacology, Hoechst AG, D-6230 Frankfurt/Main, Germany

To evaluate the role of bradykinin in the antihypertrophic effect of the angiotensin-converting enzyme (ACE) inhibitor, ramipril, we investigated the influence of HOE 140, a specific $B_2$-receptor antagonist, on the effects of ramipril on left ventricular hypertrophy (LVH) in rats with aortic banding. Ramipril at a dose of 1 mg kg$^{-1}$ day$^{-1}$ for 6 weeks prevented the increase in blood pressure and development of LVH after aortic banding; plasma ACE activity was significantly inhibited. A lower dose of ramipril (10 μg kg$^{-1}$ day$^{-1}$ for 6 weeks) had no effect on the increase in blood pressure or on plasma ACE activity, but prevented LVH after aortic banding. The antihypertrophic effects of the higher and the lower dose ramipril, as well as the antihypertensive action of the higher dose of ramipril were abolished by the coadministration of HOE 140 (500 μg kg$^{-1}$ day$^{-1}$). The present data show for the first time that the beneficial effects of an ACE-inhibitor on LVH in rats with hypertension caused by aortic banding can be prevented by a specific $B_2$-receptor antagonist.

**Keywords:** Left ventricular hypertrophy; angiotensin converting enzyme inhibition; ramipril; bradykinin; HOE 140

**Introduction** The renin angiotensin system (RAS) has been implicated in the development and maintenance of hypertension and cardiac hypertrophy (Schelling *et al.*, 1991). In the coarctation model of hypertension angiotensin-converting enzyme (ACE) inhibitors have been shown to inhibit the development, and to induce regression, of cardiac hypertrophy in doses without effects on blood pressure (Linz *et al.*, 1989). Some findings indicate that ACE inhibitors suppress the cardiac hypertrophic response by reducing the formation of angiotensin II (AII), which stimulates hypertrophy and matrix protein synthesis (Schelling *et al.*, 1991). However, since inhibition of ACE, besides reducing AII, also increases bradykinin levels, it is conceivable that bradykinin, through the subsequent generation of nitric oxide (NO) (Wiemer *et al.*, 1991), also contributes to the prevention of the hypertrophic response by ACE inhibitors.

In the present study we investigated the influence of HOE 140 (D-Arg-[Hyp$^3$,Thi$^5$, D-Tic$^7$, Oic$^8$] bradykinin), a specific $B_2$-receptor antagonist (Wirth *et al.*, 1991) on the antihypertrophic effect of ramipril in LVH in rats made hypertensive by aortic banding.

**Methods** Male Sprague Dawley rats weighing 280–300 g (Möllegaard, Skensved, Denmark) were fasted for 12 h before surgery. Anaesthesia was induced by i.p. injection of 200 mg kg$^{-1}$ hexobarbitone (Evipan). The abdomen was opened by a cut parallel to the linea alba. The abdominal aorta was exposed above the left renal artery and a silk thread was passed under it. A cannula no. 1 (0.9 × 40 mm) was placed longitudinally on the aorta and both aorta and cannula were tied. The cannula was then removed, leaving an aortic lumen determined by the diameter of the cannula. Before the abdomen was closed with catgut, the animals received 5.5 mg rolitetracycline (Reverin, Hoechst AG, Frankfurt, Germany). The skin was closed by clipping and covered with tar spray. Control animals were subjected to the same procedure, but without aortic banding.

During the first 5 days following the operation the animals received tetracycline (1 g 350 ml$^{-1}$) in the drinking water. The animals were allotted to 6 groups of 20 rats each as follows: Group I, sham-operated; Group II, aortic banding without treatment; Group III, aortic banding, and ramipril treatment with 1 mg kg$^{-1}$ day$^{-1}$; Group IV, aortic banding, ramipril treatment with 1 mg kg$^{-1}$ day$^{-1}$ and HOE 140 500 μg kg$^{-1}$ day$^{-1}$; Group V, aortic banding, and ramipril treatment with 10 μg kg$^{-1}$ day$^{-1}$; Group VI, aortic banding, ramipril treatment with 10 μg kg$^{-1}$ day$^{-1}$, and HOE 140 500 μg kg$^{-1}$ day$^{-1}$. Ramipril treatment started the day after the operation and continued for 6 weeks via the drinking water. At the end of the operation miniosmotic pumps were implanted subcutaneously in the neck of the animals to deliver HOE 140. At the end of the experiment (after 6 weeks), the animals were anaesthetized with 200 mg kg$^{-1}$ i.p. hexobarbitone and blood pressure was measured via catheters in the left carotid artery. The hearts were excised, cleaned of blood with saline, gently blotted to dryness, and left ventricular weight (LVW) including the septum as well as the remaining cardiac tissue representing the right ventricle (RVW) were determined (to the nearest 0.1 mg). Weights are given per 100 g body weight. Plasma ACE activity was determined radioenzymatically by use of [$^3$H]-Hip-Gly-Gly as substrate. Ramipril and HOE 140 were dissolved in saline.

Statistical analysis was performed with ANOVA followed by the Bonferroni test when appropriate. Differences were considered significant if $P<0.05$. Results are given as mean ± s.d.

**Results** The higher dose of ramipril (1 mg kg$^{-1}$ day$^{-1}$) prevented the increase in blood pressure after aortic banding as well as the development of LVH (Figure 1a,b). Plasma ACE activity was significantly inhibited (Table 1). The lower dose of the ACE inhibitor (10 μg kg$^{-1}$ day$^{-1}$) after six weeks neither prevented the increase in blood pressure nor lowered plasma ACE activity, but it prevented the development of LVH (Figure 1a,b; Table 1).

The antihypertrophic effects of the higher and lower dose of ramipril and the antihypertensive action of the higher dose of ramipril were both abolished by the coadministration of HOE 140 (Figure 1a,b). Plasma ACE activity was not changed by HOE 140 treatment (Table 1). Right ventricular weight (46–51 mg per 100 g body weight) and body weight (between 468 ± 8 and 475 ± 9) did not differ between groups.

**Discussion** The present data show for the first time that the beneficial effects of an ACE inhibitor on LVH in rats with

[1] Author for correspondence.

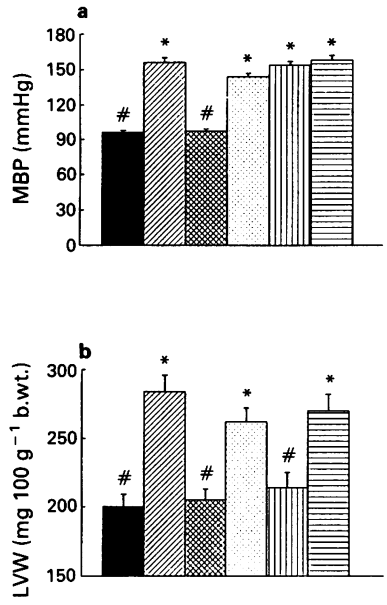


**Figure 1** Effects of orally administered ramipril (1 mg kg$^{-1}$ day$^{-1}$ and 10 μg kg$^{-1}$ day$^{-1}$), and coadministration of HOE 140 (500 μg kg$^{-1}$ day$^{-1}$ s.c.) on (a) mean blood pressure (MBP) and (b) left ventricular weight (LVW) in rats with aortic banding:( ■ ) Sham; ( ▨ ) control vehicle; ( ▩ ) ramipril 1 mg kg$^{-1}$; ( ▧ ) ramipril 1 mg+HOE 140; ( ▥ ) ramipril 10 μg kg$^{-1}$; ( ▤ ) ramipril 10 μg+HOE 140. *$P<0.05$ vs sham, #$P<0.05$ vs control.

hypertension caused by aortic banding can be prevented by a specific $B_2$-receptor antagonist. At the end of the study the blood pressure lowering effect in the group treated with the higher dose of ramipril was abolished by HOE 140, as observed for the acute antihypertensive actions of ramipril and their reversal by a $B_2$-receptor antagonist (Danckwardt *et al.*, 1990). However, under both treatment regimens, in the present study with and without blood pressure reduction, the antihypertrophic effect of ramipril was abolished by HOE 140. This observation provides evidence for a possible involvement of bradykinin in the antihypertrophic effect of ramipril in LVH.

Both ramiprilat and bradykinin have been shown to increase nitric oxide and prostacyclin release in cultured endothelial cells (Wiemer *et al.*, 1991). Nitric oxide and nitric oxide-generating vasodilators have been demonstrated to be antimitogenic (Garg & Hassid, 1989). Similar effects are known for prostacyclin and adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Shirotani *et al.*, 1991). Thus, both nitric oxide and prostacyclin when increased by BK following ACE inhibition may participate in the beneficial effects of ACE inhibitors.

On the other hand AII has been claimed to be a myocardial growth factor (Schelling *et al.*, 1991). However, the specific blockade of AII $AT_1$-receptors, seems to be less effective in this respect than ACE inhibition. In a recent study in the same experimental model, the AII $AT_1$-receptor antagonist, DuP 753 (Losartan), given at a high dose that lowered blood pressure, was less effective on cardiac hypertrophy than ramipril at a low dose which did not lower blood pressure (Linz *et al.*, 1991).

In summary our data in rats with aortic banding and left ventricular hypertrophy provide evidence that the ACE inhibitor-induced potentiation of bradykinin contributes to the beneficial effects of ACE inhibitors.

**Table 1** Effects of orally administered ramipril and subcutaneous coadministration of HOE 140 on plasma angiotensin converting enzyme (ACE) activity

| | *Sham* | *Control* | *Ramipril* (1 mg) | *Rami* (1 mg) + *HOE 140* | *Ramipril* (10 μg) | *Rami* (10 μg) + *HOE 140* |
|---|---|---|---|---|---|---|
| Plasma-ACE activity | 224 ± 0 | 219 ± 9 | 61 ± 6*# | 78 ± 6*# | 217 ± 7 | 222 ± 8 |

$P<0.05$; *, vs Sham; #, vs control; plasma ACE activity: (nmol min$^{-1}$ ml$^{-1}$). Sham: sham-operated animals; Control: untreated controls with aortic banding; Ramipril 1 mg: ramipril 1 mg kg$^{-1}$ day$^{-1}$; Rampril 10 μg: ramipril 10 μg kg$^{-1}$ day$^{-1}$; HOE 140: $B_2$-receptor antagonist (500 μg kg$^{-1}$ day$^{-1}$).

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**SPECIAL REPORT**



# Human big-endothelin-1 and endothelin-1 release prostacyclin via the activation of $ET_1$ receptors in the rat perfused lung

[1]Pedro D'Orléans-Juste, Sabine Télémaque, Audrey Claing, *Masaki Ihara & *Mitsuo Yano

Department of Pharmacology, Medical School, Université de Sherbrooke, Sherbrooke (Québec) J1H 5N4, Canada and *Central Research Laboratories, Banyu Pharmaceutical Co., Ltd., 2-9-3 Shimomeguro, Meguro-ku, Tokyo 153, Japan

Although $ET_1$ and $ET_2$ binding sites were found in rat lung membranes, a selective $ET_1$ receptor antagonist, BQ-123 (10 μM), did not displace [$^{125}$I]-endothelin-1 ([$^{125}$I]ET-1) from $ET_2$ sites, illustrating the selectivity of the angatonist for $ET_1$ receptors. In rat perfused lungs, BQ-123 (1 μM) markedly reduced the prostacyclin ($PGI_2$) releasing properties of endothelin-1 (ET-1: 5 nM) and human big-ET-1 (100 nM) suggesting that both peptides induce the release of $PGI_2$ via the selective activation of $ET_1$ receptors.

**Keywords:** Endothelin-1; human big-endothelin-1; antagonist; $ET_1$ receptors; prostacyclin; rat perfused lungs; $ET_1$ and $ET_2$ binding sites

**Introduction** The cloning and biochemical characterization of two distinct receptor subtypes for endothelins, namely $ET_1$ (ET-A, Arai *et al.*, 1990) and $ET_2$ (ET-B, Sakurai *et al.*, 1990) have prompted the development of selective antagonists which are critical for the understanding of the pharmacological properties of the endothelins *in vivo* and *in vitro*.

Ihara *et al.* (1992) have reported a novel, selective $ET_1$ receptor antagonist, BQ-123 (cyclo[D-Asp-L-Pro-D-Val-L-Leu-D-Trp]), which is a potent antagonist of endothelin-1 (ET-1)-induced hypertension in conscious rats and a competitive and specific antagonist of ET-1-induced contraction of porcine coronary arteries. Yet in conscious rats, BQ-123 did not affect the initial hypotension caused by an intravenous administration of ET-1, which has been suggested to be mediated by $ET_2$ receptors.

Endothelin-induced vasoconstriction has been associated with activation of $ET_1$ receptors and the release of the endothelium-derived relaxing factor with activation of $ET_2$ receptors (Webb, 1991; Ihara *et al.*, 1992); the receptors mediating the endothelin-induced release of prostacyclin ($PGI_2$) remain to be identified.

The present study was attempted to identify the receptors responsible for the ET-1 and big-ET-1-induced release of $PGI_2$ from the rat perfused lung (de Nucci *et al.*, 1988; D'Orléans-Juste *et al.*, 1991). Furthermore, the selectivity of BQ-123 for $ET_1$ receptors was investigated by binding studies in rat lung membranes.

**Methods** Male rats (Wistar, 250–400 g) were anaesthetized with urethane (0.75 mg kg$^{-1}$, i.p.) and their spinal cord sectioned. Following thoracotomy, the pulmonary artery was cannulated, the lungs suspended in a heated chamber (37°C) and perfused (5 ml min$^{-1}$) with an oxygenated (95% $O_2$, 5% $CO_2$) Krebs solution (concentrations in mM: NaCl 117.5, KCl 4.7, $KH_2PO_4$ 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 5.5). They were left to stabilize for 60 min before ET-1 (5 nM) or big-ET-1 (100 nM) were infused (0.1 ml min$^{-1}$ for 3 min). Subsequently, BQ-123 (1 μM) was infused through the lung 15 min prior to, and during a second challenge with ET-1 or big-ET-1. The effluent from the lungs was collected (1 min samples) before, during and after infusion of the various peptides. The samples were stored (− 20°C) until the concentration of the stable, hydrolytic metabolite of prostacyclin, 6-keto-$PGF_{1\alpha}$, was determined by radioimmunoassay (Salmon, 1978).

For the binding studies, the lung membranes were prepared as previously described (Ihara *et al.*, 1991). Non-specific binding was defined by adding 200 nM ET-1 to the assay mixture and was estimated at 5.3% of the total specific binding.

Synthetic big-ET-1, ET-1 and endothelin-3 (ET-3) were purchased from Peptide Institute Inc. (Osaka, Japan). The antagonist, BQ-123 was synthesized at Banyu Pharmaceutical (Tokyo, Japan). Urethane (ethylcarbamate) was purchased fom M.C.B. Manufacturing Chemist Inc. (Cincinatti, U.S.A.). Phosphate buffered saline (PBS, pH 7.4), 6-keto-$PGF_{1\alpha}$ and 6-keto-$PGF_{1\alpha}$-antiserum were purchased from Sigma (St-Louis, U.S.A.). The 6-keto-$PGF_{1\alpha}$ antiserum did not cross-react with ET-1, big-ET-1 or BQ-123. The tracers, [$^3$H]-6-keto-$PGF_{1\alpha}$ and [$^{125}$I]-ET-1 were purchased from Amersham (Oakville, Canada). Agents were dissolved either in saline or PBS.

Data used in the text and figures are expressed as means ± s.e. mean of $n$ observations. Statistical comparisons between groups were made by paired Student's $t$ test. $P$ values of $<0.05$ were considered to be statistically significant.

**Results** The basal release of $PGI_2$ from the rat lungs was 1.1 ± 0.2 ng ml$^{-1}$ ($n$ = 15). Infusion of ET-1 (5 nM) or big-ET-1 (100 nM) for 3 min increased the release of $PGI_2$ to a maximum of 6.6 ± 1.6 or 7.8 ± 1.8 ng ml$^{-1}$ respectively ($n$ = 6–9, $P<0.01$). BQ-123 at 1 μM did not affect the basal release of $PGI_2$ (Figure 1a and b) but abolished the release of $PGI_2$ induced by ET-1 (Figure 1a), and significantly reduced the release induced by big-ET-1 (maximum release in presence of BQ-123: 2.8 ± 0.9 ng ml$^{-1}$, $n$ = 6, $P<0.01$) (Figure 1b). The release of $PGI_2$ induced by ET-1 or big-ET-1 was restored 60 min after the cessation of the BQ-123 infusion (Figure 1a and b).

In control experiments, two successive infusions (with a time interval of 60 min) of ET-1 (5 nM) or big-ET-1 (100 nM) induced the release of equivalent amounts of $PGI_2$ (maximum release of $PGI_2$; ET-1; 9.3 ± 2.1 and 8.4 ± 2.9 ng ml$^{-1}$, $n$ = 4; big-ET-1: 10.1 ± 2.8 and 11.5 ± 1.3 ng ml$^{-1}$, $n$ = 4).

In rat lung membranes, ET-1, ET-3 and BQ-123 inhibited [$^{125}$I]-ET-1 binding with $IC_{50}$ values of 0.19 ± 0.01, 4.47 ± 0.48 and 15.7 ± 1.2 nM, respectively. High affinity binding sites for BQ-123 were estimated at 60% of the total specific [$^{125}$I]-ET-1 binding (Figure 2a). In the presence of 10 μM BQ-123, to block the high affinity sites, [$^{125}$I]-ET-1 binding to the residual sites

[1]Author for correspondence.

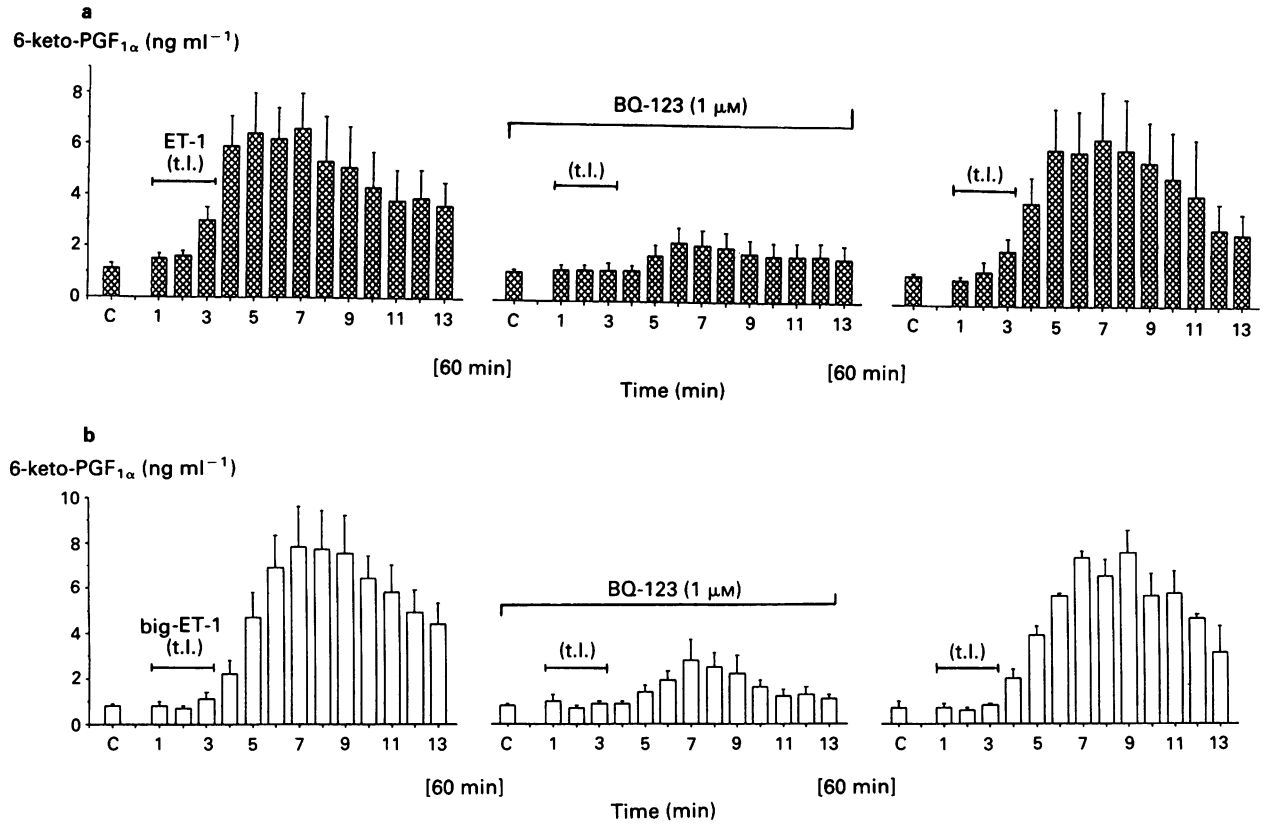


**Figure 1** Effect of a selective $ET_1$ receptor antagonist, BQ-123, on the endothelin-1 (ET-1) or big-ET-1-induced release of prostacyclin ($PGI_2$, measured as 6-keto-$PGF_{1\alpha}$) from the rat perfused lung. (a) Effect of ET-1 (hatched columns) (5 nM) infused for 3 min through the lung circulation (t.l.) in the absence or in the presence of BQ-123 (1 μM, t.l., for 15 min prior to the second administration of ET-1). (b) Effect of big-ET-1 (open columns) (100 nM) infused for 3 min in the lung circulation (t.l.) in the absence or in the presence of BQ-123 (1 μM, t.l., for 15 min prior to the second administration of big-ET-1). Sixty (60) min following the interruption of the infusion of BQ-123, the release of $PGI_2$, induced by a third infusion of ET-1 (5 nM) or big-ET-1 (100 nM), was restored (right panels, a and b). C = basal levels of $PGI_2$ measured prior to the administration of ET-1 or big-ET-1. Each result is the mean (± s.e.mean shown by vertical lines) of at least 6 determinations.

(40%) was inhibited equipotently both by ET-1 and ET-3 ($IC_{50}$ values: ET-1: 0.14 ± 0.01 nM; ET-3: 0.12 ± 0.01 nM) (Figure 2b).

**Discussion** The selective $ET_1$ receptor antagonist, BQ-123, was used to assess the role of $ET_1$ receptors in the ET-1 and big-ET-1-induced release of prostacyclin from the rat perfused lung and in the identification of both $ET_1$ and $ET_2$ binding sites in the rat lung membranes. In absence of BQ-123, ET-1 was 25 times more potent thatn ET-3 in displacing [$^{125}$I]-ET-1 binding, which reflects the order of potency of the endothelin isomers on $ET_1$ receptors, where ET-1 is more potent than ET-3 (Arai *et al.*, 1990). The $ET_1$ receptor antagonist, BQ-123, induced a concentration-dependent inhibition of [$^{125}$I]-ET-1 binding. The high affinity sites for BQ-123 were 60% of the total specific [$^{125}$I]-ET-1 binding sites, and in the presence of BQ-123 (10 μM), the binding of [$^{125}$I]-ET-1 to the remaining 40% binding sites was inhibited equipotently by ET-1 or ET-3, suggesting the displacement of the tracer from non-selective receptors for endothelins ($ET_2$ receptor; Sakurai *et al.*, 1990) and illustrating that BQ-123 (10 μM) did not affect $ET_2$ binding sites.

In contrast, a lower concentration of BQ-123 markedly reduced the ET-1 and big-ET-1-induced release of prostacyclin supporting our hypothesis, that both peptides enhance the release of prostanoid via the selective activation of $ET_1$ receptors in the rat perfused lung. The exact location of these receptors remains to be determined. In addition, it is probable that the activation of $ET_1$ receptors by big-ET-1 follows conversion to ET-1 via a phosphoramidon-sensitive endothelin

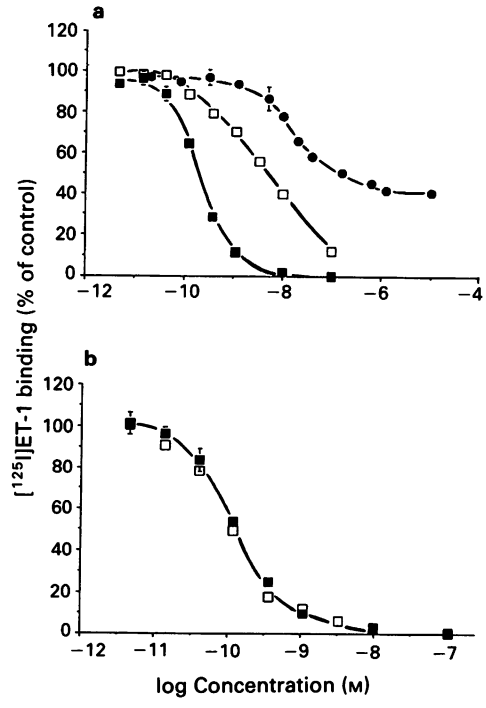


**Figure 2** Inhibition of [$^{125}$I]-endothelin-1 ([$^{125}$I]ET-1) binding to rat lung membranes by ET-1 (■), ET-3 (□) and BQ-123 (●) (a) and by ET-1 (■) and ET-3 (□) in the presence of 10 μM BQ-123 (b). Each value represents the mean of at least three experiments; vertical bars shown s.e.mean.

converting enzyme (ECE, Fukuroda *et al.*, 1990) that we have recently identified in the rat pulmonary vasculature (D'Orléans-Juste *et al.*, 1991).

The use of selective analogues such as BQ-123 will further our understanding of the receptors involved in the pharmacological effects of endothelins *in vivo* and *in vitro*.

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# Role of nitric oxide in non-adrenergic, non-cholinergic inhibitory junction potentials in canine ileocolonic sphincter

Sean M. Ward, [1]Emma S. McKeen & [2]Kenton M. Sanders

Department of Physiology, University of Nevada School of Medicine, Reno, NV 89557, U.S.A.

**1** Electrical field stimulation causes neurally-mediated relaxation of the ileocolonic sphincter that is due to activation of non-adrenergic and non-cholinergic (NANC) nerves. Recent studies have suggested that nitric oxide (NO) is the neurotransmitter that mediates relaxation.

**2** Using intracellular recording techniques, we have tested whether NANC inhibitory junction potentials (i.j.ps) in the canine ileocolonic sphincter are also mediated by NO.

**3** Electrical field stimulation elicited excitatory and inhibitory junction potentials: e.j.ps were blocked by atropine ($10^{-6}$ M) and tetrodotoxin (TTX; $10^{-6}$ M); i.j.ps were also blocked by TTX and partially blocked by apamin ($10^{-6}$ M). I.j.ps were unaffected by atropine, phentolamine and propranolol (all at $10^{-6}$ M).

**4** The arginine analogues, L-$N^G$-nitroarginine methyl ester (L-NAME) and $N^G$-monomethyl-L-arginine (L-NMMA), decreased the amplitude of i.j.ps and L-arginine, but not D-arginine, partially restored the i.j.ps.

**5** I.j.ps were also inhibited by oxyhaemoglobin (1%), but not by methaemoglobin.

**6** Exogenous NO ($10^{-7}$ M to $3 \times 10^{-5}$ M) caused concentration-dependent hyperpolarizations that were similar in amplitude to the NANC nerve-evoked i.j.ps. Hyperpolarizations to NO were unaffected by L-NAME, but were blocked by oxyhaemoglobin.

**7** Tetrodotoxin, L-NAME and oxyhaemoglobin all caused depolarization of resting membrane potential.

**8** The specific guanosine 3′:5′-cyclic monophosphate phosphodiesterase inhibitor, M&B 22948, caused hyperpolarization, increased the maximum level of hyperpolarization reached during i.j.ps, and increased the duration of i.j.ps.

**9** These data further support the hypothesis that NANC neurotransmission in the ileocolonic sphincter is mediated by NO or an NO-releasing compound. The data also suggest that tonic release of NO, possibly from spontaneous firing of NANC nerves, may regulate resting membrane potential and tone in this sphincter.

**Keywords:** Non-adrenergic, non-cholinergic nerves; gastrointestinal motility; inhibitory junction potential

## Introduction

Recent evidence has suggested that nitric oxide (NO) or a NO-releasing substance, may be the transmitter that mediates NANC inhibitory neurotransmission in the gut (Toda *et al.*, 1990; Bult *et al.*, 1990; Dalziel *et al.*, 1991). Early support for this hypothesis came from a series of mechanical studies in which strips of muscle from the canine ileocolonic sphincter relaxed in response to electrical field stimulation of NANC nerves (Boeckxstaens *et al.*, 1990a,c). These responses were blocked by arginine analogues that are known to inhibit specifically nitric oxide synthase (Palmer *et al.*, 1987; see also Figure 5 in Moncada *et al.*, 1991), the enzyme responsible for producing NO from L-arginine. The inhibition by arginine analogues was reversed by L-arginine, but not by D-arginine. Inhibitory responses were mimicked by exogenous NO, and responses to NANC nerve stimulation and exogenous NO were blocked by oxyhaemoglobin, which is known to be a scavenger of extracellular NO (Martin *et al.*, 1985). Investigations into the chemical identity of the substance that conveys the NANC inhibitory signal showed that field stimulation released a substance that behaved in a manner very similar to authentic NO in bioassay cascades (Boeckxstaens *et al.*, 1991). Taken together, these data suggest that NO may be a NANC inhibitory transmitter in the canine ileocolonic sphincter, and this work has stimulated many other investigations of this hypothesis. Recent studies of gastrointestinal (GI) muscles from nearly all levels of the GI tract and from several species have provided strong support for the notion that NO is the primary inhibitory neurotransmitter in the gut (Toda *et al.*, 1990; Bult *et al.*, 1990; Dalziel *et al.*, 1991; Thornbury *et al.*, 1991; Tottrup *et al.*, 1991).

Although NANC inhibitory nerves ultimately mediate relaxation in GI muscles, an important step in neurotransmission is the hyperpolarization response in postjunctional, smooth muscle membranes. Hyperpolarization, which inhibits electrical rhythmicity and decreases the open probability of voltage-dependent $Ca^{2+}$ channels (Smith *et al.*, 1989; Langton *et al.*, 1989) is thought to be mediated by a transient increase in $K^+$ conductance (Tomita, 1972). We tested the hypothesis that inhibitory junction potentials in the ileocolonic sphincter are mediated by NO, or an NO-releasing compound, by examining the following: (i) the effects of NO synthase inhibitors on NANC inhibitory junction potentials, (ii) the effect of authentic NO on membrane potential, (iii) the effects of NO scavengers on NANC i.j.ps and exogenous NO, (iv) and in an attempt to try and explain the mechanism involved in NANC i.j.ps, we have examined the effect of the guanosine 3′:5′-cyclic monophosphate (cyclic GMP) phosphodiesterase inhibitor M&B 22948 on the NANC i.j.p.

## Methods

Mongrel dogs of either sex were anaesthetized with sodium pentobarbitone (45 mg $kg^{-1}$). The abdomen was opened and a segment of bowel (8 cm) from the distal ileum to the proximal colon was removed. This segment contained the ileocolonic sphincter. The fascia attaching the caecum to the ileum was cut and the caecum was removed. The ileocolonic sphincter region was bisected by a longitudinal cut from small bowel to colon and the luminal contents were removed by washing

[1] Present address: University of Ulster at Coleraine, County Derry, Coleraine, BT52 1SA, Northern Ireland.
[2] Author for correspondence.

with Krebs-bicarbonate solution. The resulting sheet was pinned-out with the mucosal side up in a dish of oxygenated Krebs-bicarbonate solution. The ring of circular muscle identified as the ileocolonic sphincter was very prominent (Figure 1). Preparations for intracellular recording were made by cutting muscle strips 1–2 mm wide and 2–3 cm long in planes transverse and parallel to the sphincter. The mucosal layers were dissected away from the underlying smooth muscle. Muscle strips were placed between two parallel platinum electrodes in a 2 ml electrophysiological recording chamber and pinned to the Sylgard floor (Dalziel *et al.*, 1991). The muscles were allowed to equilibrate for approximately 1 h before intracellular experiments were performed. Throughout experiments, preparations were maintained at $37.5 \pm 0.5$°C by constant perfusion with pre-warmed, pre-oxygenated Krebs-bicarbonate solution having the following composition (mM): $Na^+$ 137.4, $K^+$ 5.9, $Ca^{2+}$ 2.5, $Mg^{2+}$ 1.2, $Cl^-$ 134, $HCO_3^-$ 15.5, $H_2PO_4^-$ 1.2, dextrose 11.5. Equilibration of the solution with 97% $O_2$:3% $CO_2$ achieved a final pH of 7.3 to 7.4.

Cells near the submucosal surface of the sphincteric ring were impaled with glass microelectrodes filled with 3 M KCl and having resistances ranging from 30 to 50 MΩ. Transmembrane potentials was measured with a standard electrometer (WPI M-7000), and outputs were displayed on an oscilloscope (Tektronix 5111). Electrical signals were recorded on magnetic tape (Hewlett-Packard 3964A) and by a chart recorder (Gould 2200). The preparations were electrically field stimulated with an electronic stimulator (Grass S44) and a stimulus isolation unit hooked to the platinum electrodes. I.j.ps were elicited by trains of 3 square wave pulses (0.5 ms duration, supramaximal voltage, 20 Hz). These short trains were delivered every 10–20 s throughout the experimental period.

### *Solution and drugs*

L-$N^G$-nitroarginine, methylester (L-NAME), $N^G$-monomethyl-L-arginine (L-NMMA), L-arginine, D-arginine, and propranolol (all Sigma) were used as hydrochloride salts. The sulphate salt of atropine (Sigma), and the mesylate salt of phentolamine (Ciba Geigy) were used. Drugs were dissolved in distilled water as stock solutions of $10^{-1}$ or $10^{-2}$ M and further serial dilutions were made in Krebs-bicarbonate solution as required. M&B 22948 (Zaprinast), a gift from Rhone-Poulenc Rorer, was dissolved in 0.1 M NaOH ($10^{-1}$ M) and diluted to the final desired concentration in Krebs-bicarbonate solution as required. Drugs were introduced to a 20 ml side reservoir from which the bath was perfused at a rate of 5 ml $min^{-1}$. The lag time for perfusion of the recording chamber from the reservoir was 1–2 s.

NO stock solution was prepared by bubbling ice-cold, deoxygenated (sonication under vacuum followed by purging with pure nitrogen gas) distilled water with NO gas (99% pure) to give a saturated solution (1–1.5 mM; Ignarro *et al.*, 1987). NO was diluted in Krebs-bicarbonate solution to the desired concentration immediately before exposing the muscles to this solution. Oxyhaemoglobin was prepared as a haemolysate of canine blood according to the method of Bowman & Gillespie (1982) with the exception that red cells were lysed by 1:1 addition of distilled water. Methaemoglobin was prepared in a similar manner, but potassium ferricyanide was used to convert oxyhaemoglobin to methaemoglobin prior to dialysis (Thornbury *et al.*, 1991).

Data are expressed as mean ± s.e. and paired or unpaired Student's *t* tests were used for determination of statistical significance where appropriate; *n* values refer to number of muscles used in each experiment.

## Results

Circular muscle cells in the ileocolonic sphincter had average resting membrane potentials of $-55 \pm 2$ mV and either displayed small spontaneous oscillations in membrane potential (55%) or were electrically quiescent ($n = 30$). Electrical field stimulation produced a transient depolarization followed by a more sustained hyperpolarization (Figure 2). The depolarization was identified as a cholinergically-mediated excitatory junction potential (e.j.p.) because it was blocked by atropine ($10^{-6}$ M). The hyperpolarization response persisted in the presence of atropine, phentolamine and propranolol (all at $10^{-6}$ M). These non-adrenergic and non-cholinergic (NANC) responses were identified as inhibitory junction potentials (i.j.ps) elicited by intramural nerves because they were reduced or abolished by tetrodotoxin ($10^{-6}$ M; Figure 3). In 50% of the preparations i.j.ps consisted of two components: an initial fast hyperpolarization phase which partially recovered within 8 s followed by a sustained hyperpolarization phase that persisted for about 20 s. In the remainder of the preparations the initial fast phase could not be distinguished from the sustained component. I.j.ps averaged $15 \pm 1$ mV in amplitude and $15 \pm 0.9$ s in duration ($4.6 \pm 0.4$ s in duration at half maximal amplitude; $n = 30$ muscles from 12 dogs).

I.j.ps could be recorded from muscles for up to 8 h under control conditions. Throughout the period of a single cell impalement the amplitude of the i.j.p. remained stable under control conditions. The duration of impalements varied considerably but recordings were maintained for up to 3 h.

Others have shown that an extract from bee venom, apamin, inhibits i.j.ps and neurally-mediated mechanical relaxations (Banks *et al.*, 1979; Shuba & Vladimirova, 1980). Apamin has been shown to block small conductance, $Ca^{2+}$-activated K channels in a variety of preparations (cf. Capoid & Ogden, 1989). There appears to be apamin-sensitive and insensitive components to the NANC inhibitory response in GI muscles (Costa *et al.*, 1986). We tested the effects of apamin

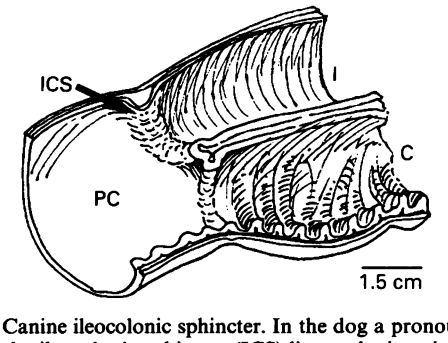


**Figure 1** Canine ileocolonic sphincter. In the dog a pronounced ring of muscle, the ileocolonic sphincter (ICS) lies at the junction between the terminal ileum (I) and proximal colon (PC). The caecum (C) lies below this region. Muscle cells within the sphincteric ring of circular muscle were impaled for electrophysiological studies.

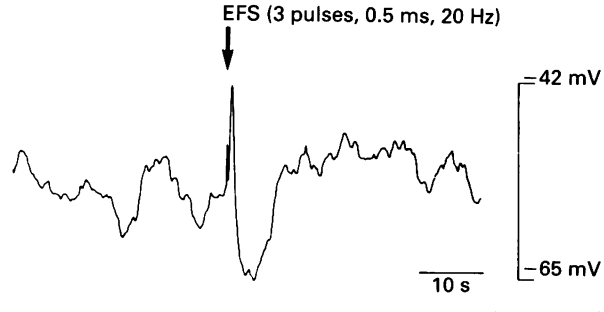


**Figure 2** Responses to electrical field stimulation (stimulus applied at arrow) were characterized by an initial depolarization (e.j.p.), followed by a more sustained hyperpolarization response (i.j.p.). I.j.ps were non-adrenergic and non-cholinergic (NANC) responses. In many muscles membrane potential spontaneously oscillated (see irregular baseline before and after response to electrical field stimulation) during the course of experiments.

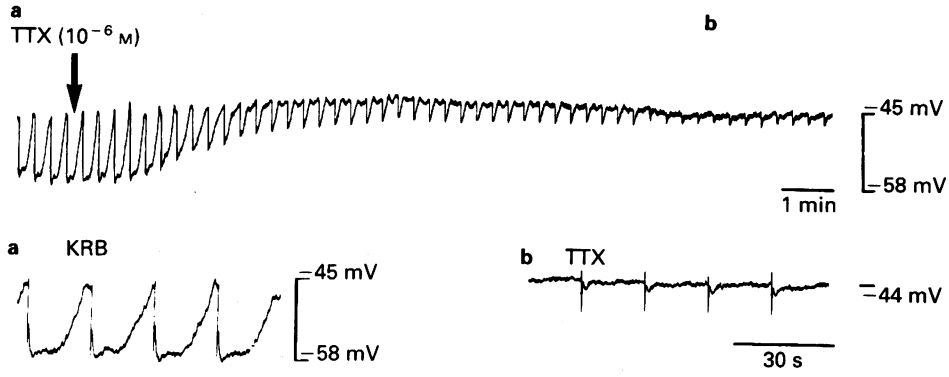


**Figure 3** Effects of tetrodotoxin (TTX; $10^{-6}$ M) on i.j.ps. Top trace shows time-course of response to TTX. I.j.ps were elicted every 20 s. TTX reduced amplitude of i.j.ps. I.j.ps during control (a) and at maximal effect of TTX (b) are shown at expanded time scale in bottom panels.

on i.j.ps in the ileocolonic sphincter. Apamin ($10^{-6}$ M) reduced the amplitude of the fast hyperpolarization phase from an average of 21 ± 3 mV to 14 ± 1.5 mV ($n = 4$, $P < 0.05$), but did not affect the duration or the slow component of the i.j.p. (Figure 4). These data suggest that different mechanisms, perhaps different K channels, mediate the slow and fast components of the i.j.p.

Nitric oxide (NO) is produced from L-arginine by the enzyme nitric oxide synthase (cf. Moncada *et al.*, 1991). NO synthesis is stereospecific and can be competitively inhibited by the L-arginine analogues, L-NAME and L-NMMA. Exposure of ileocolonic muscle strips to L-NAME ($10^{-4}$ M) caused a depolarization in resting membrane potential (from −54 ± 3 mV to −47 ± 1 mV) and decreased the amplitudes

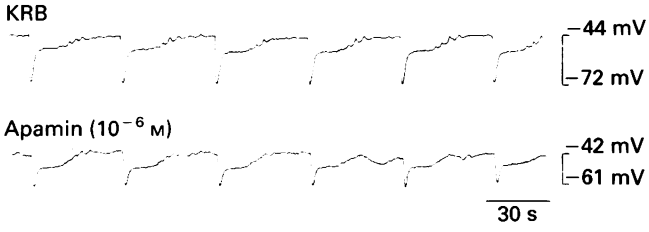


**Figure 4** Effects of apamin ($10^{-6}$ M) on i.j.ps. Top trace shows repetitive i.j.ps recorded in Krebs-bicarbonate solution. Bottom trace shows i.j.ps after exposure to apamin for 15 min. Apamin reduced amplitude of fast phase of i.j.p., but did not affect slow phase and duration of i.j.ps.

of i.j.ps (from 14 ± 1 mV to 5 ± 1 mV; $n = 10$, $P < 0.001$ respectively). In muscles with 2 phase i.j.ps, both phases were inhibited by L-NAME. Figure 5 shows an example of the effects of L-NAME ($10^{-4}$ M) on resting potential and i.j.ps. The average time for L-NAME to produce a maximal reduction in the amplitude of the i.j.p. was 19.5 ± 1 min. The effects of L-NAME were fully reversible upon washout. L-NMMA ($10^{-4}$ M) also reduced the i.j.p. amplitude from 16 ± 1 mV to 6 ± 1.5 mV ($n = 6$) and was also fully reversible upon washout.

The inhibitory action of L-arginine analogues on NANC nerve-induced relaxations of the canine ileocolonic sphincter have been shown to be reversed by L-arginine, but not by the stereoisomer D-arginine (Boeckxstaens *et al.*, 1990a). We tested whether i.j.ps inhibited by L-NAME could be restored by L-arginine, but not by D-arginine. Muscles were exposed to L-NAME ($10^{-4}$ M) for 15 min. Then L-arginine (1 mM) was added to the perfusion solution. L-Arginine partially reversed the inhibition of i.j.ps caused by L-NAME (Figure 6). In these experiments the average i.j.p. amplitude before the addition of L-NAME was 14 mV ± 1.8 mV ($n = 5$), L-NAME reduced the average i.j.p. to 5 mV ± 1.2 mV, and L-arginine reversed the L-NAME effect from 5 mV ± 1.2 mV to 8.4 mV ± 1.0 mV ($P < 0.01$). Restoration of i.j.ps in the presence of L-NAME was stereospecific, addition of D-arginine did not restore i.j.ps inhibited by L-NAME ($n = 6$; Figure 7).

Oxyhaemoglobin (1%), which has been previously shown to scavenge NO (Martin *et al.*, 1985), significantly reduced the amplitude of the NANC nerve-induced i.j.ps from an average of 16 mV ± 4 mV to 4 mV ± 2 mV ($n = 5$, $P < 0.05$; see Figure

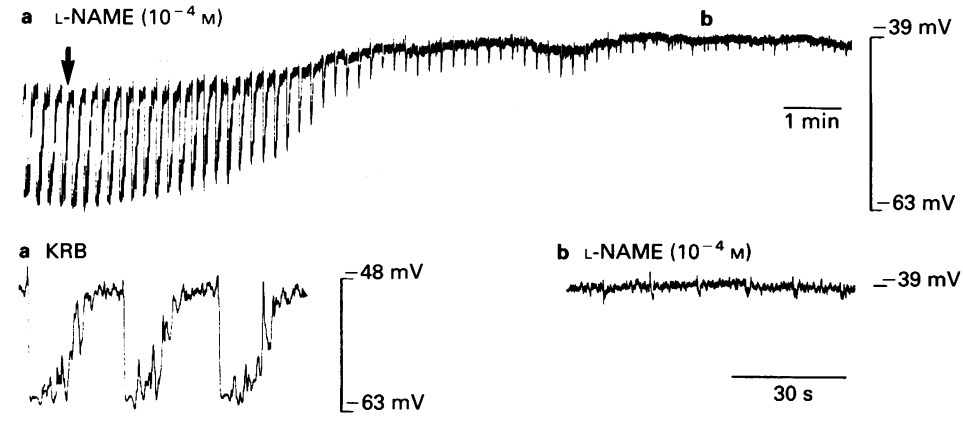


**Figure 5** Effect of L-$N^G$-nitroarginine methyl ester (L-NAME) on i.j.ps. L-NAME ($10^{-4}$ M) was added at arrow. This caused depolarization of membrane potential and a reduction in the amplitude of i.j.ps. Top panel shows time-course of effects. Bottom panels show i.j.ps before L-NAME (a) and at maximal effect of L-NAME. Similar effects occurred in response to $N^G$-monomethyl L-arginine (data not shown).

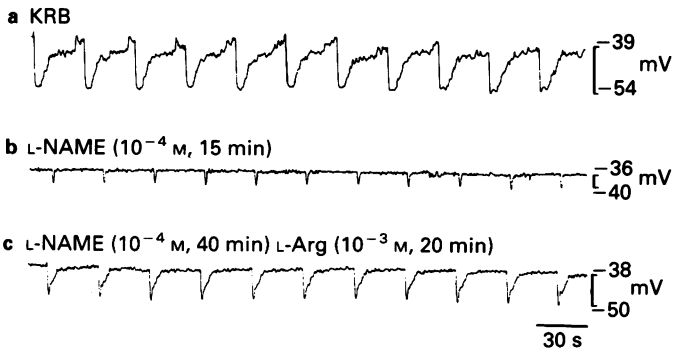


**Figure 6** Inhibitory effects of L-$N^G$-nitroarginine methyl ester (L-NAME) on i.j.ps were reversed by L-arginine: (a) repetitive i.j.ps elicited in control conditions; (b) reduction in i.j.ps in the presence of L-NAME ($10^{-4}$ M); (c) partial restoration of i.j.ps by addition of L-arginine (L-Arg, 1 mM)

8). This effect took an average of 13 ± 2 min to fully develop. Methaemoglobin (1%) did not affect i.j.ps.

Arginine analogues and oxyhaemoglobin reduced the amplitude of i.j.ps, but these agents did not abolish these events. This may have been due to: (i) the concentration of oxyhaemoglobin and arginine analogues used may have been insufficient to inhibit totally NO synthesis or sequester all NO released, or (ii) another substance, perhaps co-released with NO, could be responsible for the remaining portion of the i.j.p. In two experiments we tested the effects of L-NAME ($10^{-4}$ M) and oxyhaemoglobin (1%) together. Combination of these two agents completely abolished i.j.ps.

Experiments were also performed to determine whether exogenous NO could mimic the hyperpolarization produced by NANC nerve stimulation. NO was added to the bath at estimated concentrations ranging from $10^{-7}$ M to $3 \times 10^{-5}$ M as previously described (Thornbury *et al.*, 1991). NO caused concentration-dependent hyperpolarizations that were similar in amplitude to the NANC nerve-evoked i.j.ps. Figure 9 shows responses to several concentrations of NO obtained while maintaining a single impalement. In a series of experiments $3 \times 10^{-6}$ M NO caused hyperpolarization averaging 8.1 mV ± 1.6 mV (i.e. from −51.4 ± 2.8 mV to −59.5 ± 2.7 mV; $n = 10$; $P < 0.001$) and $10^{-5}$ M NO caused hyperpolarization averaging 11.3 mV ± 1.0 mV (from −56 ± 1.8 mV to −67.3 ± 1.9 mV; $n = 16$ from 8 dogs;

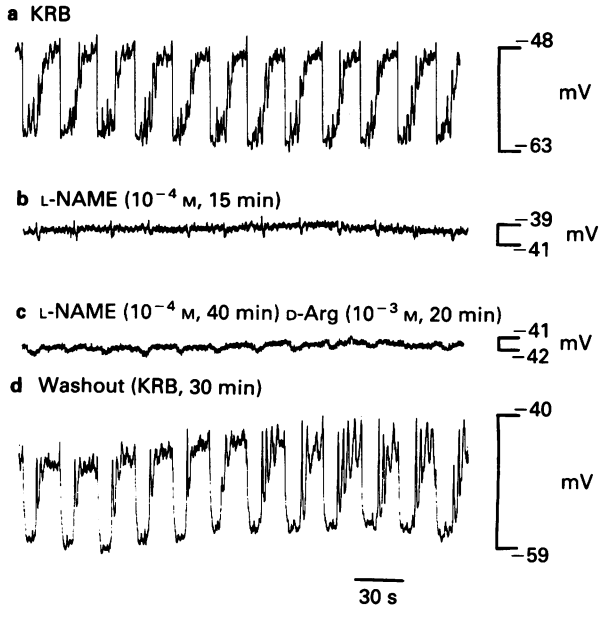


**Figure 7** I.j.ps inhibited by L-$N^G$-nitroarginine methyl ester (L-NAME) were not restored by D-arginine; (a) repetitive i.j.ps elicited in control conditions; (b) reduction in i.j.ps in the presence of L-NAME ($10^{-4}$ M); (c) failure to restore i.j.ps by addition of D-arginine (D-Arg, 1 mM); (d) washout of L-NAME and restoration of i.j.ps.

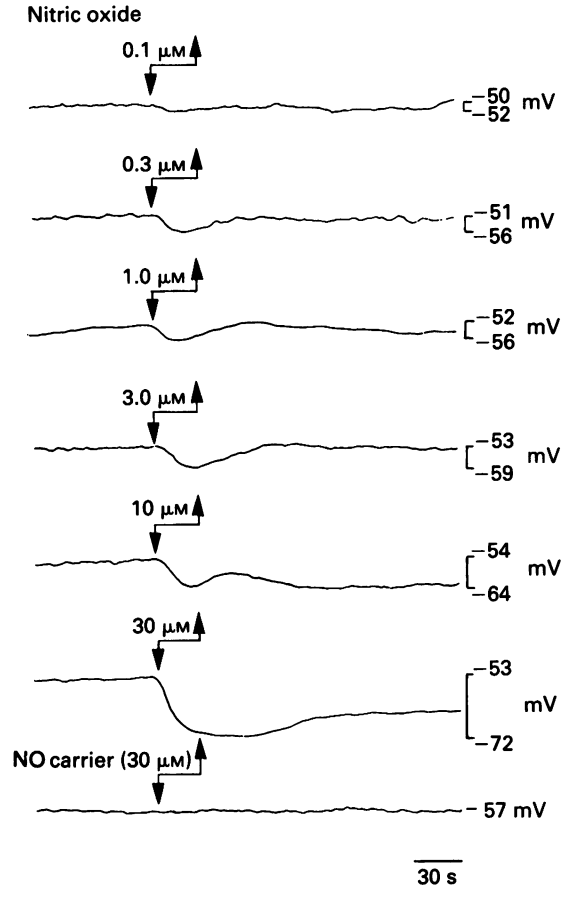


**Figure 9** Effects of exogenous nitric oxide on membrane potential. While maintaining a single impalement, the muscle was exposed to several concentrations of NO. This caused a concentration-dependent hyperpolarization response (estimated concentrations shown above each trace). Bottom panel shows that the solution that NO was dissolved in (i.e. amount of NO carrier needed to make $3 \times 10^{-5}$ M dilution of NO) had no effect alone.


**Figure 8** Effects of oxyhaemoglobin: repetitive i.j.ps were elicited, and at the arrow, oxyhaemoglobin (oxy-Hb; 1%). This caused depolarization of membrane potential and a reduction in the amplitude of i.j.ps.

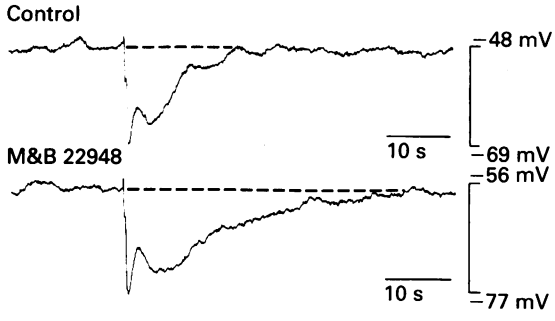


**Figure 10** Effect of M&B 22948 on i.j.ps: traces show superimposed i.j.ps recorded before (control) and in the presence of M&B 22984 ($10^{-4}$ M). The specific cyclic GMP phosphodiesterase inhibitor, M&B 22948 caused an enhancement in the duration of i.j.ps (see text for details).

$P < 0.0001$). L-NAME ($10^{-4}$ M) did not significantly affect the hyperpolarization caused by NO. NO ($3 \times 10^{-6}$ M) caused an average 9.2 mV ± 2.7 mV hyperpolarization in the presence of L-NAME (from −54.4 mV ± 6.3 to −63.6 ± 2.5 mV; $n = 5$), and $10^{-5}$ M NO caused an average 12.2 mV ± 1.0 mV hyperpolarization (from −59 mV ± 4.6 mV to −71.2 mV ± 4.3 mV; $n = 5$). Oxyhaemoglobin (1%), an NO scavenger, did however significantly reduce the NO-induced hyperpolarization. Hyperpolarizations caused by exogenous NO were reduced by oxyhaemoglobin (i.e. from 6.0 ± 1 mV with $3 \times 10^{-6}$ M NO to 1 ± 1 mV; $n = 4$, $P < 0.05$; and from 13 ± 1 mV with $10^{-5}$ M NO to 1 ± 0.4 mV; $n = 4$, $P < 0.005$).

In vascular muscles the receptor for NO appears to be the haemoprotein of soluble guanylate cyclase (Rapoport & Murad, 1983). Binding of NO increases the production of cyclic GMP (Craven & DeRubertis, 1978), and elevation of cyclic GMP is associated with relaxation in smooth muscles (Ignarro & Kadowitz, 1985). NANC effects may be mediated by cyclic GMP in GI smooth muscles. In order to determine whether cyclic GMP participates in the generation of NANC i.j.ps, we tested the effects of the specific cyclic GMP phosphodiesterase inhibitor M&B 22948 (Kukovetz *et al.*, 1982) on i.j.ps. Addition of M&B 22948 ($10^{-4}$ M) caused an 8 mV ± 3 mV hyperpolarization in resting membrane potential from −59 mV to −67 mV ($n = 5$; $P < 0.05$). Although the absolute amplitude of the fast component of the i.j.p. was not significantly affected by M&B 22948, the duration of the second component was increased from 13.4 ± 3.2 s to 20.6 ± 3.2 s ($P < 0.01$) in the presence of M&B 22948. Figure 10 shows superposition of i.j.ps before and in the presence of M&B 22948. I.j.ps are likely to be due to an increase in K conductance (Tomita, 1972). Therefore, the fact that the amplitude of the fast phase was maintained despite the 8 mV hyperpolarization in resting potential caused by M&B 22948, suggests that the increase in K conductance during i.j.ps was enhanced by M&B 22948.

## Discussion

The ileocolonic sphincter (ICS) is formed by a thickening of the circular muscle layer, and it divides the terminal ileum from the proximal colon. It serves as a true sphincter; tonic contraction maintains a region of high pressure (e.g. 66 cm$H_2O$ in dogs; see Kelley *et al.*, 1966). The ICS is thought to cause retention of ileal contents to increase absorption and to prevent reflux of colonic contents into the ileum to help prevent bacterial overgrowth of the small bowel (see Papasova, 1989). Distension of the ileum causes the pressure in the ICS to decrease (Kelley *et al.*, 1966; Kelley & De Weese, 1969), and this is a neurally-mediated reflex (Pahlin & Kewenter, 1975). Extrinsic innervation of the ICS comes from vagal nerves and from sympathetic nerves originating in the superior and inferior mesenteric ganglia, and a dense intrinsic innervation arises from the myenteric plexus (Papasova, 1989). Stimulation of either group of extrinsic nerves enhances sphincteric pressure and stops trans-sphincteric flow (Pahlin, 1975; Pahlin & Kewenter, 1976). Low frequency stimulation of vagal nerves however, inhibits sphincteric pressure (Pahlin & Kewenter, 1975), suggesting a class of low threshold inhibitory neurones running with the vagus. Electrical field stimulation of ICS muscles *in vitro* yields a pronounced relaxation in the presence of drugs to block adrenoceptors and cholinoceptors (Conklin & Christensen, 1975; Papasova & Mizhorkova, 1981; Boeckxstaens *et al.*, 1990d). These non-adrenergic, non-cholinergic inhibitory nerves relax the ICS and allow ileal contents to pass into the colon (see Papasova, 1989). The intrinsic nerves that convey NANC inhibitory input to the smooth muscle of the canine ICS can be activated by acetylcholine (ACh) via nicotinic receptors and by γ-aminobutyric acid (GABA) via $GABA_A$ receptors (Pelckmans *et al.*, 1989; Boeckxstaens *et al.*, 1990b).

Several putative mediators of NANC relaxation have been investigated; adenosine 5′-triphosphate (ATP) and vasoactive intestinal polypeptide (VIP) have generally been considered the strongest candidates (see Hoyle & Burnstock, 1989). However, studies have suggested that neither of these transmitter candidates mediate NANC relaxations in the canine ICS. For example, desensitization of receptors for ATP inhibited further relaxations in response to exogenous ATP, but did not affect relaxations induced by electrical field stimulation or ACh (i.e. acting through nicotinic receptors as described above; Boeckxstaens *et al.*, 1990d). VIP appears to be ineffective in causing relaxation or hyperpolarization in the ICS (MacKenzie & Szurszewski, 1984; Boeckxstaens *et al.*, 1990d). Recent work has shown that field stimulation of NANC nerves in ICS muscles releases a substance that was identified as NO on the basis of its effects on bioassay tissues, chemical stability, neutralization by haemoglobin, and inhibition by arginine analogues (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1991).

The membrane potentials of ICS cells is relatively positive (i.e. −55 mV in the present study; −43 mV in guinea-pig ICS (Kubota, 1983); and −55 mV in another study of the canine ICS (MacKenzie & Szurszewski, 1983), and small spontaneous oscillations in membrane potential are observed in many cells. The resting potentials and oscillations are in the range where voltage-dependent $Ca^{2+}$ channels are activated in many smooth muscle cells, including neighbouring circular muscle cells of the canine proximal colon (Langton *et al.*, 1989; Ward *et al.*, 1990). It is possible that the spontaneous mechanical tone in ICS muscles might be related to a small, continuous leak of $Ca^{2+}$ into cells. Addition of excitatory agonists, such as noradrenaline, causes depolarization (Ward & Sanders, unpublished observations), and this would be expected to increase the open probability of voltage-dependent $Ca^{2+}$ channels and increase the influx of $Ca^{2+}$. ICS muscles are also capable of generating action potentials, but these were rarely observed in the present study or in a previous study of the canine ICS (MacKenzie & Szurszewski, 1983). Therefore, this muscle appears to depend upon the resting membrane potential as an important means of regulating the influx of $Ca^{2+}$ and the force of contraction.

The transmitter released by NANC nerves produces i.j.ps and hyperpolarizes membrane potential. Previous studies have shown that the membrane hyperpolarization elicited by NANC nerve stimulation causes relaxation (MacKenzie & Szurszewski, 1983), and it is likely that this mechanism explains the NANC-induced relaxations described by Boeckxstaens and colleagues (cf. Boeckxstaens *et al.*, 1990a). In the present study we have obtained data suggesting that NANC i.j.ps in the canine ICS are due to release and the actions of NO, and it is likely that the NO-mediated i.j.ps are the mechanism behind NANC relaxations in the ICS (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1990a; 1991). The fact that exogenous NO

causes hyperpolarization and mimics the membrane response to field stimulation in ICS muscles further supports the hypothesis that NO is the NANC inhibitory transmitter in these muscles.

At present the mechanism of the NANC inhibitory junction potentials is unknown. Most investigators have concluded that i.j.ps are due to a transient increase in K conductance (cf. Tomita, 1972). We found that apamin blocks a portion of the i.j.p. in ICS muscles, suggesting the involvement of small conductance $Ca^{2+}$-activated K channels (Capoid & Ogden, 1989). However, the inability of apamin to abolish i.j.ps suggests either that the potency of this agent for the K channels mediating i.j.ps is weak or that more than a single class of channels is involved. The link between NO and K channels is poorly understood. We have found in colonic circular muscle cells that NO increases the open probability of large conductance $Ca^{2+}$-activated K channels (Thornbury *et al.*, 1991). Whether these channels have a role in NANC responses in ICS muscles will require further investigation.

The receptor for NO in smooth muscle cells appears to be soluble guanylate cyclase (Rapoport & Murad, 1983). Our data suggest that effects of NO on membrane potential are also mediated via cyclic GMP. M&B 22948, a specific inhibitor of cyclic GMP phosphodiesterase (Kukovetz *et al.*, 1982), caused hyperpolarization and prolonged the duration of i.j.ps.

Boeckxstaens and colleagues found that addition of arginine analogues (e.g. L-NNA) or oxyhaemoglobin raised basal tone (Boeckxstaens *et al.*, 1990a). These data suggest that there is tonic release of NO in ICS that may regulate basal tone. Other muscles, such as the anococcygeus (Gillespie *et al.*, 1989) and gastric antrum (Ozaki *et al.*, 1992), also appear to be influenced by the tonic release of NO. In the present study we observed an electrophysiological correlate of the increase in basal tone in response to arginine analogues and oxyhaemoglobin. L-NAME and oxyhaemoglobin caused depolarization of resting potential, and this effect may explain the rise in tone in response to these agents. These findings suggest that constant release of NO maintains membrane potential at a more negative level than would occur in the absence of NO. The source of the tonic release of NO is unknown at present but it is possible that NO could leak from nerves, endothelial cells lining blood vessels, or other cell types that express NO synthase (cf. Moncada *et al.*, 1991). The fact that TTX raises basal mechanical tone in ileocolonic muscles (Boeckxstaens, 1991) suggests that a portion of the NO may come from spontaneous neural activity. We also found that M&B 22948 caused a significant hyperpolarization of the membrane potential. If tonic release of NO stimulates production of cyclic GMP and cyclic GMP serves as the second messenger to mediate the hyperpolarization response, then inhibiting metabolism of cyclic GMP should increase the influence of NO on membrane potential and cause hyperpolarization.

In summary, experiments on the canine ICS have suggested that NO is the inhibitory transmitter that mediates the NANC relaxation. In this, and most other GI smooth muscles, relaxation is preceded by a hyperpolarization response known as an i.j.p. We have shown that i.j.ps in the canine ICS are likely to be mediated by NO. NO is presumably coupled to hyperpolarization via the enhanced production of cyclic GMP.

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# Enteral absorption of octreotide

[1]G. Fricker, *J. Drewe, J. Vonderscher, [2]T. Kissel & *C. Beglinger

Drug Delivery Systems, Sandoz Pharma Ltd., CH-4002 Basel, Switzerland, and *Depts of Anaesthesia, Int. Medicine and Research University Clinic, University of Basel, CH-4031 Basel, Switzerland

1 The somatostatin octapeptide-analogue, octreotide, is absorbed as intact peptide from the gastro-intestinal (GI) tract.

2 *In situ* absorption experiments in rats confirmed our recent intubation studies in human volunteers demonstrating that the peptide has preferential absorption sites in the small intestine. Absorption of octreotide was higher in the jejunum than in the duodenum or the ileum.

3 Experiments with bile-duct cannulated rats demonstrated that the absorption of octreotide decreased in the presence of bile, reflecting a negative influence of biliary components on the absorption of the peptide.

4 Uptake experiments using rat jejunal brush border membranes were performed to analyse the absorption mechanisms. The transport of octreotide into jejunal brush border membranes was significantly higher than the uptake into membrane vesicles isolated from rat ileum. When initial uptake (0–15 s) rates into the membrane vesicles were calculated as a function of the peptide concentration, a saturable component could be observed, indicative of transport mechanisms different from simple diffusion.

**Keywords:** Octreotide; intestinal; membrane transport; brush border membrane

## Introduction

The clinical benefit of therapeutically active peptides is often limited by their short biological half-lives, necessitating continuous intravenous infusions, or by their rapid degradation in the gastro-intestinal (GI) tract excluding oral administration. The synthetic somatostatin octapeptide-analogue octreotide (Sandostatin) partially overcomes both problems, since its structure has been stabilized against enzymatic degradation (Bauer *et al.*, 1982). Octreotide suppresses the release of growth hormone, prolactin and various hormones of the gastro-entero-pancreatic endocrine system, thereby regulating intestinal and pancreatic secretory processes. It is used clinically in the therapy of acromegaly and in the symptomatic treatment of carcinoid syndrome or endocrine tumours of the GI tract (Del Pozo, 1988; Battershill & Clissold, 1989). Recent studies in healthy volunteers have demonstrated that octreotide effectively suppresses plasma insulin levels after oral administration, indicating that functionally active peptide is absorbed (Williams *et al.*, 1986; Fuessl *et al.*, 1987), despite its rather low systemic bioavailability of about 0.3% (Köhler *et al.*, 1987). It is not known, whether this low bioavailability is due to restricted absorption sites in the intestinal tract, where the drug passes by, or due to an interaction with intestinal content such as biliary or pancreatic fluids. Recent experiments with rat jejunal brush border membranes indicated a high metabolic stability of the peptide in the small intestine of rats (Fricker *et al.*, 1991).

The goals of the present study were to localize the site of octreotide absorption in the intestinal tract, to estimate the absorbed fraction of intact peptide by studies in rats, and to investigate, whether the absorption of the peptide is influenced by local factors such as bile, pancreatic juice or the presence of specific transport systems in the enterocyte plasma membrane.

## Methods

### In situ *absorption studies in rats*

All animal studies were approved by the Committee of the Swiss Cantonal Agency for Animal Protection. Male Wistar rats, weighing approximately 300 g, (BRL, Fühlinsdorf, CH) were kept without food, but with free access to water for one day before the experiment. The animals were anaesthetized by i.p. injection of urethane (1 g $kg^{-1}$). The peritoneum was opened by a midline incision and 5 cm segments of the desired intestinal area were ligated in order to prevent transit of the administered peptide down the gut maintaining normal blood supply. The beginning of the duodenum was localised 0.5 cm distal to the pylorus, that of the jejunum 10 cm distal to the ligamentum duodenocolicum, and the ileal segment was between 2 and 7 cm proximal to the ileocecal valve. Octreotide, 50 $\mu$g, dissolved in 0.5 ml 0.9% saline was injected into the intestinal segment under investigation. Blood samples were taken by puncture of the vena cava 10 min before and 20, 60 and 120 min after drug administration and immediately centrifuged at 10,000 *g* for 5 min at 4°C. The plasma was kept frozen until the concentration of octreotide was determined by a radioimmunoassay (Bauer *et al.*, 1982). The rabbit antiserum was found to recognise only intact peptide with a very low cross-reactivity to peptide fragments, somatostatin-14 or somatostatin-28. The area under the plasma curve (AUC) was estimated by the trapezoidal rule. The absorption efficiency was calculated following the equation: $[(AUC_{intra\text{-}intestinal} \times dose_{i.v.})/(AUC_{i.v.} \times dose_{intra\text{-}intestinal})] \times 100 = \%$ [absorption efficiency].

When bile-duct cannulated rats were used, the common bile duct of each animal was cannulated one day prior to the kinetic experiment under i.p. pentobarbitone (10 mg $kg^{-1}$) anaesthesia. After cannulation, the rats were kept under fasting conditions until the kinetic experiment. Sham-operated rats were treated like the bile-duct cannulated rats except that the bile-duct was not cannulated. All animals were allowed to move freely after the surgery; the biliary cannula was protected by a fixing-tape around the body.

### In vitro *absorption studies*

*In vitro* intestinal absorption of octreotide was studied with brush border membrane vesicles obtained from rat jejunum and ileum. The membrane vesicles were isolated by the divalent cation precipitation method (Hopfer *et al.*, 1973). As indicated by the marker enzyme leucineaminopeptidase, the membrane vesicles were enriched over the starting homogenate 21 ± 5 (s.d.) fold ($n = 20$). In control experiments the uptake of D-glucose and L-alanine revealed a sodium-dependent overshoot (Murer & Hopfer, 1974; Satoh *et al.*,

[1] Author for correspondence.
[2] Present address: Department of Pharmaceutics, University of Marburg, D-3500 Marburg, Germany.

1989), indicating that the membranes had maintained their functional integrity. The absorption of radiolabelled peptide started after adding 20 μl of membrane vesicles in suspension medium (300 mM mannitol, 20 mM HEPES-Tris, pH 7.5), to 180 μl incubation medium (final concentrations: 100 mM mannitol, 100 mM NaCl or 100 mM KCl, 20 mM HEPES-Tris, pH 7.5, and 20 μM [$^{14}$C]-octreotide). When the membrane potential dependency of the transport was investigated, the membrane vesicles were preloaded with 100 mM KCl and incubated in a medium containing 100 mM tetramethylammoniumchloride instead of NaCl subsequent to treatment with valinomycin (10 μg mg$^{-1}$ protein). The absorption was stopped by adding 2.5 ml of ice-cold suspension medium. Vesicle associated ligand was separated from free ligand by rapid filtration of the suspension through 0.45 μm nitrocellulose filters (Sartorius, Göttingen, FRG) and subsequent washing twice with 2 ml of ice-cold suspension medium. Membrane associated radioactivity was determined by liquid scintillation counting after dissolving the filters and membranes in 5 ml Filtercount (Canberra Packard International SA, Zurich, CH). To determine the uptake of octreotide into an osmotically active space (=intravesicular lumen) the absorption was measured after incubation of the vesicles at varying extra-vesicular osmolarity, adjusted by addition of mannitol. Statistical differences between the vesicle absorption data were calculated by Student's *t* test. Curve analysis was performed with the data analysis programme, Enzfitter (Elsevier Publishers, Amsterdam, NL).

### *Chemicals*

[$^{14}$C]-octreotide with a specific activity of 40.99 Ci mg$^{-1}$ and unlabelled peptide were synthesized in the Preclinical Research Department, Sandoz Pharma Ltd., Basle, Switzerland (Bauer *et al.*, 1982). The radiochemical purity of labelled peptide was checked by high performance liquid chromatography (h.p.l.c.) analysis before the experiments and was found to be greater than 98%. All other chemicals were purchased in reagent grade from commercial sources.

## Results

*In situ* studies with rats were performed to evaluate rate, extent and the site of intestinal absorption of octreotide. Octreotide, 50 μg was administered to either the duodenum, the jejunum or the ileum of the animals. Octreotide plasma levels were determined for up to 2 h after administration. The AUC values were calculated only for this time period, because the plasma concentrations reached a plateau after 2 h. Therefore, extrapolation to infinity was not possible. The determination of octreotide plasma levels showed that the $AUC_{0-2h}$ and the $C_{max}$ were highest after intra-jejunal peptide administration (Table 1). The absorption efficiency$_{(0-2h)}$ in the duodenum was 1.56% compared to 3.16% in the jejunum and 0.19% in the ileum.

To investigate, whether the lower absorption efficiency in the duodenum compared to the jejunum might be caused by the presence of high concentrations of bile, the plasma levels of octreotide in control animals were compared with the plasma levels of the peptide in sham-operated and bile-duct cannulated rats after intestinal peptide administration. Octreotide plasma levels in bile-duct cannulated animals exceeded several fold those in control animals. The maximum plasma concentration of octreotide observed in bile-duct cannulated rats was 8.73 ng ml$^{-1}$ compared to 2.23 ng ml$^{-1}$ in control animals, resulting in an almost 6 fold increased AUC (Table 2).

Additional experiments with brush border membranes of rat intestine were performed, to evaluate, whether the higher absorption in the jejunum can be explained as the result of a selective absorption mechanism. The brush border membrane vesicles were isolated from either the jejunal or the ileal part of rat small intestine. To determine, whether the uptake of octreotide by jejunal brush border membranes is due to transport into the intravesicular lumen or binding to the membrane surface, the effect of increasing incubation medium osmolarity on the uptake at 60 min incubation was investigated. Extrapolation to infinite osmolarity (i.e., no intravesicular lumen) showed ~75% uptake compared to control conditions (300 mosmol), indicating that actual transport under iso-osmotic standard conditions is ~25% of total uptake. The uptake rates of the peptide in the initial phase of absorption (0–15 s) were significantly higher with membrane vesicles isolated from the jejunum as compared to the uptake observed with membrane vesicles prepared from the ileum (Figure 1). In addition, the uptake of octreotide into jejunal brush border membranes was stimulated, when the peptide was administered in the presence of an inwardly directed $Na^+$-gradient ($[Na]_o$: 100 mM; $[Na]_i$: 0 mM, Figure 1)

The influence of the membrane potential was tested by creating electrical valinomycin-induced $K^+$-diffusion potentials. Preloading the vesicles with 100 mM KCl, subsequent treatment with valinomycin and incubation in a tetramethylammoniumchloride (TMA-chloride, 100 mM) containing medium should generate a relatively negative intravesicular potential. The experiments showed a higher uptake of octreotide under these conditions compared to $K^+$-equilibrated conditions or an intra-vesicular positive potential created by preloading the vesicles with $TMA^+$ and subsequent incubation in a $K^+$-containing medium after treatment with valinomycin.

In addition, the absorption of the peptide by jejunal brush border membrane vesicles was measured at different concentrations of octreotide. Plotting of the initial uptake rates versus the peptide concentration (0–15 s) revealed a non-linear functional relationship between the initial absorption rate and the peptide concentration (Figure 2). Subtraction of a linear component, representative for passive diffusion, from the total uptake rate suggested an additional saturable absorption

**Table 2** Effect of bile on intestinal octreotide absorption in rats

| *Animal model* | $AUC_{0-2h}$ (ng h$^{-1}$ ml$^{-1}$) | $C_{max}$ (ng ml$^{-1}$) | $t_{max}$ (h) |
|---|---|---|---|
| Control rats | 2.26 ± 1.13 | 2.23 ± 1.34 | 1.2 ± 0.6 |
| Sham operated | 5.10 ± 6.17 | 4.14 ± 3.68 | 0.5 ± 0.2 |
| Bile-duct cannulated | 12.94 ± 4.66 | 8.73 ± 3.06 | 1.2 ± 0.5 |

In different rat models after administration of 50 μg octreotide ( mean values ± s.d.; $n = 6$).

**Table 1** Pharmacokinetic parameters of octreotide after administration at different sites of rat small intestine

| *Administration site* | $AUC_{0-2h}$ (ng h$^{-1}$ ml$^{-1}$) | $C_{max}$ (ng ml$^{-1}$) | $t_{max}$ (h) | *Absorption efficiency* (%) |
|---|---|---|---|---|
| Duodenum | 4.39 ± 2.05 | 3.20 ± 1.54 | 0.5 ± 0.1 | 1.56 |
| Proximal jejunum | 8.89 ± 2.63 | 7.10 ± 1.75 | 0.4 ± 0.1 | 3.16 |
| Ileum | 0.52 ± 0.09 | 2.37 ± 1.17 | 0.7 ± 0.3 | 0.19 |
| i.v. reference | 14.04 ± 1.16 | 23.27 ± 1.31 | 0.1 ± 0.0 | 100 |

In anaesthetized rats after administration of 50 μg octreotide in 0.5 ml saline at different sites in the small intestine; reference animals received an i.v. dose of 2.5 μg octreotide (mean values ± s.d.; n = 6).

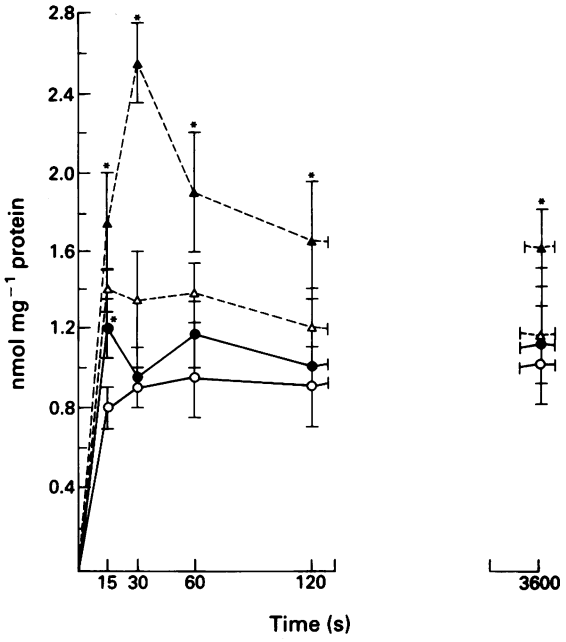


**Figure 1** Uptake of octreotide by intestinal brush border membrane vesicles of rat. The vesicles were resuspended in 300 mM mannitol, 20 mM HEPES-Tris, pH 7.5 and incubated in a medium containing 100 mM mannitol, 100 mM NaCl (▲, ●) or 100 mM KCL (△, ○) 20 mM HEPES-Tris, pH 7.5 and 20 $\mu$M [$^{14}$C]-octreotide. Each point represents the mean of 4 determinations from 3 different membrane preparations (▲, △; jejunal membrane vesicles; ●, ○: ileal membrane vesicles); vertical bars show s.e. mean.
* Significantly different from controls (KCl-incubation, $P < 0.05$).

component with an apparent $K_M$ of 45 $\mu$M and a $V_{max}$ of 1.8 nmol min$^{-1}$ mg$^{-1}$ protein. This occurrence of a saturable transport component is an indication of a direct interaction of the peptide with membrane constituents.

## Discussion

The prevalent methods of administration for peptide drugs are still the subcutaneous or intravenous route. Oral administration indisputably offers therapeutic advantages, but its use is controversial, mainly because of the known enzymatic lability of peptides. Our interest was focused on the intestinal absorption of octreotide, which is stabilised against proteolytic degradation in GI fluids (Pless *et al.*, 1986).

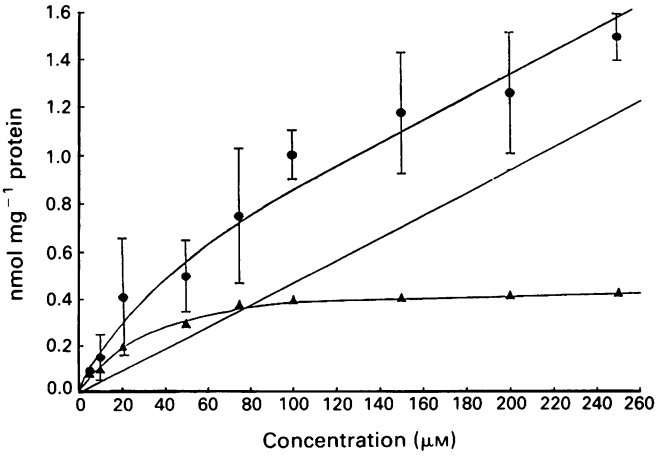


**Figure 2** Concentration-dependent uptake of octreotide by jejunal brush border membrane vesicles of rat. Uptake of various concentrations of [$^{14}$C]-octreotide was measured at 25°C for 15 s by incubating the vesicles in 100 mM mannitol, 100 mM NaCl, 20 mM HEPES/Tris, pH 7.5, and octreotide. Each point represents the mean of 20 determinations from 5 separate membrane preparations: vertical bars show s.e. mean. (●) = total uptake; (▲) = calculated saturable component.

The results of these investigations can be summarised as follows:

(1) Octreotide showed marked differences in the extent and rates of absorption at the various sites of the intestine. Highest plasma levels were achieved after peptide administration into the jejunum.

(2) The absorption of octreotide was increased in bile-duct cannulated rats and was diminished in the presence of high concentrations of biliary or pancreatic juice, in rats and probably in man.

(3) The uptake of octreotide by rat brush border membrane vesicles was higher in jejunal than in ileal membrane vesicles. It exhibited a saturable component indicative of a specific membrane-peptide interaction.

The studies imply in accordance with recent investigations (Fuessl *et al.*, 1987; Köhler *et al.*, 1987; Fricker *et al.*, 1991) that octreotide is absorbed from the GI tract as an intact peptide. Comparison with our previous data obtained by intubation of human volunteers (Köhler *et al.*, 1987) demonstrates that both in rats and man a single jejunal dose of $\sim$110 $\mu$g kg$^{-1}$ resulted in similar plasma concentrations, which were above 0.3 ng ml$^{-1}$ up to 3 h. Previous studies have described plasma concentrations of intact octreotide above 0.3 ng ml$^{-1}$ to be efficient in suppressing growth hormone secretion (Lamberts *et al.*, 1985) or in suppressing plasma insulin levels (Fuessl *et al.*, 1987) or prolactin secretion (Fricker *et al.*, 1991).

The different plasma levels of octreotide after administration in various parts of the small intestine of rats point to the existence of absorption sites in the jejunum, recognising octreotide. The present data confirm the recent findings of site-dependent absorption of octreotide in healthy volunteers (Köhler *et al.*, 1987), indicating the action of a selective absorption mechanism in the jejunum. Support for this hypothesis was provided by the finding of a saturable, curvilinear uptake, when the peptide absorption was measured in rat jejunal brush border membranes. The functional properties of the human intestine cannot be unequivocally deduced from the results obtained with rat intestinal brush border membranes, but peptide absorption was significantly higher in jejunal than in ileal membrane vesicles, which parallels the results in the whole animal and the intubation study with man.

It is known that the small intestine is able to absorb biologically significant quantities of intact oligopeptides (Amoss *et al.*, 1972; Lundin & Vilhardt, 1986; Takaori *et al.*, 1986) but none of the studies could demonstrate conclusively a specific membrane-peptide interaction or the presence of an active transport system for oligopeptides with more than four amino acids. It cannot be excluded, that such oligopeptides may also be recognised by one of the physiological peptide carriers, which have been described for dipeptides, tri- or tetrapeptides. For those peptides pH-dependent absorption systems are discussed (Gardner, 1984; Humphrey & Ringrose, 1986; Tsuji *et al.*, 1987; Wilson *et al.*, 1989), whereas our experiments suggest a $Na^+$-dependent or potential-dependent mechanism for the uptake of octreotide. The observed $Na^+$-dependency may be an indirect effect via the $Na^+/H^+$-exchange system by producing a proton gradient as previously discussed for dipeptides (Ganapathy *et al.*, 1984). However, variation of intra- or extravesicular pH in the absence of $Na^+$ had only minor effects upon the uptake rates of octreotide. The potential sensitivity indicates, that octreotide transport is associated with the translocation of a net positive charge (e.g. $Na^+$/octreotide or positively charged octreotide alone).

It has also to be mentioned, that octreotide exhibits a complex modulation of the electric surface properties of lipid membranes by changing the lipid headgroup conformation, as demonstrated by fluorescence spectroscopy and nuclear magnetic resonance studies (Beschiaschvili & Seelig, 1991). Therefore, the evaluation of the exact uptake mechanism, the

identification of potential binding sites, as well as the interaction of octreotide with charged cell surfaces is the subject of current investigations.

The finding of the low plasma levels of octreotide after intra-intestinal administration does not contradict the membrane permeability rates found in the membrane vesicle experiments. It can partially be explained by the presence of high local concentrations of bile and/or pancreatic juice in the intestinal lumen. In addition, the positive net charge of octreotide might contribute to a high binding of the peptide to mucus constituents in the intestinal lumen, thereby decreasing the bioavailability of the peptide after oral administration. Recent studies using a fluorescent analogue of octreotide (Fricker *et al.*, 1991) showed a relatively high concentration of this analogue in the enterocytes of rat jejunum. This supports evidence that the further transport through the cells and the basolateral plasma membrane might include rate limiting steps and therefore also contribute significantly to the low bioavailability of these peptides.

Considered together, our findings demonstrate that octreotide can be enterally absorbed as an intact peptide, preferentially in the jejunal part of the small intestine. Presumably, other peptide drugs may be susceptible to intestinal absorption as well, once they have been stabilised against proteolytic degradation.

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# Characterization of a novel aquaretic agent, OPC-31260, as an orally effective, nonpeptide vasopressin $V_2$ receptor antagonist

[1]Yoshitaka Yamamura, Hidenori Ogawa, Hiroshi Yamashita, Tomihiko Chihara, Hisashi Miyamoto, Shigeki Nakamura, Toshiyuki Onogawa, Tatsuya Yamashita, Tetsumi Hosokawa, Toyoki Mori, Michiaki Tominaga & Youichi Yabuuchi

Second Tokushima Institute of New Drug Research, Otsuka Pharmaceutical Co., 463-10, Kagasuno, Kawauchi-cho, Tokushima 771-01, Japan

**1** OPC-31260, a benzazepine derivative, has been studied for its ability to antagonize the binding of arginine vasopressin (AVP) to receptors in rat liver ($V_1$) and kidney ($V_2$) plasma membranes, for antagonism of the antidiuretic action of AVP in alcohol-anaesthetized rats and for diuretic action in conscious normal rats.

**2** OPC-31260 caused a competitive displacement of [$^3$H]-AVP binding to both $V_1$ and $V_2$ receptors with $IC_{50}$ values of $1.2 \pm 0.2 \times 10^{-6}$ M and $1.4 \pm 0.2 \times 10^{-8}$ M, respectively.

**3** OPC-31260 at doses of 10 to 100 $\mu g\,kg^{-1}$, i.v., inhibited the antidiuretic action of exogenously administered AVP in water-loaded, alcohol-anaesthetized rats in a dose-dependent manner. OPC-31260 did not exert an antidiuretic activity suggesting that it is not a partial $V_2$ receptor agonist.

**4** After oral administration at doses of 1 to 30 $mg\,kg^{-1}$ in normal conscious rats, OPC-31260 dose-dependently increased urine flow and decreased urine osmolality. The diuretic action of OPC-31260 was characterized as aquaresis, the mode of diuretic action being different from previously known diuretic agents such as furosemide, hydrochlorothiazide and spironolactone.

**5** The results indicate that OPC-31260 is a selective $V_2$ receptor antagonist and behaves as an aquaretic agent. OPC-31260 will be a useful tool in studying the physiological role of AVP and in the treatment of various conditions characterized by water retention.

**Keywords:** OPC-31260; vasopressin; nonpeptide antagonist; aquaresis; $V_2$ receptor

## Introduction

Arginine vasopressin (AVP) may contribute to cardiovascular regulation by causing vasoconstriction and stimulating renal water absorption through $V_1$ and $V_2$ receptors, respectively. This classification of AVP receptor subtypes was originally proposed by Michell *et al.* (1979) and was based on intracellular mechanisms: adenosine 3′: 5′-cyclic monophosphate (cyclic AMP)-independent ($V_1$) and cyclic AMP-dependent ($V_2$) pathways. AVP antagonists, are of potential importance in the treatment of water-retaining disorders. Although several potent peptide vasopressin antagonists are currently available (for reviews see Kinter *et al.*, 1988; Manning & Sawyer, 1989; Laszlo *et al.*, 1991), the therapeutic usefulness of such compounds is limited because of their low oral bioavailability and partial agonist activity (Mah & Hofbauer, 1988; Brooks *et al.*, 1988; Albrightson-Winslow *et al.*, 1989). Therefore, none has emerged as a clinically useful antidiuretic antagonist.

Recently, we reported on an orally effective, nonpeptide $V_1$ receptor antagonist, OPC-21268, that specifically antagonized responses to AVP *in vitro* and *in vivo* (Yamamura *et al.*, 1991). Subsequently, via a series of structural conversions of OPC-21268, the selective $V_2$ receptor antagonist OPC-31260 was discovered. This paper describes the ability of OPC-31260 to inhibit AVP receptor binding in rat liver and kidney plasma membranes and to antagonize exogenous AVP in water-loaded, alcohol-anaesthetized rats. Furthermore, we examined the aquaretic effects of orally administered OPC-31260 in normal conscious rats. The chemical structure of OPC-31260, [5-dimethylamino-1-{4-(2-methylbenzoylamino)benzoyl}-2,3,4,5-tetrahydro-1*H*-benzazepine], is shown in Figure 1.

[1] Author for correspondence.

## Methods

### In vitro *arginine vasopressin binding assay in rat liver and kidney plasma membranes*

Liver and kidney plasma membranes were prepared from Sprague-Dawley rats (300–400 g) (Charles River, Japan). Details of the methods used have been published elsewhere (Nakahara *et al.*, 1978; Nakamura *et al.*, 1983). Briefly, liver or kidney was first homogenized in a Potter-Elvehjem smooth glass homogenizer in a medium containing 250 mM sucrose, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5. The homogenate was centrifuged at 1500 *g* for 15 min and the pellet was rehomogenized in a loose Dounce homogenizer. Liver plasma membrane fraction was obtained from the 1500 *g* pellet with an iso-osmotic Percoll method after 10,000 *g* centrifugation for 1 h. Kidney plasma membrane was obtained with a PEG-dextran double phase system as interfacial membrane fraction after 10,000 *g* centrifugation for 10 min. The membranes were washed 3 times with Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl. Protein was measured by the method of Lowry *et al.* (1951).

Various concentrations of AVP and OPC-31260 were incubated with 100 $\mu g$ (protein equivalent) of liver membranes or 300 $\mu g$ of kidney membranes in 0.25 ml of 100 mM Tris-HCl buffer, pH 8.0 containing 5 mM $MgCl_2$, 1 mM EDTA, 0.1% bovine serum albumin, and 1.9 nM [$^3$H]-AVP (53.6 Ci $mmol^{-1}$, New England Nuclear) in the liver or 3.8 nM

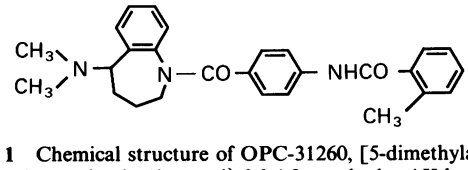


**Figure 1** Chemical structure of OPC-31260, [5-dimethylamino-1-{4-(2-methylbenzoylamino)benzoyl}-2,3,4,5-tetrahydro-1*H*-benzazepine].

[$^3$H]-AVP in the kidney. After incubation for 10 min at 37°C (liver membranes) or 4 h at 4°C (kidney membranes), 3 ml of ice cold buffer was added to each assay tube, and bound and free ligands were separated by filtration through a glass microfibre filter (Whatman, GF/B) and then washed three times. The radioactivity trapped on the filters was counted in a liquid scintillation counter. Specific binding was determined by subtraction of the nonspecific binding which was measured in the presence of 1 $\mu$M unlabelled AVP.

To determine binding kinetic constants, $K_d$ and $B_{max}$, liver or kidney plasma membranes were incubated with increasing concentrations of [$^3$H]-AVP with or without excess ($10^{-6}$ M) unlabelled AVP to obtain a saturation curve. To investigate whether OPC-31260 interacts competitively or noncompetitively, the saturation binding of [$^3$H]-AVP was examined in the absence and presence of OPC-31260 at concentrations of $3 \times 10^{-7}$ M and $1 \times 10^{-6}$ M in liver membranes and $3 \times 10^{-9}$ M, and $1 \times 10^{-8}$ M in kidney membranes. Data on the saturation curve were plotted according to the method of Scatchard and fitted by a regression analysis (Scatchard, 1979).

### In vivo *antagonism to exogenous arginine vasopressin in water-loaded, alcohol-anaesthetized rats*

The bioassay method for antidiuretic activity was used (for details see Bisset & Chowdrey, 1984). This method was originally developed by Dicker (1953) and subsequently modified by Bisset & Lewis (1962), and Clark & Rocha e Silva (1967). Male Sprague-Dawley rats (300–350 g) (Charles River Labs), deprived of food for 18 h but allowed free access to water, were given water (5% body weight) by stomach tube, followed 1 h later by 13% ethanol (5% body weight). After full anaesthesia, the trachea was cannulated for ventilation and then the femoral artery, vein and bladder were cannulated for measurement of arterial pressure, for i.v. injections, and for urine collections, respectively. The rat was placed on a set of scales, balanced by a counterweight and received a constant infusion of 3% ethanol (v/v), 1.67% glucose (w/v) and 0.3% NaCl (w/v) into its stomach, controlled automatically by a servo-controlled pump. Urine flow was measured by a photo-transistor connected with a staircase integrator having a 1 min time base, and urine was collected for 5 min. Urine osmolality was determined by freezing point depression with a Fiske osmometer. After constant urine flow was established, AVP (50 $\mu$u kg$^{-1}$) and OPC-31260 (10, 30, 100 $\mu$g kg$^{-1}$) were given intravenously.

### *Evaluation of antidiuretic agonist activities of OPC-31260 and d(CH$_2$)$_5$Tyr(Et)VAVP in Brattleboro rats*

Female Brattleboro rats homozygous for hypothalamic Diabetes Insipidus and weighing between 180 and 280 g were used. OPC-31260 (30 mg kg$^{-1}$) and vehicle (5% gum arabic) were administered orally in a volume of 2 ml kg$^{-1}$ and d(CH$_2$)$_5$Tyr(Et)VAVP (10 $\mu$g kg$^{-1}$) was administered subcutaneously in a volume of 1 ml kg$^{-1}$. Spontaneously voided urine was collected for 6 h with metabolic cages. Both before and during the study, the rats received water and food *ad libitum*.

### *Aquaretic effects of oral administered OPC-31260 in normal conscious rats*

Hydrated conscious rats (weighing 300–350 g) were orally dosed with vehicle (5% gum arabic, 2 ml kg$^{-1}$) or OPC-31260 at doses of 1 to 30 mg kg$^{-1}$ and were then placed individually in metabolic cages. Both before and during the study, the rats received water and food *ad libitum*. Spontaneously voided urine was collected every 2 h for an 8 h period.

### *Comparison of OPC-31260 and other diuretic agents in normal conscious rats*

OPC-31260 (1 to 30 mg kg$^{-1}$), furosemide (10 to 300 mg kg$^{-1}$), hydrochlorothiazide (1 to 30 mg kg$^{-1}$) and spironolactone (50 to 150 mg kg$^{-1}$) were administered orally in normally hydrated conscious rats ($n = 4$). Urine was collected for 4 h with metabolic cages. Urinary sodium was determined by a flame photometer (Hitachi 750, Japan).

### *Statistics*

The results are presented as means ± s.e.mean. One way analysis of variance (ANOVA) followed by Dunnett's test (Dunnett, 1955) was used. $P < 0.05$ was taken to be significant.

### *Drugs*

In vitro *study:* AVP (Peptide Institute Inc., Japan) was dissolved in 0.1 M Tris-HCl buffer, pH 8.0 containing 5 mM $MgCl_2$, 1 mM EDTA, 0.1% bovine serum albumin (Sigma Chemical Co.) and OPC-31260 was dissolved in dimethylsulphoxide (DMSO) (Wako Pure Chemicals, Japan) at a concentration of $10^{-3}$ M and diluted with assay buffer. The final concentration of DMSO in the binding assay was 2% and had no effect on [$^3$H]-AVP binding.

In vivo *study:* AVP and d(CH$_2$)$_5$Tyr(Et)VAVP (Sigma Chemical Co.) were dissolved in isotonic saline and OPC-31260 dissolved in dimethylformamide (DMF) (Wako). Bolus injections were given in 30 $\mu$l and then flushed in with 0.2 ml isotonic saline. Injection of vehicle (DMF) alone in these volumes had no consistent cardiovascular effects. For oral administration, furosemide, hydrochlorothiazide, spironolactone (Sigma Chemical Co.) and OPC-31260 were suspended with 5% gum arabic in water and administered in a volume of 2 ml kg$^{-1}$.

## Results

### *Arginine vasopressin binding in rat liver and kidney plasma membranes*

Both AVP and OPC-31260 displaced [$^3$H]-AVP binding to rat liver ($V_1$ receptor) and kidney ($V_2$ receptor) plasma membranes (Figure 2). The $IC_{50}$ value of OPC-31260 was $1.4 \pm 0.2 \times 10^{-8}$ M for $V_2$ receptors and $1.2 \pm 0.2 \times 10^{-6}$ M for $V_1$ receptors ($n = 3$); the corresponding $IC_{50}$ values for AVP were $4.0 \pm 0.4 \times 10^{-9}$ M at $V_2$ and $2.3 \pm 0.5 \times 10^{-9}$ M at $V_1$ receptors. OPC-31260 is therefore two orders of magnitude more selective for $V_2$ receptors than for $V_1$ receptors.

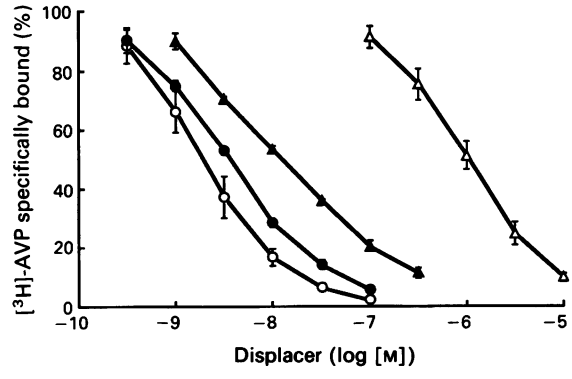


**Figure 2** Displacement curves of [$^3$H]-arginine vasopressin ([$^3$H]-AVP) by unlabelled AVP (○, ●) and OPC-31260 (△, ▲) in rat liver (open symbols) and kidney (closed symbols) plasma membranes. Values represent means with s.e.mean shown by vertical lines of 3 or 4 separate determinations performed in duplicate.

**Table 1** Effects of OPC-31260 on number and apparent affinity of [$^3$H]-arginine vasopressin ([$^3$H]-AVP) binding sites in rat liver and kidney plasma membranes

| *Rat liver membranes* ($V_1$) | | | *Rat kidney membranes* ($V_2$) | | |
|---|---|---|---|---|---|
| *Concentration of OPC-31260* | $B_{max}$ (pmol mg$^{-1}$) | $K_d$ (nM$^{-1}$) | *Concentration of OPC-31260* | $B_{max}$ (pmol mg$^{-1}$) | $K_d$ (nM$^{-1}$) |
| None | 1.96 ± 0.30 | 1.10 ± 0.14 | None | 0.40 ± 0.07 | 1.38 ± 0.08 |
| $3 \times 10^{-7}$ M | 2.23 ± 0.38 | 2.47 ± 0.26* | $3 \times 10^{-9}$ M | 0.41 ± 0.08 | 2.40 ± 0.26* |
| $1 \times 10^{-6}$ M | 2.42 ± 0.39 | 5.51 ± 0.51** | $1 \times 10^{-8}$ M | 0.38 ± 0.06 | 4.03 ± 0.45** |

Values are means ± s.e.mean, $n = 4$–5; * $P < 0.05$, ** $P < 0.01$ versus control (absence of OPC-31260, none). The $B_{max}$ values of both receptors are not significantly different at each concentration of OPC-31260.

Scatchard plots of [$^3$H]-AVP saturation binding in the absence and presence of OPC-31260 were determined. $K_d$ and $B_{max}$ values for [$^3$H]-AVP in rat liver were $1.10 \pm 0.14 \times 10^{-9}$ M and $1.96 \pm 0.30 \times 10^{-12}$ mol mg$^{-1}$ protein respectively; in rat kidney the corresponding values were $1.38 \pm 0.08 \times 10^{-9}$ M, $0.40 \pm 0.03 \times 10^{-12}$ mol mg$^{-1}$ protein. The $K_d$ of [$^3$H]-AVP was reduced significantly in both rat liver and kidney in the presence of OPC-31260 although $B_{max}$ values did not change in either tissue (Table 1). The results indicate that OPC-31260 inhibits [$^3$H]-AVP binding to both $V_1$ and $V_2$ receptors in a competitive manner.

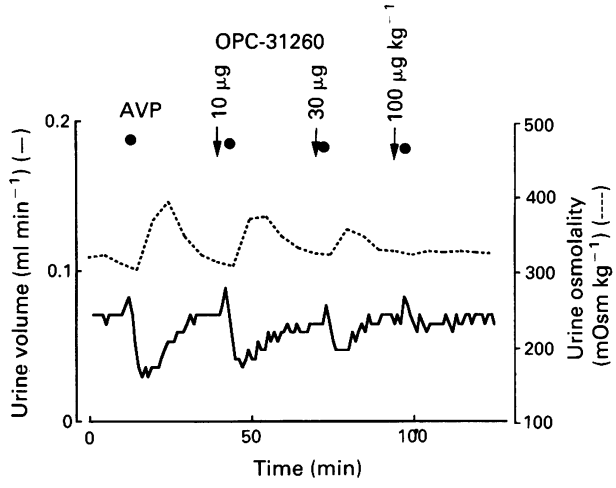


**Figure 3** *In vivo* antagonism of exogenous arginine vasopressin (AVP) by OPC-31260 in water-loaded, alcohol-anaesthetized, rats. AVP (50 μu kg$^{-1}$, ●) and OPC-31260 (arrows) were administered intravenously. Typical traces of three experiments are shown.

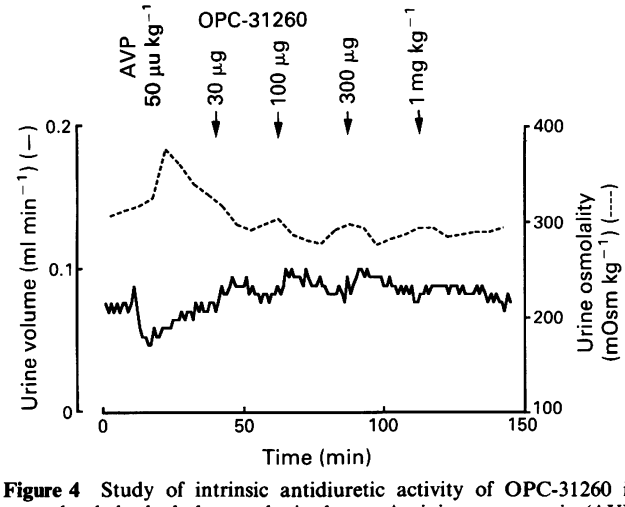


**Figure 4** Study of intrinsic antidiuretic activity of OPC-31260 in water-loaded, alcohol-anaesthetized rats. Arginine vasopressin (AVP, 50 μu kg$^{-1}$, ●) and OPC-31260 (arrows) were administered intravenously. Typical traces of three experiments are shown.

### *Antagonism of exogenous arginine vasopressin in water-loaded, alcohol-anaesthetized rats*

Bolus injections of AVP (50 μu kg$^{-1}$) in water-loaded, alcohol-anaesthetized rats, induced a reproducible decrease in urine flow and increase in urine osmolality. After intravenous administration of OPC-31260 at doses of 10 to 100 μg kg$^{-1}$, the antidiuretic action of exogenous AVP was inhibited in a dose-dependent manner (Figure 3).

OPC-31260 (0.03 to 1 mg kg$^{-1}$, i.v.) had no effect on urine flow and urine osmolality (Figure 4). d(CH$_2$)$_5$Tyr(Et)VAVP, which inhibits the antidiuretic action of AVP in normal rats, was observed also to have an antidiuretic effect in Brattleboro rats, i.e. it significantly decreased urine volume and water intake, and increased urine osmolality (Figure 5). However, OPC-31260 did not decrease urine volume and water intake, and did not increase urine osmolality (Figure 5).

### *Aquaretic effects of orally administered OPC-31260 on hydrated conscious rats*

OPC-31260 was administered orally at doses of 1 to 30 mg kg$^{-1}$ in hydrated conscious rats ($n = 4$) and spontaneously voided urine was collected every 2 h for an 8 h period (Figure 6). OPC-31260 caused a dose-dependent increase in urine volume and a drop in urine osmolality. At a dose of 30 mg kg$^{-1}$, the aquaretic effect of OPC-31260 was significant for up to 8 h.

### *Comparison of urine volume and urinary sodium excretion with OPC-31260 and other diuretics*

We administered OPC-31260 (1 to 30 mg kg$^{-1}$), hydrochlorothiazide (1 to 30 mg kg$^{-1}$), furosemide (10 to 300 mg kg$^{-1}$) and spironolactone (50 to 150 mg kg$^{-1}$) in normally hydrated con-

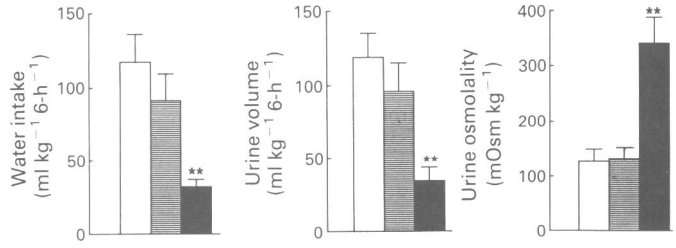


**Figure 5** Water intake, urine volume and urine osmolality in Brattleboro rats over a 6 h collection period after oral administrations of OPC-31260 (30 mg kg$^{-1}$, $n = 7$, hatched column) and vehicle (5% gum arabic, $n = 6$, open column) or subcutaneous administration of d(CH$_2$)$_5$Tyr(Et)VAVP (10 μg kg$^{-1}$, $n = 6$, closed column). Values are means with s.e.mean shown by vertical lines. ** $P < 0.01$, d(CH$_2$)$_5$Tyr(Et)VAVP-treated groups vs. the vehicle-treated groups. The values of OPC-31260-treated groups are not significantly different from vehicle-treated groups (by Dunnett).

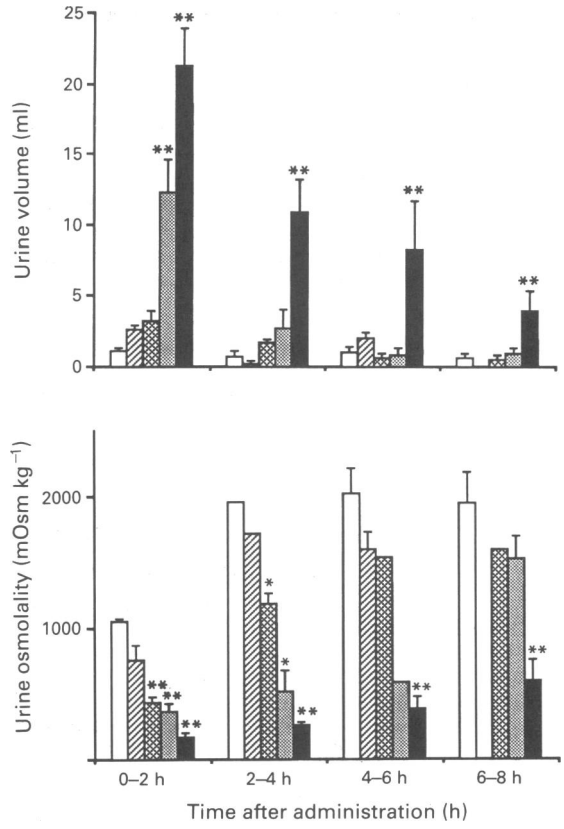


**Figure 6** Antagonism by OPC-31260 to endogenous arginine vasopressin (AVP) in hydrated conscious rats. OPC-31260 at 1 (hatched), 3 (cross-hatched), 10 (stippled) and 30 (closed) mg kg$^{-1}$ and vehicle (5% gum arabic, open column) were administered orally. Values are means with s.e.mean shown by vertical lines. $n = 4$. * $P < 0.05$; ** $P < 0.01$, OPC-31260-treated groups vs. the vehicle-treated group. Statistical analysis of urine osmolality was performed using calculated values from the urine volume and urine osmolality relationship obtained in this experiment when spontaneously voided urine was zero.

scious rats ($n = 4$). All tested compounds increased urine volume and urinary sodium excretion and decreased urine osmolality (Table 2). With OPC-31260 as compared to furosemide, urinary sodium excretion was rather low. Furthermore, the urine osmolality level dropped below the plasma osmolality level; this did not occur with other diuretics. The diuretic effects of traditional diuretic agents were closely associated with urinary sodium excretion, but OPC-31260 selectively increased the excretion of water rather than that of sodium.

## Discussion

Our results demonstrate that OPC-31260 inhibited AVP binding to $V_1$ and $V_2$ receptors in a competitive manner. $IC_{50}$ values calculated from displacement curves of OPC-31260 showed that OPC-31260 is about 100 times more selective for $V_2$ receptors than for $V_1$ receptors. Although peptide $V_2$ antagonists have been developed by Manning & Sawyer (1989), these antagonists also inhibit AVP binding to $V_1$ receptors at the same concentrations.

Like smooth muscle cells, mesangial cells in kidney possess $V_1$ receptors (Bonventre *et al.*, 1986) and recently, using autoradiography, Gertsberger & Fahrenholz (1989) showed $V_1$ binding sites in the kidney. Thus, the kidney plasma membranes used in this study might have also contained $V_1$ receptors. However, Scatchard analysis of [$^3$H]-AVP saturation binding to the kidney membranes exhibited a straight line (regression coefficient, $0.955 \pm 0.012$, $n = 5$), consistent with the existence of a single class of [$^3$H]-AVP binding sites in the kidney membranes. Indeed, the cyclic AMP generation induced by AVP which is a consequence of $V_2$ receptor stimulation, was also inhibited by OPC-31260 at the same doses (unpublished data). Furthermore, OPC-21268, which we recently reported to be a highly selective $V_1$ receptor antagonist (Yamamura *et al.*, 1991), inhibited AVP binding to kidney membranes only at concentrations over $10^{-4}$ M. These results indicate that the number of $V_1$ receptors present in the kidney preparation, if any, is very small and that therefore the AVP binding was to $V_2$ receptors.

Recently, it was reported that, the $V_2$ receptor antagonist SK&F 101926 is devoid of diuretic activity and is a full antidiuretic in human volunteers (Dubb *et al.*, 1987). One reason for these results is that SK&F 101926 may possess partial antidiuretic agonist activity. Indeed, other $V_2$ receptor antagonists such as d(CH$_2$)$_5$Tyr(Et)VAVP have antidiuretic agonist activities in some cases. For example, in water-loaded, alcohol-anaesthetized rats, d(CH$_2$)$_5$Tyr(Et)VAVP produced a transient antidiuretic activity and then inhibited antidiuretic responses to exogenous AVP. This transient antidiuretic action is derived from its intrinsic partial agonist activity.

To study whether OPC-31260 possesses partial agonist activity, we examined the effect of OPC-31260 in water-loaded, alcohol-anaesthetized rats and in Brattleboro rats. OPC-31260 at doses from 0.03 to 1 mg kg$^{-1}$ in water-loaded, alcohol-anaesthetized rats, did not change urine flow or urine osmolality. Furthermore, urine flow, water intake and urine osmolality of OPC-31260-treated Brattleboro rats did not demonstrate significant differences from vehicle-treated rats. Similar results were obtained in indomethacin-treated hydrated dogs (our unpublished data). Thus, these results suggest that OPC-31260 does not possess partial agonist activity.

**Table 2** Comparison of effects of OPC-31260 and standard diuretics on urine volume, osmolality and urinary sodium excretion in normally hydrated conscious rats

| *Compound* | *Dose* (mg kg$^{-1}$) | *Urine volume* (ml 4-h$^{-1}$) | *Urine osmolality* (mOsm kg$^{-1}$) | *Urinary sodium excretion* (mEq 4-h$^{-1}$) |
|---|---|---|---|---|
| Control (5% Arabic gum) | | 1.8 ± 0.5 | 1045 ± 252 | 0.165 ± 0.088 |
| OPC-31260 | 1 | 2.8 ± 0.2 | 755 ± 116 | 0.153 ± 0.038 |
| | 3 | 4.8 ± 0.6 | 426 ± 43** | 0.304 ± 0.018 |
| | 10 | 15.0 ± 3.4** | 300 ± 26** | 0.550 ± 0.073** |
| | 30 | 31.9 ± 5.5** | 173 ± 27** | 0.905 ± 0.075** |
| Furosemide | 3 | 2.8 ± 0.2 | 504 ± 52** | 0.159 ± 0.030 |
| | 10 | 14.1 ± 1.9** | 393 ± 37** | 1.388 ± 0.205** |
| | 30 | 19.4 ± 1.9** | 343 ± 16** | 2.032 ± 0.174** |
| | 100 | 24.2 ± 1.2** | 337 ± 14** | 2.530 ± 0.138** |
| Hydrochlorothiazide | 1 | 4.2 ± 0.6 | 871 ± 59 | 0.534 ± 0.073* |
| | 10 | 7.0 ± 1.1* | 833 ± 105 | 0.922 ± 0.056** |
| | 30 | 7.5 ± 0.8* | 756 ± 66 | 1.092 ± 0.092** |
| Spironolactone | 50 | 2.7 ± 0.5 | 952 ± 136 | 0.339 ± 0.042 |
| | 150 | 6.6 ± 2.3* | 634 ± 107 | 0.554 ± 0.129** |

Values are means ± s.e.mean, $n = 4$ or 5: * $P < 0.05$; ** $P < 0.01$ versus control.

OPC-31260 is effective by oral administration. In normal hydrated conscious rats, oral OPC-31260 administrations at doses of 1 to 30 mg kg$^{-1}$ produced increases in urine volume and decreases in urine osmolality, dose-dependently. This result demonstrated that OPC-31260 is the first active oral aquaretic.

When compared with other traditional diuretic agents, the diuretic effect of OPC-31260 is strikingly different. Traditional diuretics increase sodium excretion concomitant with water excretion. While OPC-31260 selectively increased the excretion of water rather than that of sodium and urine, osmolality fell below the plasma osmolality level at some doses. This result may be therapeutically useful for treatment of disease states associated with excessive vasopressin and/or excessive body water e.g. syndrome of inappropriate excretion of ADH (SIADH), hyponatremia and congestive heart failure.

In conclusion, the present studies confirm that OPC-31260 inhibits AVP binding to $V_1$ and $V_2$ receptors in a competitive manner and that it is about 100 times more selective for $V_2$ receptors. OPC-31260 antagonizes endogenous and exogenous AVP *in vivo*, and its diuretic mode is water diuresis. OPC-31260 differs from peptide-derivative $V_2$ receptor antagonists in that it has no antidiuretic agonist activity and can be absorbed after oral administration. It is expected that OPC-31260 will be a useful probe for studying the physiological and pathophysiological role of AVP and treatment of diseases associated with water imbalanced states.

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# Formoterol and salbutamol inhibit bradykinin- and histamine-induced airway microvascular leakage in guinea-pig

[1]C. Advenier, Y. Qian, J-D. Law Koune, M. Molimard, *M-L. Candenas & E. Naline

Laboratoire de Pharmacologie, Faculté de Médecine Paris-Ouest, 15, rue de l'Ecole de Médecine, F-75270 Paris Cedex 06, France and *Centro de Productos naturales Organicos "Antonio Gonzalez", Instituto Universitario de Bio-Organica, Universidad de La Laguna, Carretera de la Esperanza, 2, 38206 La Laguna, Tenerife, Spain

**1** The effects of the $\beta_2$-adrenoceptor agonists, salbutamol and formoterol, on the increase of microvascular permeability induced by histamine or bradykinin in guinea-pig airways have been studied *in vivo*. Extravasation of intravenously injected Evans blue dye was used as an index of permeability. The effects of salbutamol and formoterol on the increase in pulmonary airway resistance induced by histamine or bradykinin have also been studied.

**2** The increase in pulmonary airway resistance induced by histamine or bradykinin was totally inhibited by salbutamol and formoterol. The $ED_{50}$ of the two mediators were $0.59 \pm 0.21$ ($n = 5$) and $0.20 \pm 0.14$ ($n = 5$) $\mu g\,kg^{-1}$ respectively for salbutamol, and $0.13 \pm 0.12$ ($n = 6$) and $0.02 \pm 0.01$ ($n = 6$) $\mu g\,kg^{-1}$ respectively for formoterol.

**3** Salbutamol (10 and $30\,\mu g\,kg^{-1}$) and formoterol (1 and $10\,\mu g\,kg^{-1}$) inhibited the increase of microvascular permeability induced by histamine ($30\,\mu g\,kg^{-1}$) in the guinea-pig airways. The inhibitory effect was predominant in the trachea and the main bronchi, with a maximum inhibition of 20 to 50%. The two drugs had little or no inhibitory effect on the other structures studied, viz. nasal mucosa, larynx, proximal and distal intrapulmonary airways.

**4** Salbutamol and formoterol (1 and $10\,\mu g\,kg^{-1}$) abolished the increase in microvascular permeability induced by bradykinin ($0.3\,\mu g\,kg^{-1}$). This inhibitory effect of two $\beta$-adrenoceptor stimulants was predominant in the trachea and the nasal mucosa where it was observed with $1\,\mu g\,kg^{-1}$ of the $\beta$-adrenoceptor agonists. In the main bronchi, and in the proximal and distal intrapulmonary airways, the effects of bradykinin were abolished by $10\,\mu g\,kg^{-1}$ of formoterol and salbutamol.

**5** The effects of bradykinin, but not those of histamine, were significantly reduced (nasal mucosa, main bronchi and distal intrapulmonary airways) or abolished (trachea, proximal intrapulmonary airways) by morphine $10\,mg\,kg^{-1}$, i.v. These results suggest that an indirect effect, through non-adrenergic non-cholinergic (NANC) nerves is involved in the action of bradykinin on the microvascular permeability.

**6** In conclusion, intravenously injected $\beta$-adrenoceptor stimulants can inhibit, partially or totally, the increase of airways microvascular permeability induced by intravenous histamine or bradykinin. However, these effects require doses that are higher than those that inhibit the increase in pulmonary airway resistance induced by these mediators. As suggested by the results obtained with morphine, the higher efficacy of $\beta_2$-adrenoceptor agonists versus bradykinin may occur through activation of presynaptic receptors of the non-adrenergic non-cholinergic (NANC) nerves preventing release of inflammatory neuropeptides such as substance P and neurokinin A.

**Keywords:** Histamine; bradykinin; salbutamol; formoterol; vascular permeability; plasma exudation; plasma leakage; airway oedema; asthma

## Introduction

Bradykinin (BK) is a naturally occurring inflammatory nonapeptide which is generated either by cleavage of a low molecular weight kininogen by tissue kallikrein or by activation of the plasma proteolytic cascade (Regoli & Barabe, 1980); it may be an important mediator of inflammatory airway diseases such as asthma (Fuller *et al.*, 1987; Barnes *et al.*, 1988) or allergic rhinitis (Proud *et al.*, 1983; 1988). Asthmatic patients have elevated kinin concentrations in plasma (Abe *et al.*, 1967) and in nasal and bronchoalveolar lavage fluid after antigen challenge (Baumgarten *et al.*, 1985; Christiansen *et al.*, 1987). Both intravenous and inhaled bradykinin cause bronchoconstriction in asthmatic, but not in normal subjects (Simonsson *et al.*, 1973; Newball *et al.*, 1975; Fuller *et al.*, 1987; Polosa *et al.*, 1990). Bradykinin causes not only bronchoconstriction but also stimulation of sensory nerves (Kaufman *et al.*, 1980; Dixon & Barnes, 1989), increase of mucus secretion (Davies *et al.*, 1982) and airway microvascular leakage with oedema formation (Saria *et al.*, 1983; Rogers *et al.*, 1990), which may be relevant to asthma (Persson, 1987; Chung *et al.*, 1990). Concerning the upper airways, bradykinin is the mediator that is most consistently increased in nasal lavages from patients with nasal symptoms associated with rhinovirus colds (Naclerio *et al.*, 1987). In addition bradykinin and histamine levels are increased in nasal secretions from individuals with allergic rhinitis (Proud *et al.*, 1983).

Conflicting results have been reported concerning the effects of $\beta$-adrenoceptor stimulants on the increase of microvascular permeability induced in the airways by various mediators. Erjefält & Persson (1986) have shown that terbutaline attenuates histamine- and leukotriene $D_4$ ($LTD_4$)-induced microvascular leakage in cat and guinea-pig trachea, whereas Boschetto *et al.* (1989) have found that salbutamol has no effect on the action of the platelet activating factor (PAF). More recently, Norris *et al.* (1990) have reported that salbutamol inhibits neurogenic inflammation in the rat trachea, while Tokuyama *et al.* (1991) have demonstrated that inhaled formoterol and salbutamol reduce the histamine-induced airway microvascular leakage in guinea-pigs.

The purpose of this study was to evaluate and compare the effects on airway microvasculature of two $\beta$-adrenoceptor stimulants, salbutamol and formoterol, against the effects of intravenous bradykinin and histamine. Formoterol is a new $\beta_2$-adrenoceptor agonist which has been shown to be more

[1] Author for correspondence.

potent than salbutamol and, when given by inhalation, acts for 12 h compared with 4 h for salbutamol (Löfdahl & Svedmyr, 1989; Becker & Simons, 1989; Wallin *et al.*, 1990; Malo *et al.*, 1990). We have also compared the effects exerted by salbutamol and formoterol on increased microvascular permeability with their effects on increase of airway resistance induced by histamine and bradykinin.

## Methods

### *Measurement of airway resistance*

Tricoloured guinea-pigs weighing from 0.4 to 0.6 kg were anaesthetized with urethane (1.25 g kg$^{-1}$, i.p.). A cannula was inserted into the trachea and the animals were allowed to breath spontaneously. Pulmonary airway resistance ($R_{AW}$) was determined according to the methods of Amdur & Mead (1958) and Advenier *et al.* (1972). Transpulmonary pressure was measured by needle pleural puncture, and airflow and tidal volume by plethysmography. All values were continuously recorded on a 7700 Hewlett Packard recorder.

The action of formoterol or salbutamol on the bronchoconstriction induced by histamine or bradykinin was tested on groups of 4 to 6 guinea-pigs, one group for each bronchoconstrictor and each substance. Each animal received 2–3 i.v. doses of histamine (30 $\mu$g kg$^{-1}$) or bradykinin (0.3 $\mu$g kg$^{-1}$) at intervals of 15 min and the mean response was calculated. Formoterol (0.03 to 1 $\mu$g kg$^{-1}$) or salbutamol (0.1 to 3 $\mu$g kg$^{-1}$) were then administered i.v., and one of the mediators was injected once more 5 min after each dose. The results are expressed as percentage changes of the initial effects.

Basal values of pulmonary resistance were 0.47 $\pm$ 0.07 cm$H_2O$ ml$^{-1}$ s$^{-1}$ ($n$ = 22). Histamine (30 $\mu$g kg$^{-1}$) and bradykinin (0.3 $\mu$g kg$^{-1}$) doses were chosen to raise airway resistance by 80 to 200%.

In experiments with bradykinin, all animals were pretreated with captopril (5 mg kg$^{-1}$) 15 min before the beginning of the experiment to block bradykinin metabolism (Ichinose & Barnes, 1990a) and potentiate its effects.

### *Measurement of airway microvascular leakage*

Vascular permeability was quantified by the extravasation of Evans blue dye, which correlates well with extravasation of radiolabelled albumin in the skin (Udaka *et al.*, 1970) and airways (Rogers *et al.*, 1989). Tricoloured guinea-pigs weighing 0.4 to 0.6 kg were anaesthetized with urethane (1.25 g kg$^{-1}$, i.p.). A jugular vein was cannulated to inject drugs.

At time 0, saline (1 ml kg$^{-1}$), formoterol (1 or 10 $\mu$g kg$^{-1}$), salbutamol (1 to 30 $\mu$g kg$^{-1}$) or morphine (10 mg kg$^{-1}$) was injected followed, 4 min later, by Evans blue dye (30 $\mu$g kg$^{-1}$). After a further 1 min, saline (1 ml kg$^{-1}$), bradykinin (0.3 $\mu$g kg$^{-1}$) or histamine (30 $\mu$g kg$^{-1}$) were injected; 5 min later, the thorax was opened and a blunt-ended, 13-gauge needle passed into the aorta. The ventricles were cross-clamped and blood was expelled through an incision in the right atrium at 80 mmHg pressure with about 100 ml saline (pH 5.5), in order to remove the intravascular dye from the systemic and pulmonary circulations (Evans *et al.*, 1988; Jin *et al.*, 1988; Ichinose & Barnes, 1990b) until the perfusate was clear. The lungs were then removed. The connective tissues, vasculature, and parenchyma were gently scraped, and the airways were divided into four components: lower part of trachea, main bronchi, and proximal (the proximal 3 mm portion) and distal intrapulmonary airways (Udaka *et al.*, 1970; Rogers *et al.*, 1989). Samples of mucosa and of larynx were also removed and prepared under similar conditions. The tissues were blotted dry, placed in preweighed tubes, and reweighed, and their dye content was extracted in formamide at 37°C for 18 h. Dye concentration was quantified by light absorbance at 620 nm (DCP spectrophotometer, Vital, 6907 AC Dieren, Holland) and its tissue content (ng dye mg$^{-1}$ wet wt tissue) was calculated from a standard curve of dye concentrations in the 0.5 to 10 $\mu$g ml$^{-1}$ range.

The doses of histamine (30 $\mu$g kg$^{-1}$) and bradykinin (0.3 $\mu$g kg$^{-1}$) were chosen from preliminary experiments and gave 30 to 70% of maximal effect (Table 1). In experiments with bradykinin, all animals were pretreated with captopril (5 mg kg$^{-1}$) 15 min before the beginning of experimentation to block bradykinin metabolism (Ichinose & Barnes, 1990a) and potentiate its effects (Table 1). Time to remove tissues was chosen in agreement with Boschetto *et al.* (1989) who have shown that the maximal (but non significant) effect of $\beta_2$ agonist (salbutamol) against PAF (platelet-activating factor) was observed 5 min after administration and with Rogers *et al.* (1990) who have shown that bradykinin-induced leakage was maximal in all airways after 5 min.

### *Drugs*

The drugs used were: bradykinin (gift of Professor Regoli, Sherbrooke, Canada), formoterol (Ciba Geigy, Paris, France), salbutamol sulphate, histamine hydrochloride, formamide, Evans blue dye (Sigma, St. Louis, U.S.A.), captopril (Squibb, Paris, France), urethane (Prolabo, Paris, France), morphine hydrochloride (Pharmacie Centrale des Hôpitaux, Paris, France). All drugs were dissolved in distilled water and then diluted in saline.

### *Statistical analysis of results*

Data are expressed as mean $\pm$ s.e.mean. Statistical analysis of the results was performed by analysis of variance and/or

**Table 1** Effects of histamine (30 and 150 $\mu$g kg$^{-1}$) and bradykinin (BK) (0.1 to 1 $\mu$g kg$^{-1}$) on vascular permeability in guinea-pig airways

| | n | *Nasal mucosa* | *Larynx* | *Trachea* | *Main bronchi* | *Proximal intrapulmonary airways* | *Distal intrapulmonary airways* |
|---|---|---|---|---|---|---|---|
| NaCl solution (0.9%) | 6 | 11.1 ± 1.2 | 2.6 ± 0.4 | 12.6 ± 1.0 | 29.4 ± 3.5 | 29.4 ± 4.6 | 25.1 ± 1.9 |
| Histamine (30 $\mu$g kg$^{-1}$) | 7 | 26.7 ± 4.3‡ | 19.1 ± 2.3‡ | 77.8 ± 2.7‡ | 100.3 ± 5.8‡ | 61.3 ± 3.5‡ | 43.8 ± 2.2‡ |
| Histamine (150 $\mu$g kg$^{-1}$) | 6 | 77.6 ± 16.8§ | 65.8 ± 9.8‡ | 123.3 ± 11.5‡ | 143.5 ± 14.3‡ | 129.7 ± 9.2‡ | 116.7 ± 8.2‡ |
| BK (0.3 $\mu$g kg$^{-1}$) | 6 | 8.1 ± 1.3 | 11.8 ± 2.5§ | 32.0 ± 3.3† | 51.2 ± 7.1§ | 55.4 ± 6.4§ | 30.5 ± 2.6 |
| Captopril (5 mg kg$^{-1}$) | 8 | 20.0 ± 1.4‡ | 31.3 ± 2.8‡ | 54.0 ± 3.7‡ | 63.3 ± 4.3‡ | 62.2 ± 3.2‡ | 60.6 ± 3.9‡ |
| Captopril (5 mg kg$^{-1}$) + BK (0.1 $\mu$g kg$^{-1}$) | 4 | 28.7 ± 2.3[a] | 24.2 ± 2.5 | 50.1 ± 1.8 | 70.3 ± 8.5 | 63.4 ± 8.7 | 57.1 ± 5.6 |
| Captopril (5 mg kg$^{-1}$) + BK (0.3 $\mu$g kg$^{-1}$) | 6 | 50.7 ± 6.0[b] | 33.9 ± 6.9 | 89.1 ± 7.5[a] | 138.2 ± 10.7[b] | 144.3 ± 12.3[b] | 114.7 ± 11.2[b] |
| Captopril (5 mg kg$^{-1}$) + BK (1 $\mu$g kg$^{-1}$) | 6 | 63.6 ± 7.7[b] | 66.7 ± 9.0[a] | 114.4 ± 8.3[b] | 153.5 ± 13.1[b] | 162.1 ± 21.3[a] | 136.7 ± 18.1[a] |

Results are mean $\pm$ s.e.mean. Values expressed as ng of Evans blue dye per mg of wet tissue.
$n$ = number of guinea-pigs per group.
Significant differences from NaCl 0.9% solution (histamine, bradykinin or captopril) are shown as: §$P < 0.05$, †$P < 0.01$ and ‡$P < 0.001$.
Significant differences from captopril alone (captopril + bradykinin) are shown as: [a]$P < 0.01$ and [b]$P < 0.001$.

Student's $t$ test. Probability values of $P < 0.05$ were considered significant.

## Results

### *Pulmonary airway resistance*

Figure 1 shows the dose-response curves of salbutamol and formoterol against the increases of pulmonary airway resistance induced by bradykinin ($0.3\,\mu g\,kg^{-1}$ in the presence of captopril) or histamine ($30\,\mu g\,kg^{-1}$). Both $\beta$-adrenoceptor stimulants exerted dose-dependent inhibitory effects of up to 100% on bradykinin- and histamine-induced bronchoconstriction. In terms of potency, the $ED_{50}$ values of salbutamol and formoterol (Table 2) showed that these drugs were respectively 3.0 and 6.3 times (but non significantly) more active against bradykinin than against histamine and that formoterol was more potent than salbutamol (but non significantly) against histamine (4.5 times) and bradykinin (10 times).

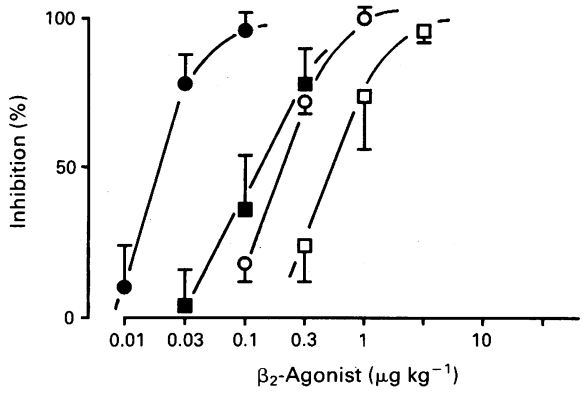


**Figure 1** Inhibition by salbutamol (□, ○) and formoterol (■, ●) of the pulmonary airway resistance increase induced by histamine (□, ■) ($30\,\mu g\,kg^{-1}$, i.v.) or bradykinin (○, ●) ($0.3\,\mu g\,kg^{-1}$, i.v.) in the anaesthetized guinea-pig. Experiments with bradykinin were performed in the presence of captopril ($5\,mg\,kg^{-1}$). Points represent means (s.e.mean shown by vertical bars) of 4 to 6 animals.

**Table 2** $ED_{50}$ of salbutamol and formoterol on the effects of histamine and bradykinin on airway resistance in guinea-pigs

| | n | *Histamine* $ED_{50}$ ($\mu g\,kg^{-1}$) | n | *Bradykinin* $ED_{50}$ ($\mu g\,kg^{-1}$) |
|---|---|---|---|---|
| Salbutamol | 5 | 0.59 ± 0.21 | 5 | 0.21 ± 0.14 |
| Formoterol | 6 | 0.13 ± 0.12 | 6 | 0.02 ± 0.01 |

$n$ = number of experiments. Values are mean ± s.e.mean.

### *Microvascular leakage*

Captopril $5\,mg\,kg^{-1}$ induced a significant increase of microvascular leakage in guinea-pig airways; this effect was not modified by salbutamol; it was slightly, but significantly, reduced by formoterol $10\,\mu g\,kg^{-1}$ at the nasal mucosa level (Table 3).

The effects of salbutamol and formoterol on the increases of microvascular permeability induced by histamine and bradykinin at different sites of the respiratory tract are shown on Figures 2 and 3.

In experiments conducted with histamine (Figure 2), the most pronounced inhibitory effects of the two $\beta$-adrenoceptor stimulants were observed in the trachea and the main bronchi. In the trachea, formoterol (1 and $10\,\mu g\,kg^{-1}$) and salbutamol (10 and $30\,\mu g\,kg^{-1}$) exerted similar effects, reducing by a maximum of about 30 to 40% the increase in microvascular permeability induced by histamine. In the main bronchi, the effects of formoterol ($10\,\mu g\,kg^{-1}$) were significantly ($P < 0.01$) more pronounced than those of salbutamol ($30\,\mu g\,kg^{-1}$) with maximum inhibitions of 50% and 19% respectively. In the proximal intrapulmonary airways the inhibitory effect of salbutamol was observed only with the higher dose. Both drugs were devoid of inhibitory effects in the distal intrapulmonary airways and in the larynx. In the nasal mucosa, only salbutamol in doses of $30\,\mu g\,kg^{-1}$ showed a significant inhibitory effect against histamine whereas formoterol $10\,\mu g\,kg^{-1}$ significantly increased the effects of histamine in a dramatic manner.

The inhibitory effects of salbutamol and formoterol on the bradykinin-induced increase of microvascular permeability (Figure 3) were more widely distributed through the respiratory tract than when histamine was the mediator, since these effects were observed not only in the trachea, the main bronchi and the proximal intrapulmonary airways, but also in the distal intrapulmonary airways and the nasal mucosa. Furthermore, the maximum effect of formoterol and salbutamol versus bradykinin was greater than that observed with histamine since the increase in microvascular leakage induced by bradykinin was abolished by the $\beta$-adrenoceptor agonists at $1\,\mu g\,kg^{-1}$ in the nasal mucosa and trachea, and at $10\,\mu g\,kg^{-1}$ in the bronchi. However at a concentration of $1\,\mu g\,kg^{-1}$, these drugs potentiated the effects of bradykinin in the larynx. In contrast with the results obtained in experiments with histamine, salbutamol and formoterol were almost equally potent since they exerted their inhibitory effect in equivalent doses (1 and/or $10\,\mu g\,kg^{-1}$); the only exception was the main bronchi where salbutamol, but not formoterol, was significantly active at a dose of $1\,\mu g\,kg^{-1}$.

In order to evaluate the influence of the non-adrenergic non-cholinergic system on the effects of histamine and bradykinin, we studied the effects of morphine on the increase of microvascular leakage induced by these two mediators (Table 4). In contrast with $\beta_2$-adrenoceptor agonists (formoterol and salbutamol), morphine $10\,mg\,kg^{-1}$ did not modify the effects of histamine or captopril alone on microvascular leakage in guinea-pig airways. Conversely, morphine, except at larynx

**Table 3** Effects of formoterol ($10\,\mu g\,kg^{-1}$), salbutamol ($10\,\mu g\,kg^{-1}$) on the effects of captopril on vascular permeability in guinea-pig airways

| | n | *Nasal mucosa* | *Larynx* | *Trachea* | *Main bronchi* | *Proximal intrapulmonary airways* | *Distal intrapulmonary airways* |
|---|---|---|---|---|---|---|---|
| NaCl solution (0.9%) | 6 | 11.1 ± 1.2 | 2.6 ± 0.4 | 12.6 ± 1.0 | 29.4 ± 3.5 | 29 ± 4.6 | 25.1 ± 1.9 |
| Captopril ($5\,mg\,kg^{-1}$) | 8 | 20.0 ± 1.4‡ | 31.3 ± 2.8‡ | 54.0 ± 3.7‡ | 63.3 ± 4.3‡ | 62.2 ± 3.2‡ | 60.6 ± 3.9‡ |
| Captopril ($5\,mg\,kg^{-1}$) + formoterol ($10\,\mu g\,kg^{-1}$) | 4 | 14.6 ± 1.4[a] | 32.9 ± 6.0 | 50.3 ± 1.9 | 64.2 ± 9.1 | 61.4 ± 3.4 | 52.8 ± 4.9 |
| Captopril ($5\,mg\,kg^{-1}$) + salbutamol ($10\,\mu g\,kg^{-1}$) | 4 | 16.8 ± 1.8 | 31.5 ± 5.3 | 54.0 ± 8.8 | 66.6 ± 5.7 | 66.3 ± 8.8 | 69.8 ± 11.1 |

Results are mean ± s.e.mean. Values expressed as ng of Evans blue dye per mg of wet tissue.
$n$ = number of guinea-pigs per group.
Significant differences from NaCl 0.9% solution (captopril alone) are shown as: ‡ $P < 0.001$.
Significant differences from captopril alone (formoterol) are shown as: [a] $P < 0.05$.

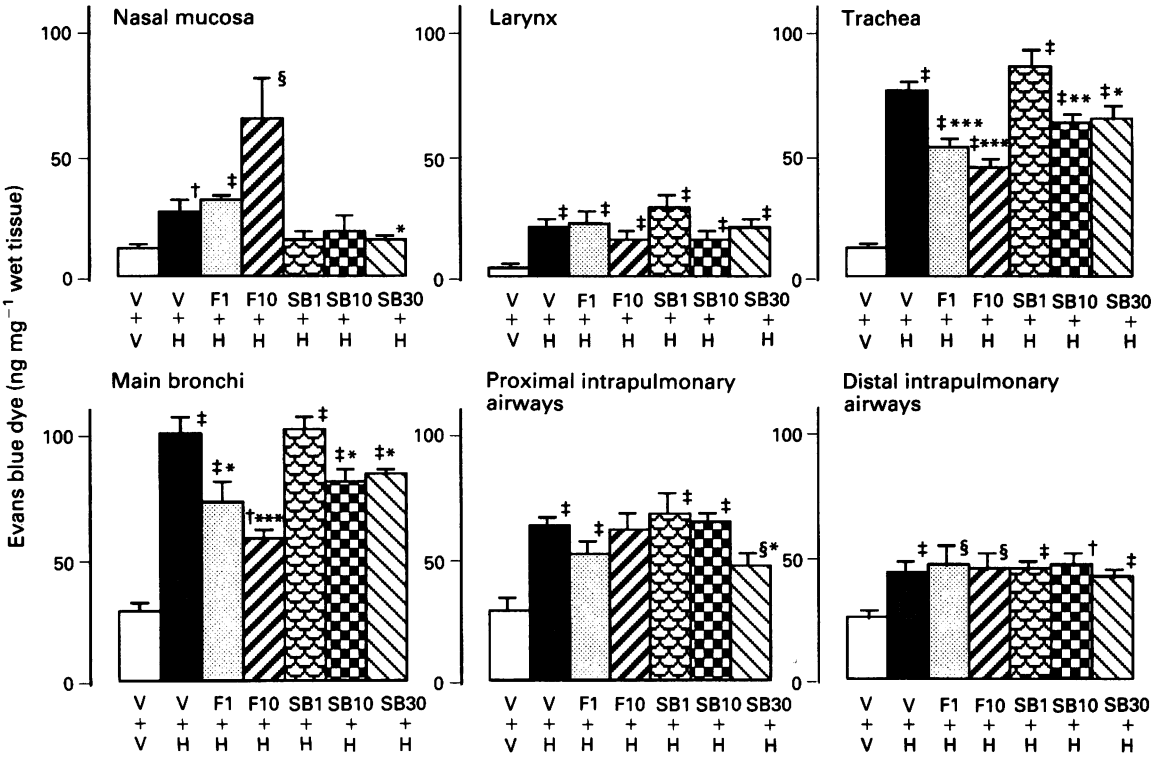


**Figure 2** Histograms illustrating histamine ($30\,\mu g\,kg^{-1}$, i.v.)-induced plasma exudation in guinea-pig airways. Responses to saline (V) or to histamine (H) (1 min after Evans blue dye injection) after saline (V), formoterol (F) (1 and $10\,\mu g\,kg^{-1}$, i.v.) and salbutamol (SB) (1 to $30\,\mu g\,kg^{-1}$, i.v.) (4 min before Evans blue dye injection). Means (s.e.mean shown by vertical bars) of 4 to 6 animals are shown. Significant differences from saline values: § $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$. Significant differences from control histamine, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

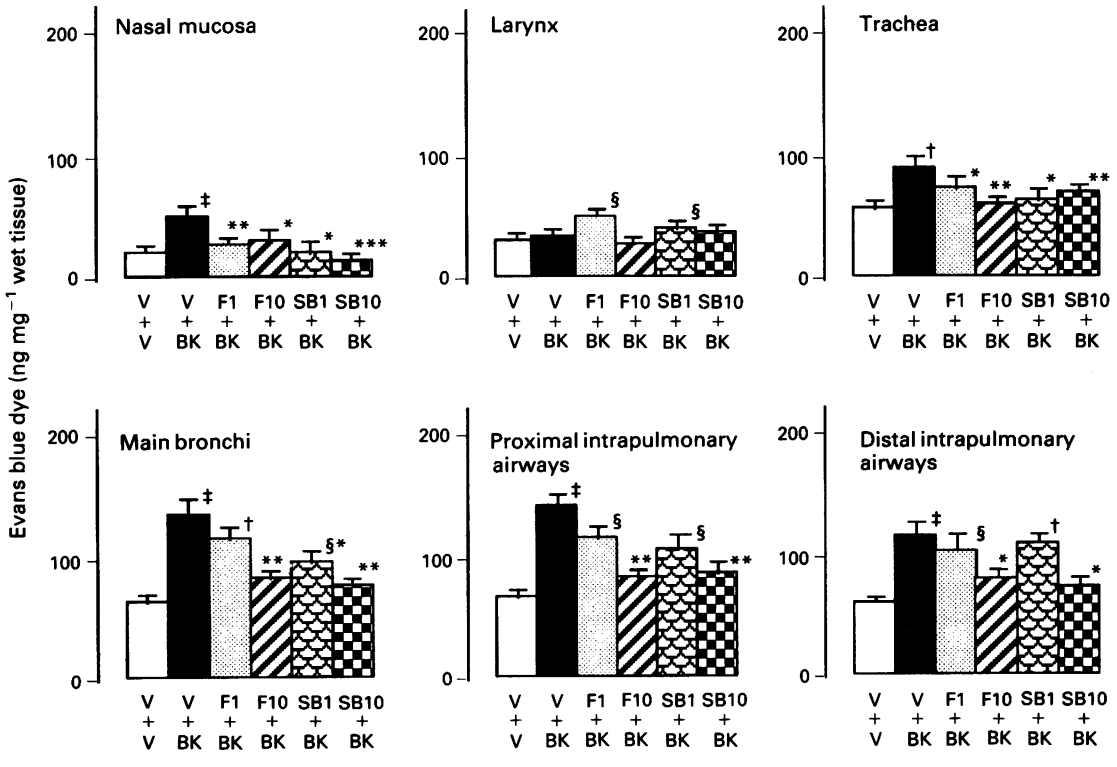


**Figure 3** Histograms illustrating bradykinin ($0.3\,\mu g\,kg^{-1}$, i.v.)-induced plasma exudation in guinea-pig airways. Responses to saline (V) or to bradykinin (BK) (1 min after Evans blue dye injection) after saline (V), formoterol (F) (1 and $10\,\mu g\,kg^{-1}$, i.v.) and salbutamol (SB) (1 and $10\,\mu g\,kg^{-1}$, i.v.) (4 min before Evans blue dye injection). Experiments with bradykinin were performed in the presence of captopril ($5\,mg\,kg^{-1}$). Means (s.e.mean shown by vertical bars) of 4 to 6 animals are shown. Significant differences from saline values: § $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$. Significant differences from control bradykinin, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

**Table 4** Effects of morphine on histamine, captopril and captopril plus bradykinin (BK)-induced plasma exudation in guinea-pig airways

| | n | *Nasal mucosa* | *Larynx* | *Trachea* | *Main bronchi* | *Proximal intrapulmonary airways* | *Distal intrapulmonary airways* |
|---|---|---|---|---|---|---|---|
| NaCl solution (0.9%) | 6 | 11.1 ± 2 | 2.6 ± 0.4 | 12.6 ± 1.0 | 29.4 ± 3.5 | 29.4 ± 4.6 | 25.1 ± 1.9 |
| Histamine (30 $\mu g kg^{-1}$) | 11 | 21.1 ± 4.4 | 24.0 ± 3.2 | 78.9 ± 6.1 | 113.8 ± 8.1 | 87.5 ± 12.7 | 53.5 ± 6.0 |
| Histamine (30 $\mu g kg^{-1}$) + morphine (10 $mg kg^{-1}$) | 6 | 23.2 ± 4.8 | 33.2 ± 5.8 | 68.8 ± 6.0 | 102.3 ± 10.1 | 69.0 ± 13.6 | 36.9 ± 3.1[a] |
| Captopril (5 $mg kg^{-1}$) | 8 | 20.0 ± 1.4 | 31.3 ± 2.8 | 54.0 ± 3.7 | 63.3 ± 4.3 | 62.2 ± 3.2 | 60.6 ± 3.9 |
| Captopril (5 $mg kg^{-1}$) + morphine (10 $mg kg^{-1}$) | 4 | 23.1 ± 3.1 | 34.4 ± 1.8 | 62.5 ± 6.3 | 69.7 ± 6.8 | 73.3 ± 2.2[a] | 68.4 ± 2.9 |
| Captopril (5 $mg kg^{-1}$) + BK (0.3 $\mu g kg^{-1}$) | 6 | 50.7 ± 6.0 | 33.9 ± 6.9 | 89.1 ± 7.5 | 138.2 ± 10.7 | 144.3 ± 12.3 | 114.7 ± 11.2 |
| Captopril (5 $mg kg^{-1}$) + morphine (10 $\mu g kg^{-1}$) + BK (0.3 $\mu g kg^{-1}$) | 6 | 11.0 ± 3.4[c] | 38.2 ± 3.6 | 67.3 ± 3.2[b] | 86.0 ± 2.4[c] | 96.8 ± 4.2[b] | 66.9 ± 4.4[b] |

Results are mean ± s.e.mean. Values expressed as ng of Evans blue dye per mg of wet tissue.
$n$ = number of guinea-pigs per group.
Significant differences from histamine, captopril or captopril plus bradykinin are shown as: [a] $P < 0.05$, [b] $P < 0.01$ and [c] $P < 0.001$.

**Table 5** Effects of propranolol on the effects of formoterol, salbutamol and bradykinin (BK) on vascular permeability in guinea-pig airways

| | n | *Nasal mucosa* | *Larynx* | *Trachea* | *Main bronchi* | *Proximal intrapulmonary airways* | *Distal intrapulmonary airways* |
|---|---|---|---|---|---|---|---|
| Captopril (5 $mg kg^{-1}$) + BK (0.3 $\mu g kg^{-1}$) | 4 | 26.6 ± 4.0 | 30.8 ± 3.0 | 61.6 ± 4.8 | 74.7 ± 4.2 | 77.0 ± 3.8 | 63.4 ± 4.8 |
| Propranolol (1 $mg kg^{-1}$) + captopril (5 $mg kg^{-1}$) + BK (0.3 $\mu g kg^{-1}$) | 5 | 18.4 ± 4.3 | 35.3 ± 4.5 | 62.6 ± 3.8 | 87.7 ± 4.0 | 110.4 ± 5.8† | 94.3 ± 4.2† |
| Propranolol (1 $mg kg^{-1}$) + captopril (5 $mg kg^{-1}$) + formoterol (10 $\mu g kg^{-1}$) + BK (0.3 $\mu g kg^{-1}$) | 5 | 20.9 ± 1.9 | 38.4 ± 2.3 | 71.1 ± 5.4 | 87.3 ± 4.6 | 96.4 ± 4.3 | 76.4 ± 7.0 |
| Propranolol (1 $mg kg^{-1}$) + captopril (5 $mg kg^{-1}$) + salbutamol (10 $\mu g kg^{-1}$) + BK (0.3 $\mu g kg^{-1}$) | 5 | 20.1 ± 3.6 | 42.7 ± 5.8 | 70.3 ± 7.0 | 70.3 ± 7.0 | 106.1 ± 5.1 | 96.3 ± 6.2 |

Results are mean ± s.e.mean. Values expressed as ng of Evans blue dye per mg of wet tissue.
$n$ = number of guinea-pigs per group.
Significant differences from captopril plus bradykinin are shown as: † $P < 0.01$.

level, reduced (trachea, proximal intrapulmonary airways) or abolished (nasal mucosa, main bronchi and distal intrapulmonary airways) the effects of bradykinin on microvascular leakage (Table 4).

Finally, Table 5 shows that propranolol (1 $mg kg^{-1}$) significantly potentiated the effects of bradykinin on vascular permeability in the proximal and distal intrapulmonary airways and abolished the protective effects of formoterol 10 $\mu g kg^{-1}$ and salbutamol 10 $\mu g kg^{-1}$.

## Discussion

Our results confirmed that bradykinin induces a considerable increase of microvascular permeability as already noted by several authors (Saria *et al.*, 1983; Erjefält & Persson, 1986; Laitinen *et al.*, 1987; Ichinose & Barnes, 1990a,b; Rogers *et al.*, 1990). The effect of bradykinin on microvascular permeability in the trachea and intrapulmonary airways is immediate and reaches its peak 5 min after injection (Rogers *et al.*, 1990). It is triggered off by stimulation of bradykinin $BK_2$ receptors, as indicated by the fact that it is inhibited by NPC 359 (D-Arg-[Hyp$^3$-Thi$^{5,6}$-D-Phe$^7$]-BK), a specific antagonist of these receptors (Ichinose & Barnes, 1990b). However, the effect of bradykinin must be more complex, being partially inhibited by indomethacin, WEB 2086 and the diazepam-hypnorm combination, which implies that cyclo-oxygenase derivatives, PAF and stimulation of opioid receptors are concomitantly involved (Rogers *et al.*, 1990). Bradykinin also exerts a delayed effect with a peak at 1 h, involving PAF (Rogers *et al.*, 1990). The bradykinin-induced increase of pulmonary airway resistance in the guinea-pig or in asthmatic subjects is also mediated by $BK_2$ receptors, but it implies, at least in the guinea-pig, indirect effects through arachidonic acid derivatives and cholinergic and NANC systems (Ichinose *et al.*, 1990; Polosa & Holgate, 1990). The effects of bradykinin in the nasal mucosa are probably $BK_2$ mediated, as suggested by the study of Lyon *et al.* (1990) on sheep nasal turbinate membranes.

In this study, two $\beta_2$-adrenoceptor stimulants, salbutamol and formoterol, inhibited the increase of microvascular permeability induced by histamine or bradykinin in the airways of guinea-pigs. This inhibitory effect was found to be more important versus bradykinin than versus histamine since the effects of bradykinin were abolished by formoterol or salbutamol, 1 and 10 $\mu g kg^{-1}$, whereas those of histamine were only partially inhibited. Furthermore, when bradykinin was the mediator, significant inhibition extended to the nasal mucosa and the distal intrapulmonary airways, which was not the case with histamine as mediator. However, the effects of salbutamol or formoterol on microvascular permeability were obtained with doses that were much higher than those required for inhibition of histamine- or bradykinin-induced increase of pulmonary airway resistance.

Thus, the $\beta$-adrenoceptor stimulants, salbutamol and formoterol, exerted unequal effects against the increase of the microvascular permeability induced by the two mediators. It has recently been shown that even in doses of 150 $\mu g kg^{-1}$, intravenous salbutamol does not significantly reduce the effects of PAF on microvascular permeability in the guinea-pig (Boschetto *et al.*, 1989). As regards histamine, our results are very similar to those of Tokuyama *et al.* (1991) who showed that inhaled salbutamol and formoterol can reduce by 20 to 50% the increase of microvascular permeability induced by inhaled histamine, and that this action of $\beta$-adrenoceptor stimulants is predominant in the trachea, the main bronchi and the proximal intrapulmonary airways. In contrast, the effects of bradykinin on microvascular permeability can be inhibited completely by salbutamol and formoterol, as shown in this study. Our results in this respect show that $\beta$-adrenoceptor agonists are more efficient in the lung than in the skin since Beets & Paul (1980) found that the maximal

effect of local injections of β-adrenoceptor stimulants (isoprenaline, salbutamol, fenoterol, terbutaline, orciprenaline) versus the bradykinin-induced increase of microvascular permeability in guinea-pig skin was in the 40–50% range.

In order to explain why β-adrenoceptor stimulants inhibit bradykinin completely and histamine partially, it may be suggested that bradykinin acts directly on the vascular smooth muscle and indirectly by releasing inflammatory mediators from nerve fibres, and that β-adrenoceptor stimulants inhibit this release. In support of the first point is the fact that bradykinin has been shown to act partly via the non-adrenergic non-cholinergic (NANC) system with selective stimulation of C-fibre afferent nerve endings and to induce the release of the neuropeptide substance P and neurokinins (Ueda *et al.*, 1984; Geppeti *et al.*, 1988; Saria *et al.*, 1988), all substances which increase the bradykinin-induced bronchoconstriction in dogs and guinea-pigs (Kaufman *et al.*, 1980; Saria *et al.*, 1988; Ichinose *et al.*, 1990). In asthmatic subjects, the ability of sodium cromoglycate and nedocromil sodium to attenuate the response of airways to bradykinin (Dixon & Barnes, 1989) has been interpreted as an effect mediated by the C-fibres (Dixon *et al.*, 1980).

Concerning the increase of vascular permeability induced by bradykinin, Rogers *et al.* (1990) have previously suggested that it involves the neuromediators of the NANC system, since this increase can be attenuated partially by opioid (Diazepam-Hypnorm) anaesthesia which inhibits sensory nerve activation. Our results with morphine, which reduced or abolished the effects of bradykinin on microvascular permeability but did not modify those of histamine, clearly support this hypothesis. Substance P and neurokinins A and B released by the NANC system have proved to be potent stimulants of microvascular leakage (Rogers *et al.*, 1988; Andrews *et al.*, 1989).

As regards our second point (i.e. inhibition of the NANC system by β-adrenoceptor stimulants), this mechanism is suggested by the experiments of Norris *et al.* (1990) who showed that intravenous salbutamol in doses of 10 and $100\,\mu g\,kg^{-1}$ significantly inhibited the plasma protein extravasation induced by antidromic stimulation of the cervical vagus nerve in the rat trachea. Salbutamol $100\,\mu g\,kg^{-1}$ was as efficacious as morphine $3\,mg\,kg^{-1}$, the inhibition of oedema reaching $61 \pm 14\%$ and $66 \pm 14\%$ respectively. These effects of morphine and salbutamol might occur through activation of presynaptic receptors resulting in an inhibition of neuropeptide release from sensory nerves, as shown for opioids by Frossard & Barnes (1987) or by Belvisi *et al.* (1989). Inhibition of neuropeptide release through β-adrenoceptor stimulation can be suggested but not proven, since β-adrenoceptor agonists may inhibit the release of acetylcholine from postganglionic cholinergic nerves in the airways (Vermiere & Vanhoutte, 1979; Rhoden *et al.*, 1988).

The effects of formoterol and salbutamol versus bradykinin in animals pretreated with captopril seem to be bradykinin-specific since salbutamol has no effect on the action of captopril itself and since formoterol in doses of $10\,\mu g\,kg^{-1}$ only has a modest, although significant, effect on the action of captopril on the nasal mucosa and the trachea.

Concerning the quantitative comparison between formoterol and salbutamol, Tokuyama *et al.* (1991) observed that when these drugs were administered by inhalation formoterol was about 35 times more potent than salbutamol in inhibiting microvascular leakage induced by histamine but this activity ratio was determined only by a dose-for-dose comparison and not by dose-action curves. Our results suggest that when formoterol and salbutamol are given intravenously the difference between these drugs is not so great.

Another result of our study is to show that, while formoterol and salbutamol were able to inhibit the effects of bradykinin and, to a lesser extent and in high doses for salbutamol, those of histamine on the guinea-pig nasal mucosa, formoterol $10\,\mu g\,kg^{-1}$ conversely increased the effects of histamine on the guinea-pig nasal mucosa and both β-adrenoceptor stimulants significantly increased the effects of bradykinin in the larynx. The increase of the effects of histamine or bradykinin in these organs might be due to a vasodilator effect of these drugs in accordance with the results reported by Williams (1977) who showed that in rabbit skin, isoprenaline potentiates the histamine-induced albumin leakage.

Finally, the high doses of salbutamol or formoterol used in our study to inhibit microvascular leakage, compared with the low doses required to inhibit the increase of airway resistance suggest that when these drugs are administered intravenously the increase of airway resistance is primarily due to contraction of the bronchial smooth muscle and, to a very small extent, to an increase of vascular permeability in the bronchial mucosa. Conversely, Tokuyama *et al.* (1991) have shown that when formoterol or salbutamol are given by inhalation the inhibitory effects of the $\beta_2$-adrenoceptor stimulants on the increase of pulmonary airway resistance correlate with their inhibitory effects on the increase of microvascular permeability, which means that in such experiments vascular exudation plays a major role in the increase of pulmonary resistance.

In conclusion, we have shown that high doses of formoterol or salbutamol administered intravenously partially or totally inhibit histamine- or bradykinin-induced increase of microvascular permeability at various levels of the upper and lower respiratory tract. The higher efficacy of the $\beta_2$-agonists versus bradykinin might be due to activation of presynaptic receptors of the NANC nerves preventing release of inflammatory neuropeptides such as substances P and neurokinin A.

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# Evidence that epithelium-dependent relaxation of vascular smooth muscle detected by co-axial bioassays is not attributable to hypoxia

[1]D. Spina, *L.B. Fernandes, *J.M.H. Preuss, †D.W.P. Hay, †R.M. Muccitelli, C.P. Page & *R.G. Goldie

Department of Pharmacology, King's College London, Manresa Road, London SW3 6LX, UK; *Department of Pharmacology, University of Western Australia, Nedlands 6009, Australia and †Department of Pharmacology, L-532, SmithKline Beecham Pharmaceuticals, P.O. Box 1539, King of Prussia, PA 19406-0939, U.S.A.

**1** The present study was undertaken to examine further the contribution of hypoxia to airway epithelium-dependent relaxation of rat aorta in the co-axial bioassay.

**2** Endothelium-denuded rat aorta contracted with phenylephrine (0.05 μM) relaxed in a time-dependent manner ($t_{\frac{1}{2}} = 8.3 \pm 0.4$ min, $n = 38$) when the bathing solution was bubbled with 95% $N_2$ and 5% $CO_2$. In co-axial bioassays, the $t_{\frac{1}{2}}$ for histamine (100 μM; guinea-pig trachea)- and methacholine (100 μM; rabbit bronchus)- induced relaxation was $1.9 \pm 0.2$ min ($n = 14$) and $1.2 \pm 0.1$ min ($n = 26$), respectively.

**3** Hypoxia-induced relaxation was not associated with a rise in intracellular guanosine 3':5'-cyclic monophosphate (cyclic GMP). This contrasts with previous findings of an elevation in cyclic GMP associated with epithelium-dependent relaxation of rat aorta in co-axial bioassays.

**4** Hypoxia-induced vascular relaxation was antagonized by the ATP-sensitive $K^+$ channel blocker, glibenclamide (100 μM). In contrast, glibenclamide (100 μM) failed to inhibit histamine (100 μM; guinea-pig trachea)- and methacholine (0.1–100 μM; rabbit bronchus)-induced release of epithelium-derived inhibitory factor (EpDIF), in co-axial bioassays. Glibenclamide (100 μM) antagonized BRL 38227 (lemakalin), but not isoprenaline-induced relaxation of phenylephrine-contracted rat aorta.

**5** These data strongly suggest that the airway epithelium-dependent relaxant responses observed in co-axial bioassays cannot be attributed to hypoxia.

**Keywords:** Epithelium-derived inhibitory factor (EpDIF); hypoxia; co-axial bioassay; glibenclamide

## Introduction

It is well established that the sensitivity of airway smooth muscle preparations from various animal species to some spasmogens, is significantly increased by removal of the epithelium (Flavahan *et al.*, 1985; Barnes *et al.*, 1985; Goldie *et al.*, 1986; Hay *et al.*, 1986). Similar data have also been obtained in human tracheal and bronchial preparations (Raeburn *et al.*, 1986; Aizawa *et al.*, 1988; Fernandes *et al.*, 1990; Knight *et al.*, 1990). Furthermore, Jeffery *et al.* (1989) have recently demonstrated a positive correlation between the extent of bronchial epithelial damage and the degree of airway hyperresponsiveness to inhaled methacholine in asthmatic patients, although not all asthmatics demonstrate epithelial cell damage (Lozewicz *et al.*, 1990). Such epithelium-dependent responsiveness has, in part, been attributed to the epithelium acting as a significant barrier to mucosal penetration of airway spasmogens (Holroyde, 1986; Undem *et al.*, 1988). It has also been suggested that the loss of epithelium-derived inhibitory factor(s) (EpDIF) may contribute to this phenomenon (Vanhoutte, 1988; Farmer, 1988; Goldie *et al.*, 1990; Raeburn, 1990).

Attempts to bioassay a non-prostanoid airway smooth muscle relaxant, EpDIF, from superfused guinea-pig trachea have failed (Holroyde, 1986; Undem *et al.*, 1988), although such a factor has been reported to be released from canine bronchus (Vanhoutte, 1988). In contrast, the release of a vasoactive EpDIF in response to histamine and methacholine has been detected in several studies using a co-axial bioassay technique (Ilhan & Sahin, 1986; Guc *et al.*, 1988; Fernandes *et al.*, 1989; Spina & Page, 1991). Extensive pharmacological assessments of this factor released by guinea-pig trachael epithelium (Fernandes & Goldie, 1990) and rabbit bronchial epithelium (Spina & Page, 1991) have failed to define its nature, although a preliminary study indicated that guinea-pig tracheal EpDIF-induced relaxation of rat aorta was mediated via activation of guanylate cyclase and the subsequent increase in the intracellular levels of guanosine-3':5'-cyclic monophosphate (cyclic GMP; Hay *et al.*, 1989).

The precise nature of this non-prostanoid, vasoactive EpDIF remains the subject of debate. The present investigation was designed primarily to assess the possibility that vascular relaxation responses produced in co-axial bioassay assemblies utilizing guinea-pig trachea or rabbit bronchus are artifactual. In particular, we were interested to investigate whether such responses might be due to hypoxia-induced decreases in vascular tone as recently suggested by Gunn & Piper (1990a,b). The influence of hypoxia on cyclic nucleotide levels in rat aorta in such co-axial assemblies was also assessed.

## Methods

### *Vascular tissue preparation*

Male Wistar rats (200–300 g) were killed by stunning and exsanguination and the thoracic aorta removed, opened and cross-cut or spiral preparations (7–8 mm in length) were obtained as previously described (Fernandes *et al.*, 1989). The endothelium was removed by gentle rubbing of the mucosal surface with a cotton wool coated probe. Initially, pharmacological verification of the removal of the endothelium, as assessed by the absence of relaxation in response

[1] Author for correspondence

to histamine (100 μM), was performed. Cross-cut and spirally cut preparations were suspended under 500 mg and 1 g tension respectively, in an organ bath containing Krebs-Henseleit solution aerated with 95% $O_2$ and 5% $CO_2$ at 37°C. Before the start of each experiment tissues were equilibrated for 45–60 min with changes of bath fluid every 15 min. Changes in the isometric tension were measured with a Grass force-displacement transducer (FTO3C) and recorded on Rikadenki pen recorders, Grass polygraph recorders or Lectromed recorders. In all experiments, the Krebs-Henseleit solution also contained the cyclo-oxygenase inhibitor, indomethacin (5 μM). The β-adrenoceptor antagonist, propranolol (1 μM) was also present in experiments involving spiral preparations.

### *Effects of hypoxia*

*Functional studies* Endothelium-denuded cross-cut preparations of rat aorta were contracted with (−)-phenylephrine (0.05 μM). Once the contractile response had reached plateau, tissues were bubbled with 95% $N_2$ and 5% $CO_2$ and changes in isometric tension monitored over a 25 min period. Preparations were then washed 3 times over a 15 min period and bubbled with 95% $O_2$ and 5% $CO_2$ for 30 min. Following this period, tissues were incubated with 0.5% dimethylsulphoxide (DMSO) or glibenclamide (100 μM) for 30 min before re-exposure to phenylephrine (0.05 μM). It was noted that the response to phenylephrine was reduced after glibenclamide (3.5 fold (confidence limits, CL: 2.7–4.4) increase in $EC_{50}$; small decrease in maximum response: control = 352 ± 16, test = 330 ± 20 mg, $n = 15$, $P < 0.05$) and to a lesser extent after DMSO (2.1 fold (CL, 1.6–2.8) increase in $EC_{50}$; no effect on maximum response). Accordingly, if required, the bath concentration of phenylephrine was increased until the contractile response was of a magnitude similar to that observed before incubation with DMSO or glibenclamide. At plateau response, the preparations were once again bubbled with 95% $N_2$ and 5% $CO_2$ and responses monitored over a 25 min period, after which the baths were bubbled with 95% $O_2$ and 5% $CO_2$ and the tissues washed 5 times over a 15 min period, then allowed to equilibrate for a further 45 min. This procedure was repeated a third time in Krebs-Henseleit solution free of DMSO or glibenclamide

*Biochemical experiments* Spiral aortic strip preparations were initially exposed to phenylephrine (0.2 μM) and then washed. Following a 30 min equilibration period, vascular preparations were exposed to phenylephrine (0.05 μM) and at a plateau response, a 200 μl aliquot of bath fluid was taken up in a plastic syringe which was sealed and kept on ice for up to 30 min before the pH and $Po_2$ was determined with a blood gas analyzer (Radiometer ABL 330, Copenhagen, Denmark). In some baths, a bathing fluid sample was also taken after bubbling with 95% $O_2$ and 5% $CO_2$ for 30 min, while samples were taken from other baths after tissues were bubbled with 95% $N_2$ and 5% $CO_2$ for 3, 10 or 30 min. The use of a drop-away bath chamber enabled spiral strips to be flash-frozen quickly at each of the time points, with Wollenberger tongs that had been precooled in liquid $N_2$. Isometric force was recorded until freezing. Frozen tissues was stored at −70°C until required for the determination of vascular cyclic GMP. Briefly, 1.5 ml of cold (0°C) 10% trichloroacetic acid, containing approximately 4000 c.p.m. [$^3$H]-cyclic GMP added as a tracer for recovery determinations, was added to a cold ground glass homogenizing tube containing frozen tissue. The tissue was then homogenized with a motor-driven ground glass pestle. Precipitated protein was separated from the soluble extract by centrifugation at 3000 *g* for 20 min at 4°C and the supernatants collected. Trichloroacetic acid was removed from the sample with 5 successive ether extractions (Brooker *et al.*, 1979). Concentrations of cyclic GMP in the tissue extracts were determined with commerically available assay kits (DuPont, New England Nuclear, Boston, MA, U.S.A.) after acetylation by the radioimmunoassay method of Brooker *et al.* (1979). Cyclic nucleotide content was corrected for percentage of recovery (70–95%) and expressed as fmol of cyclic nucleotide per mg of protein. A separate standard curve was run in duplicate with each set of samples.

### *Co-axial bioassay experiments*

*Guinea-pig trachea* Male guinea-pigs (SR/C Tricolour) weighing 450–500 g were killed by cervical dislocation and the trachea removed, dissected free of surrounding tissue and cut into tube segments approximately 7 mm in length. Aortic preparations were contracted with phenylephrine (0.05 μM) and challenged with histamine (100 μM). The failure of these preparations to relax in response to histamine provided pharmacological verification of the absence of endothelium. After equilibration for 45 min, the aortic preparations were contracted with phenylephrine (0.05 μM) and once the contraction had reached plateau, the aortae were mounted in co-axial assemblies within guinea-pig trachael tube segments as previously described (Fernandes *et al.*, 1989). Histamine (100 μM) was then added to the bath in the absence or presence of DMSO or glibenclamide (100 μM). In some preparations, a further challenge with phenylephrine (0.05 μM) was required to achieve a level of tone similar to that which was observed before glibenclamide treatment.

*Rabbit bronchus* White New Zealand rabbits of either sex were killed with an overdose of pentobarbitone (90 mg $kg^{-1}$). The lungs were quickly removed and placed in oxygenated ice-cold Krebs-Henseleit solution. Intrapulmonary bronchi (2–3 mm, i.d.) were removed from the lung, cleared of alveolar parenchymal tissue and blood vessels and cut into 7 mm long tube segments.

Bronchial segments were positioned in the organ bath above the aortic preparations which were suspended under 500 mg tension as described above and allowed to equilibrate for 30 min. Rat aorta was exposed to phenylephrine (0.2 μM) and the absence of a functional endothelium verified pharmacologically as outlined above. Following a further 35 min equilibration period, the aorta was positioned inside the bronchial tube and allowed to equilibrate for a further 10 min as described previously (Spina & Page, 1991). The vascular preparation was then precontracted with phenylephrine (10 μM) in the absence or presence of 0.5% DMSO or glibenclamide (100 μM) and challenged with cumulative doses of methacholine (0.1–100 μM). In some preparations, a further challenge with phenylephrine (10 μM) was required to achieve a level of tone similar to that which was observed before glibenclamide treatment. A third cumulative concentration-effect curve to methacholine was constructed in the same tissues 45 min after DMSO or glibenclamide (100 μM) had been removed from the bathing fluid.

### *Functional experiments*

Concentration-effect curves to the contractile agonist phenylephrine (1 nM–1 μM) and the relaxant agonists isoprenaline (0.3 nM–10 μM) and BRL 38227 (10 nM–3 μM) were constructed in the absence or presence of DMSO or glibenclamide (100 μM). In the case of the relaxant agonists, concentration-effect curves were superimposed on phenylephrine (0.05 μM)-contracted tissues.

### *Statistical analysis*

Unless otherwise stated, numerical data are expressed as arithmetic mean ± s.e.mean. The effects of glibenclamide and DMSO on hypoxia-induced relaxation were assessed by computer assisted planimetry measurements of the area under the % relaxation vs time curve (AUC mm$^2$). Two-way analysis of variance with the Greenhouse-Geisser correction for repeated measures (Wallenstein *et al.*, 1980) was used to assess the

data for AUC and hypoxia-induced reversal of phenylephrine tone. The time taken for relaxation responses to achieve 50% of maximal relaxation ($t_l$) was also measured. The effect of hypoxia on cyclic nucleotide levels, bath $Po_2$ and pH were assessed by one-way analysis of variance. Agonist potency was expressed as geometric mean concentrations producing 50% maximal response ($EC_{50}$), with 95% confidence limits (CL). The magnitude of contractile respones is expressed as a % of the maximum contraction, whereas the magnitude of relaxation responses is expressed as % reversal of spasmogen-induced tone (% $E_{max}$). Paired or unpaired Student's $t$ test and Mann-Whitney U test were used to analyze differences in means. Where multiple comparisons were made, either a Bonferroni correction was used or the Newman-Keuls test was used (Wallenstein *et al.*, 1980) and considered significant at the 0.05 level.

### *Drugs*

Drugs used in this study were obtained from the following sources: histamine hydrochloride, indomethacin, isoprenaline hydrochloride, methacholine hydrochloride, glibenclamide, dimethylsulphoxide (DMSO), propranolol hydrochloride (Sigma); BRL 38227 (lemakalim, SmithKline Beecham Pharmaceuticals, U.K.). Krebs-Henseleit solution consisted of (mM): NaCl 117.6, KCl 5.4, $NaHCO_3$ 25, $KH_2PO_4$ 1.03, $MgSO_4$ 0.57, D-glucose 11.1 and $CaCl_2$ 2.5. Isoprenaline was dissolved in 0.9% NaCl containing ascorbic acid 20 $\mu g\ ml^{-1}$. Indomethacin was dissolved in 0.5% $Na_2CO_3$. BRL 38227 was dissolved in 50% ethanol/polyethylene glycol. Glibenclamide was dissolved in 100% DMSO. All other drugs were dissolved in Krebs-Henseleit buffer or distilled water.

## Results

### *Hypoxia and spasmagon-induced tone in rat aorta*

*Cross-cut aortic strips* A time-dependent reversal of phenylphrine (0.05 $\mu$M)-induced tone in rat aorta was observed when the bathing solution was bubbled with 95% $N_2$ and 5% $CO_2$ (Figure 1). The onset of relaxation had a latency of approximately 5 min. Area under the % relaxation versus time curve over a 25 min period (AUC) was found to be $2868 \pm 135\ mm^2$ ($n = 24$) and the mean maximal relaxation was $81 \pm 2\%$ ($n = 24$) reversal of phenylephrine-induced tone (Table 1). In the presence of glibenclamide, the hypoxia-induced relaxation was inhibited as demonstrated by a significant reduction in AUC ($F_{2,44} = 16.1$, corrected $P < 0.01$) and maximum % relaxation ($F_{2,44} = 88.6$, corrected $P < 0.001$; Figure 1, Table 1). In contrast, DMSO treatment failed to alter hypoxia-induced relaxation as assessed by measurements of AUC ($F_{2,44} = 2.5$, corrected $P > 0.05$), although a small inhibitory effect on maximum % relaxation was observed ($F_{2,44} = 14.2$, corrected $P < 0.01$, Figure 1, Table 1). Furthermore, attenuation of the hypoxia-induced relaxation persisted following the removal of glibenclamide from the bath (Figure 1, Table 1).

*Spiral aortic strips* Oxygen tension ($Po_2$) in tissue baths bubbled with 95% $O_2$ and 5% $CO_2$ was $580 \pm 13$ mmHg ($n = 39$). However, a significant reduction in bath $Po_2$ was observed 3, 10 and 30 min after bubbling with 95% $N_2$ and 5% $CO_2$ ($F_{3,35} = 195$, $P < 0.001$, Table 2) but this was not associated with changes in bath fluid pH ($F_{3,35} = 2.8$, $P > 0.05$, Table 2). After 3 min hypoxia, there was no effect on phenylephrine (0.05 $\mu$M)-induced tone, despite an approximately 80% reduction in bath $Po_2$ (Table 2). In contrast, significant reversal of phenylephrine-induced tone was observed after 10 min ($23 \pm 7\%$, $P < 0.01$, Table 2) and 30 min ($19 \pm 4\%$, $P < 0.01$, Table 2) of hypoxia, when bath $Po_2$ was reduced by approximately 80% also. No significant alteration in the level of intracellular cyclic GMP was observed at any time point after induction of hypoxia ($F_{3,35} = 0.8$, $P < 0.05$, Table 2). In contrast, histamine or methacholine-induced relaxation of rat aorta in co-axial bioassay was associated with an elevation in cyclic GMP (Hay *et al.*, 1989; Spina *et al.*, 1991).

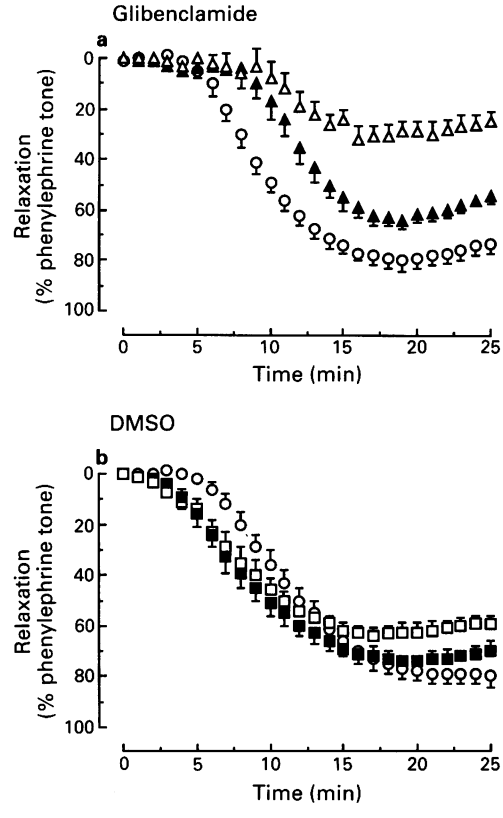


**Figure 1** Time course of hypoxia-induced relaxation of phenylephrine contracted endothelium-denuded aortic cross-cut preparations of the rat in the absence (○), or presence of (a) glibenclamide (100 $\mu$M, ▲) or (b) dimethylsulphoxide (DMSO, ■). Responses were also obtained in tissues which had been exposed to glibenclamide (△) or DMSO (□) followed by washout (after 45 min). Each point is the mean of 12 observations; verticle bars shown s.e.mean.

**Table 1** The effect of glibenclamide (100 $\mu$M) or solvent dimethylsulphoxide (DMSO) on hypoxia-induced reversal of phenylephrine-induced tone in endothelium-denuded cross-cut aorta strips

| | *AUC* ($mm^2$) | *% reversal of tone* |
|---|---|---|
| Control | $2683 \pm 195$ | $82 \pm 4$ |
| DMSO | $3065 \pm 194$ | $75 \pm 3$ |
| Washout | $2645 \pm 183$ | $65 \pm 3$*# |
| Control | $3053 \pm 178$ | $80 \pm 4$ |
| Glibenclamide | $2042 \pm 157$** | $64 \pm 3$** |
| Washout | $1278 \pm 217$**† | $35 \pm 5$**† |

Results expressed as mean ± s.e.mean with $n = 12$ preparations for each group. Significantly different compared with control (*$P < 0.05$; **$P < 0.01$), DMSO (#$P < 0.05$) or glibenclamide (†$P < 0.01$) (Neuman-Keuls test).

### *Co-axial bioassay*

*Guinea-pig trachea* When endothelium-denuded cross-cut rat aortic preparations were suspended within the lumen of epithelium-intact tubular segments of guinea-pig trachea in a co-axial bioassay, histamine caused a $81 \pm 5\%$ ($n = 14$), reversal of phenylephrine-induced tone. There was no signi-

**Table 2** Effect of bubbling with 95% $O_2$ and 5% $CO_2$ for 30 min or 95% $N_2$ and 5% $CO_2$ for 3, 10 or 30 min on bath $PO_2$, % reversal of phenylephrine-induced tone and intracellular cyclic GMP levels in endothelium-denuded rat aortic spiral strips

| | *95% $O_2$ and 5% $CO_2$* | *95% $N_2$ and 5% $CO_2$* | | |
|---|---|---|---|---|
| Time | 30 min | 3 min | 10 min | 30 min |
| *Bath $PO_2$ (mmHg)* | | | | |
| Pre[a] | 552 ± 118 | 614 ± 31 | 583 ± 22 | 586 ± 29 |
| Post[b] | 573 ± 27 | 108 ± 13* | 85 ± 11* | 88 ± 7* |
| *Bath pH (units)* | | | | |
| Pre[a] | 7.52 ± 0.02 | 7.49 ± 0.02 | 7.45 ± 0.02 | 7.47 ± 0.02 |
| Post[b] | 7.51 ± 0.02 | 7.41 ± 0.01 | 7.41 ± 0.01 | 7.42 ± 0.01 |
| *% reversal of phenylephrine-induced tone* | | | | |
| | 0.8 ± 0.8 | 1 ± 1 | 23 ± 7# | 19 ± 4# |
| *Cyclic GMP (fmol $mg^{-1}$ protein)* | | | | |
| | 291 ± 104 | 375 ± 175 | 150 ± 43 | 596 ± 306 |
| | (12) | (7) | (8) | (12) |

Results expressed as mean ± s.e.mean. Number of preparations are shown in parentheses.
[a] Pre refers to time 0, when the baths were bubbled with 95% $O_2$ and 5% $CO_2$.
[b] Post refers to the time period for which the baths were bubbled with 95% $O_2$ and 5% $CO_2$ or 95% $N_2$ and 5% $CO_2$.
Significantly lower bath $PO_2$ cf. pre control (* $P<0.001$, paired $t$ test). Significantly greater reversal of phenylephrine-induced tone in tissue bubbled with 95% $N_2$ and 5% $CO_2$ cf. tissue bubbled with 95% $O_2$ and 5% $CO_2$ (30 min control; #$P<0.01$, Mann-Whitney U test).

ficant difference between the histamine-induced reversal of phenylephrine-induced tone in glibenclamide (100 μM, 45 min) compared with DMSO-treated preparations (DMSO 89 ± 8%, $n = 7$, vs glibenclamide 73 ± 8%, $n = 7$, $P>0.05$).

The $t_{\frac{1}{2}}$ for relaxation of rat aorta under hypoxic conditions was 8.8 ± 0.5 min ($n = 38$) which was significantly longer than that for the relaxation observed in co-axial bioassays ($t_{\frac{1}{2}} = 2.0 \pm 0.2$ min, $n = 14$, $P<0.001$, Table 3). Furthermore, glibenclamide treatment failed to alter the $t_{\frac{1}{2}}$ of relaxation in co-axial bioassay (Table 3). In contrast, under conditions of hypoxia, the $t_{\frac{1}{2}}$ for the relaxation of rat aorta was significantly increased by glibenclamide ($P<0.05$, Table 3).

*Rabbit bronchus* Methacholine (100 μM) caused a 77 ± 6% ($n = 33$) reduction in phenylephrine-induced tone in rat aorta mounted within the lumen of epithelium-intact rabbit bronchus. There was no significant difference between the methacholine-induced reversal of phenylephrine-induced tone in glibenclamide-treated (100 μM, 30 min) compared with DMSO-treated preparations (DMSO 68 ± 7%, $n = 17$, vs glibenclamide 66 ± 8%, $n = 16$, $P>0.05$). Nor was there a significant difference between the relaxant potency of methacholine ($EC_{50}$) in glibenclamide-treated compared with DMSO-treated preparations (DMSO: $EC_{50} = 2.27$ μM; 95% confidence limits, 1.6–3.3, $n = 14$, vs glibenclamide: $EC_{50} = 2.0$ μM; 95% confidence limits, 1.5–2.0, $n = 14$, $P>0.05$). Furthermore, compared with control, the relaxation to methacholine was not altered after DMSO (61 ± 4%, $n = 10$, $P>0.05$) and glibenclamide (73 ± 11%, $n = 11$, $P>0.05$) had been washed out. In addition, relaxation of rat aorta in co-axial bioassay ($t_{\frac{1}{2}} = 1.2 \pm 0.1$ min, $n = 26$) was significantly shorter than relaxation of rat aorta under hypoxic conditions (cf. 8.8 ± 0.5 min, $P<0.001$).

## *Functional studies: rat aorta*

*Effect of glibenclamide on isoprenaline- and BRL 38227-induced relaxation* Isoprenaline caused concentration-dependent relaxation of phenylephrine (0.05 μM)-contracted rat aorta ($EC_{50} = 100$ nM; % $E_{max} = 86 \pm 3$%, Table 4, Figure 2a). There was no significant difference in the potency or % $E_{max}$ of isoprenaline in glibenclamide (100 μM)-treated preparations (Table 4, Figure 2a). No loss of tissue sensitivity to isoprenaline was observed after removal of glibenclamide (Table 4, Figure 2a).

The $K^+$ channel activator, BRL 38227, also caused concentration-dependent relaxation of phenylephrine (0.05 μM)-induced tone ($EC_{50} = 46$ nM; % $E_{max} = 78 \pm 4$%, Table 4, Figure 2b). In the presence of glibenclamide (100 μM) the maximum relaxation response to BRL 38227 (100 μM) was significantly reduced compared with control (20 ± 9%, $n = 6$, $P<0.05$, Table 4, Figure 2b). In contrast, following removal of glibenclamide (100 μM) the maximum relaxation response to BRL 38227 was not significantly different from control (75 ± 7%, $n = 6$, $P>0.05$, Table 4, Figure 2b), although the relaxant potency of BRL 38227 remained markedly reduced, by 354 fold (CL, 155–813, $n = 6$, $P<0.001$, Table 4, Figure 2b), compared with control.

**Table 3** Time to half maximal relaxation ($t_{\frac{1}{2}}$) in endothelium-denuded rat aorta induced by histamine in co-axial assemblies with guinea-pig trachea or by bubbling with 95% $N_2$ and 5% $CO_2$ in rat endothelium-denuded aorta

| | $t_{\frac{1}{2}}$ (min) | | |
|---|---|---|---|
| | *Control* | *DMSO* | *Glibenclamide (100 μM)* |
| Co-axial preparation | | | |
| Histamine (100 μM) | 2.0 ± 0.2 | 2.3 ± 0.4[NS] | 1.8 ± 0.2[NS] |
| *n* | 14 | 7 | 7 |
| Rat aorta alone | | | |
| 95% $N_2$ and 5% $CO_2$ | 8.8 ± 0.5 | 7.1 ± 0.6[NS] | 11.9 ± 0.6* |
| *n* | 38 | 17 | 21 |

Results expressed as mean ± s.e.mean. $n$ = number of preparations tested.
*Significantly different cf. respective control ($P<0.001$). Student's paired (glibenclamide) or non-paired (DMSO) $t$ test.
[NS]Not significantly different from respective control ($P>0.01$).

**Table 4** Effect of glibenclamide (100 μM) on the relaxant potency ($EC_{50}$) and % reversal of phenylephrine-induced tone (% $E_{max}$) to isoprenaline and BRL 38227 in endothelium-denuded aorta of rat

| | *Control* | *Glibenclamide* | *Washout* | n |
|---|---|---|---|---|
| *Isoprenaline* | | | | |
| $EC_{50}$ | 100 nM (59–168) | 116 nM (51–261) | 108 nM (60–360) | 9 |
| % $E_{max}$ | 86 ± 3% | 80 ± 2% | 82 ± 1% | 9 |
| *BRL 38227* | | | | |
| $EC_{50}$ | 46 nM (28–76) | # | 16.4 μM* (10–26) | 6 |
| % $E_{max}$ | 78 ± 4% | 20 ± 9%* | 76 ± 7% | 6 |

Relaxant potency ($EC_{50}$) expressed as geometric mean and values in parentheses represent 95% confidence limits. Relaxant agonist-induced reversal of phenylephrine-contracted aorta (% $E_{max}$) expressed as arithmetic mean and s.e.mean of *n* preparations.

# – No $EC_{50}$ value for BRL 38227 was obtained in the presence of glibenclamide (100 μM).

* Significant increase in the relaxant potency to BRL 38227 cf. control ($P<0.001$, paired *t* test).

* Significant reduction in reversal of phenylephrine-induced tone (% $E_{max}$) cf. control ($P<0.05$, Mann-Whitney U test).

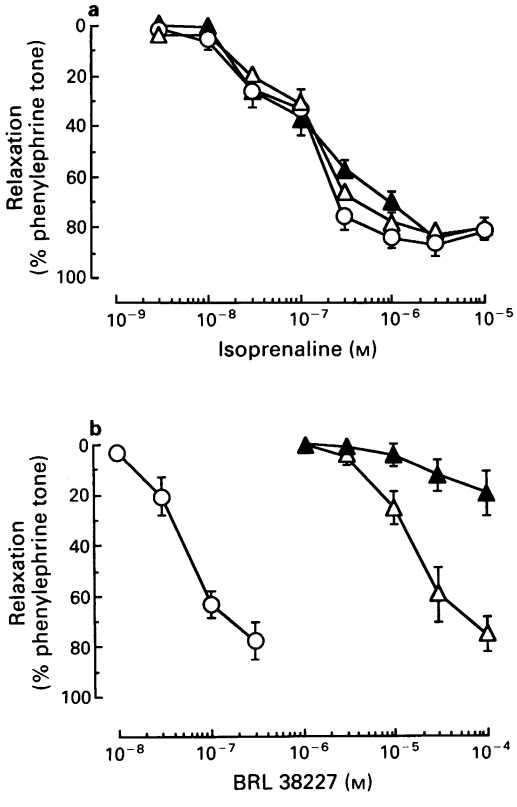


**Figure 2** Effect of glibenclamide (100 μM) on relaxation to (a) isoprenaline or, (b) BRL 38227 in phenylephrine-contracted endothelium-denuded aortic cross-cut preparations of the rat. Responses were also obtained in tissues which had previously been exposed to glibenclamide followed by washout (after 45 min). Each point is the mean of 9 (isoprenaline) and 6 (BRL 38227) observations; vertical bars show s.e.mean. Control (○); glibenclamide (▲); washout (△).

## Discussion

We and others have previously demonstrated the apparent release of a non-prostanoid inhibitory factor from airway epithelium (EpDIF) using a co-axial bioassay (Ilhan & Sahin, 1986; Guc *et al.*, 1988; Fernandes *et al.*, 1989; 1990; Fernandes & Goldie, 1990; Spina & Page, 1991). This has been confirmed in the present study. However, controversy surrounds the existence of EpDIF, due to the equivocal results concerning the release and transfer of this factor using superfusion techniques (Holroyde, 1986; Undem *et al.*, 1988; Vanhoutte, 1988). While the identity of EpDIF remains elusive, it has been suggested that relaxant responses observed in co-axial assemblies may be due to luminal hypoxia induced by epithelial $O_2$ utilization which is exacerbated by spasmogen-mediated tracheal narrowing (Gunn & Piper, 1990a,b). However, this hypothesis is not consistent with the inability of certain airway smooth muscle spasmogens including leukotrienes $C_4$ and $D_4$, and the calcium ionophore A23187, to release EpDIF (Fernandes & Goldie, 1990). The present study provides additional evidence to suggest that luminal hypoxia does not appear to account for the spasmogen-induced vascular smooth muscle relaxation observed in co-axial bioassay assemblies using guinea-pig trachea or rabbit bronchus. Thus, the $K^+$ channel blocker, glibenclamide, inhibited vascular relaxation caused by hypoxia (95% $N_2$ and 5% $CO_2$), but not the epithelium-dependent relaxation of rat aorta in co-axial preparations. Furthermore, an elevation of cyclic GMP levels in rat aorta associated with the relaxation was observed in co-axial bioassay preparations (Hay *et al.*, 1989; Spina *et al.*, 1991) but not under hypoxic conditions (present study). In addition, the $t_{\frac{1}{2}}$ for relaxation of rat aorta induced by hypoxia (95% $N_2$ and 5% $CO_2$) and in co-axial bioassay are significantly different.

The results of this study demonstrate that phenylephrine-contracted endothelium-denuded rat aorta relaxed in a time-dependent manner when bubbled with 95% $N_2$ and 5% $CO_2$. Within 3 min of bubbling with 95% $N_2$ and 5% $CO_2$, bath $Po_2$ fell by 82% and remained unaltered for the next 30 min. However, no effect on tension was observed at 5 min, although maximal relaxation was achieved by 25–30 min. In sharp contrast, relaxation in co-axial assemblies started within 1 min of challenge with histamine (100 μM) or methacholine (25 μM) and was maximal within 10 min (Fernandes *et al.*, 1989). Similar data were obtained in co-axial assemblies using both guinea-pig trachea and rabbit bronchus in the present study, suggesting that different mechanisms are responsible for hypoxia- and co-axial-induced relaxation. The hypoxia-induced reversal of vascular smooth muscle tone has previously been observed in cerebral arteries (Pearce *et al.*, 1989) and aorta (Coburn *et al.*, 1986). In this study, the magnitude of the hypoxia-induced reversal of tone was less in spiral than in cross-cut preparations. The reasons for this are not clear. The observation that in spiral preparations the hypoxia-induced reversal of tone was not associated with significant changes in intracellular levels of cyclic GMP is consistent with a number of studies demonstrating no increase or a reduction in intracellular levels of cyclic GMP during hypoxia-induced vasodilatation in endothelium-denuded vessels (Coburn *et al.*, 1986; Burke-Wolin & Wolin, 1989; Pearce *et al.*, 1989; Rodman *et al.*, 1990). Importantly, these findings contrast with the increase in intracellular cyclic GMP observed in vessels in co-axial bioassay (Hay *et al.*, 1989; Spina *et al.*, 1991).

It has recently been reported that the hypoxia-induced coronary artery vasodilatation was inhibited by the $K^+$-channel blocker glibenclamide *in vitro* (Daut *et al.*, 1990). We have demonstrated that glibenclamide attenuated hypoxia-induced relaxation of phenylephrine-contracted endothelium-denuded rat aorta as assessed by % reversal of phenylephrine-induced tone, $t_{\frac{1}{2}}$ of relaxation and area under the % relaxation versus time curve. Unlike the inhibitory effects on vascular responses to hypoxia induced by bubbling with 95% $N_2$ and 5% $CO_2$, glibenclamide failed to alter relaxant responses observed in co-axial bioassays as assessed by measurements of % $E_{max}$ or $t_{\frac{1}{2}}$ of relaxation when either guinea-pig trachea and rabbit bronchus was used. These results also suggest that EpDIF does not activate glibenclamide-sensitive $K^+$ channels.

A surprising observation for which we have no explanation, was that glibenclamide produced greater inhibition of the hypoxia-induced response after this drug had been washed from the bath. No such washout response was observed in coronary artery (Daut *et al.*, 1990) or in CA3 hippocampal neurones (Mourre *et al.*, 1989). The much higher concentration of glibenclamide used and the longer incubation period employed in the present study may account for this apparent discrepancy.

In summary, the data from the present series of experiments suggest that the mechanisms of hypoxia-induced vascular smooth muscle relaxation and the relaxant responses observed to spasmogens in co-axial bioassay assemblies can be distinguished. Thus, this study provides further evidence that the relaxant response observed in co-axial assemblies is not attributable to hypoxia.

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# Tachykininergic transmission to the circular muscle of the guinea-pig ileum: evidence for the involvement of $NK_2$ receptors

Lorand Bartho, *Paolo Santicioli, *Riccardo Patacchini & [1]*Carlo Alberto Maggi

Department of Pharmacology, University Medical School of Pecs, H-7643 Pecs Hungary and *Pharmacology Department, A. Menarini Pharmaceuticals, Via Sette Santi 3, 50131 Florence, Italy

**1** The effect of newly developed, receptor-selective tachykinin antagonists (GR 71,251 for $NK_1$ receptors, MEN 10,376 and L 659,877 for $NK_2$ receptors) on noncholinergic transmission to the circular muscle of the guinea-pig ileum has been investigated.

**2** In circular muscle strips of the ileum, electrical field stimulation in the presence of atropine (2 μM) and apamin (0.1 μM) evoked a complex motor response. The tonic primary contraction in this response was reduced by GR 71,251 (10 μM) and MEN 10,376 (3–10 μM) but not by L 659,877 (up to 10 μM). The presence of apamin was necessary in this experimental arrangement to unmask an atropine-resistant primary contraction, sensitive to tachykinin antagonists. The motor response was abolished by tetrodotoxin.

**3** In circular strips of the ileum GR 71,251 (10 μM) inhibited the tonic contraction produced by [$Sar^9$] substance P sulphone, a selective $NK_1$ receptor agonist but not that produced by [β $Ala^8$] neurokinin A (4–10), a selective $NK_2$ receptor agonist. By contrast, MEN 10,376 antagonized the effect of the $NK_2$ agonist while leaving the response to the $NK_1$ agonist unaffected.

**4** In whole segments of the ileum, distension of the gut wall by an intraluminal balloon placed at about 1 cm from the point of recording of mechanical activity of the circular muscle produced atropine-sensitive phasic contractions (ascending enteric reflex). In the presence of atropine (2 μM), a noncholinergic response was elicited, which required larger volumes of distension that the cholinergic one. The atropine-resistant ascending enteric reflex was enhanced by apamin (0.1 μM) and abolished by tetrodotoxin, either in the presence or absence of apamin.

**5** MEN 10,376 (3–10 μM) inhibited the atropine-resistant ascending enteric reflex in the presence of apamin while GR 71,251 or L 659,877 (10 μM each) were ineffective. MEN 10,376 inhibited the atropine-resistant ascending enteric reflex to a larger extent in the absence than in the presence of apamin and also slightly inhibited the ascending enteric reflex in the absence of atropine.

**6** These findings provide evidence for an involvement of $NK_2$ tachykinin receptors in excitatory transmission to the circular muscle of the guinea-pig ileum. $NK_2$ receptors are also involved in the physiological-like circular muscle activation produced by stimulation of intramural neuronal pathways which subserve the atropine-resistant ascending enteric reflex.

**Keywords:** Tachykinins; tachykinin receptors; guinea-pig ileum; circular muscle; ascending enteric reflex

## Introduction

Ample evidence, based on pharmacological, physiological, anatomical and neurochemical findings, indicates that tachykinins (TKs) play a physiological role as excitatory transmitters in the guinea-pig ileum (Franco *et al.*, 1979; Costa *et al.*, 1981; 1985a,b; Bartho *et al.*, 1982a,b; Donnerer *et al.*, 1984; Smith & Furness, 1988; Llewellyn-Smith *et al.*, 1988; 1989; Bartho *et al.*, 1989; Holzer, 1989; see also Bartho & Holzer, 1985 for review). In the guinea-pig small intestine, substance P and other TKs, such as neurokinin A (NKA) are present (Too *et al.*, 1989).

The longitudinal contractile response of the guinea-pig ileum to TKs is mediated by specific receptors (Lee *et al.*, 1982). There is evidence that $NK_1$ receptors mediate the direct response of muscle cells while $NK_3$ receptors activate intramural effector neurones which in turn release acetylcholine and possibly endogenous TKs (Kilbinger *et al.*, 1986; Laufer *et al.*, 1986; 1988; Guard & Watson, 1987). In spite of its importance for the genesis of propulsive activity and persistalsis, the effects of TKs on the circular muscle have been studied less (Holzer *et al.*, 1980; Costa *et al.*, 1985a,b). By use of natural TKs and receptor-selective TK agonists, evidence has been presented that $NK_1$, $NK_2$ and $NK_3$ receptors mediate the spasmogenic action of TKs in the circular muscle of the guinea-pig ileum (Maggi *et al.*, 1990a); the response to $NK_2$ receptor stimulation seems to be entirely ascribable to direct smooth muscle activation, that produced by $NK_3$ receptor stimulation is entirely neurogenic while that produced by $NK_1$ receptor stimulation has intermediate characteristics.

Pharmacological evidence for an involvement of *endogenous* TKs in atropine-resistant peristalsis of the ileum comes from studies using TK antagonists of the first generation, such as Spantide I or [D-$Pro^2$, D-$Trp^{7,9}$] substance P. These substances have been shown to inhibit atropine-resistant, nerve-mediated longitudinal contractions (Björkroth, 1983; Bartho *et al.*, 1983) as well as the atropine-resistant components of the peristaltic reflex and of the ascending enteric reflex (Bartho *et al.*, 1982a,b; Costa *et al.*, 1985a,b; Grider, 1989; Holzer, 1989).

However, TK antagonists of the first generation have little if any ability to discriminate between different TK receptors (Regoli, 1985; Buck & Shatzer, 1988) and consequently, the relative role of substance P (SP) and NKA in the overall response cannot be deduced. With the introduciton of new, second generation TK antagonists, specific for different types of TK receptors, it has become possible to obtain more information about the relative contributions of different receptor types and, consequently, of the various TKs, in nerve-mediated responses in different tissues (Maggi *et al.*, 1991a,b). The aim of this study was to address the question

[1] Author for correspondence.

of the relative role of $NK_1$ and $NK_2$ receptors in the physiological activation of the circular muscle of the guinea-pig ileum by using some of the newly developed receptor-selective antagonists to block atropine-resistant motor responses produced by nerve activation.

## Methods

### General

Male albino guinea-pigs (250–300 g) were stunned and bled. A segment of the ileum was excised and placed in oxygenated (95% $O_2$ and 4% $CO_2$) Krebs solution.

### Circular muscle strips

The ileum was opened along the mesenteric border and pinned flat on a Petri dish. A small strip (approximately 3 mm wide) was then dissected along the circular axis as described by Costa *et al.* (1985b) with the only exception that no attempt was made to remove the mucosal layer, in order to avoid any possible damage to the inner circular muscle. The strips were transferred to 5 ml baths for isotonic recording (load 3 mN) of mechanical activity via a Basile 7050 Unirecord. In some experiments, the strips were electrically stimulated by means of two platinum wire electrodes (distance between electrodes, 5 cm) placed at the top and bottom of the organ bath, connected to a GRASS S88 stimulator. All experiments started after a 120 min equilibration period. In a first series of experiments, three different receptor-selective tachykinin antagonists, GR 71251 ($NK_1$ receptor selective, Hagan *et al.*, 1990; Hall & Morton, 1991), MEN 10,376 ($NK_2$ receptor selective, Maggi *et al.*, 1991c) and L 659,877 ($NK_2$ receptor selective, Williams *et al.*, 1988) were studied on the noncholinergic (atropine-resistant) contraction produced by electrical stimulation (5 Hz for 30 s, 0.15 ms pulse width, maximal voltage) in the presence of atropine (2 μM) and apamin (0.1 μM). Apamin was added to the bath in order to unmask a primary atropine-resistant contraction to field stimulation (see Results).

After having recorded two or more reproducible responses to electrical stimulation at 20 min intervals, the stated concentration of the antagonist was added to the bath and left in contact for 15 min.

In a second series of experiments, the selectivity of antagonists was tested against circular muscle contractions produced by the $NK_1$ receptor-selective agonist [$Sar^9$] substance P sulphone (Drapeau *et al.*, 1987) or the $NK_2$ receptor-selective agonist, [β $Ala^8$] neurokinin A (4–10) (Rovero *et al.*, 1989; Maggi *et al.*, 1990b). The concentration of the two agonists (300 nM) was selected in order to produce a contraction comparable to that produced by electrical stimulation. Experiments were performed in the presence of atropine (2 μM) and apamin (0.1 μM).

In a third series of experiments the effects of GR 71,251 and MEN 10,376 toward field stimulation-induced contraction were investigated in the absence of atropine and apamin. Stimulation of 10 Hz for 30 s resulted in a primary contraction comparable to that observed in the presence of atropine and apamin. All contractile responses were expressed as % of maximal response to 160 mM KCl.

### Ascending enteric reflex

The effect of TK antagonists on the ascending enteric reflex produced by radial stretch (balloon distension) was studied in 6–7 cm long segments of whole ileum by use of an experimental arrangement similar to that described by Holzer (1989). The ileal segment was secured horizontally at the bottom of a 6 ml flat glass organ bath by a metal bar passed through the lumen. The bath was filled with oxygenated Krebs solution and warmed to 37°C. The mechanical activity of the circular muscle was recorded via a thread secured to the mesentery close to the intestinal wall. The thread connected the ileum to an auxotonic transducer and the preparation was kept under a resting load of 5 mN. The ascending enteric reflex was elicited either in the absence or in the presence of atropine (2 μM) and, in atropine-treated preparations either in the presence or absence of apamin (0.1 μM). Distension was carried out by inflating a latex balloon (Hugo Sachs, size 5, unloaded volume 100 μl) placed at about 1 cm caudal to the recording site; for experiments without atropine a volume of 0.1 ml was sufficient to elicit maximal responses, while in the presence of atropine larger volumes (0.2–0.3 ml) were required. After having recorded at least 3 reproducible responses to balloon distension (10–15 min apart from each other) drugs were added to the bath. The neurogenic nature of the evoked response was checked at the end of experiments by addition of tetrodotoxin (1 μM). Amplitude of evoked responses was expressed as % of the response to 80 mM KCl which produced maximal circular shortening of the preparation. All the responses evoked by balloon distension, either in the presence or absence of atropine or apamin were abolished by tetrodotoxin.

### Statistical analysis

All data in the text are mean ± standard error (s.e.) of the mean. Statistical analysis was performed by means of Student's *t* test for paired or unpaired data or by analysis of variance, when applicable.

### Drugs

Drugs used were: atropine HCl (Serva, Heidelberg, Germany), tetrodotoxin (Sankyo, Tokyo, Japan), apamin (Sigma). The $NK_2$-selective antagonist L 659,877 (cyclo(Leu-Met-Gln-Trp-Phe-Gly)) was purchased from Cambridge Research Biochemicals, England. [β$Ala^8$]-NKA (4–10) and MEN 10,376 were synthesized by Dr P. Rovero, Chemistry Department, Menarini Pharmaceuticals, by conventional solid-phase methods. GR 71,251 ([D-$Pro^9$[Spiro-γ-lactam]$Leu^{10}$,$Trp^{11}$]SP (1-11)) was kindly provided by Dr R. Hagan, Department of Neuropharmacology, Glaxo Group Research, Ware, England.

Concentrated solutions (1–10 mM) of L 659,877, MEN 10,376 and [β$Ala^8$]-neurokinin A(4–10) were prepared in dimethylsulphoxide (DMSO) and diluted in Krebs solution. Control experiments showed that DMSO alone (0.1–0.3% final concentration) had no effect on the responses under study. The other peptides were dissolved in Krebs solution. Concentrations of L 659,877 greater than 10 μM were not tested because of precipitation in the bath.

## Results

### Circular muscle strips: response to electrical stimulation

In the presence of atropine (2 μM), electrical field stimulation (1–20 Hz) usually failed to evoke a primary contraction of circular muscle strips (Figures 1 and 2). During stimulus delivery either a slight relaxation was observed in some strips or no effect at all. A large 'off' contraction was observed at the end of the stimulus period (30 s). Preliminary experiments showed that the 'off' contraction was hardly affected by tachykinin antagonists, although it was suppressed by tetrodotoxin. Since apamin has been reported to exert a facilitatory action on the putatively tachykininergic excitation of circular muscle (Holzer *et al.*, 1989), its effect was investigated. Apamin (0.1 μM) induced or increased submaximal phasic contractions in nearly all preparations tested; this effect was long-lasting.

In the presence of apamin, an atropine-resistant primary contraction to field stimulation was unmasked. A typical response to 1–10 Hz field stimulation (see Figures 1 and 2)

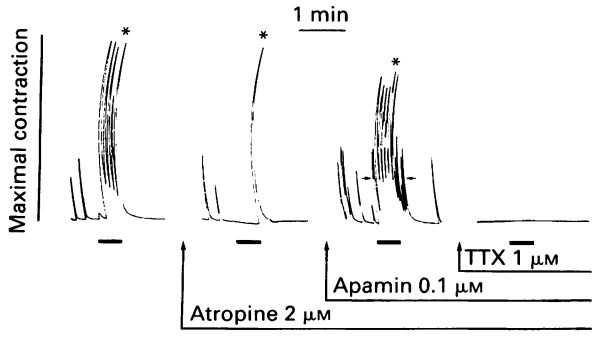


**Figure 1** Typical tracing showing the response of circular muscle strips from the guinea-pig ileum to electrical field stimulation (5 Hz for 30 s, horizontal bars) in the absence and presence of atropine and unmasking of an atropine-resistant primary contraction by apamin. Tetrodotoxin (TTX) abolished all responses to electrical stimulation, demonstrating their neural origin. Vertical scale indicates maximal response to KCl (160 mM). Horizontal arrowheads show the height of the primary tonic contraction recorded in the presence of atropine and apamin which has been used for quantitative evaluation. Asterisks mark the 'off' contraction.

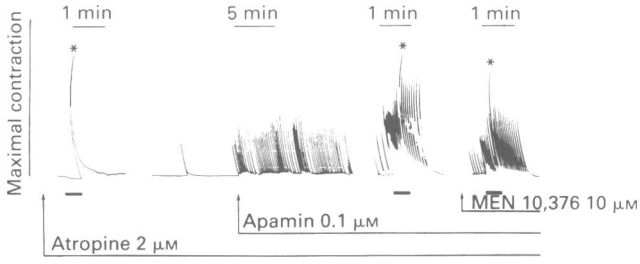


**Figure 2** Typical tracing illustrating the effect of apamin on spontaneous activity of circular muscle strips from the guinea-pig ileum, unmasking of an atropine-resistant primary contraction to electrical stimulation (5 Hz) and its inhibition by MEN 10,376 (15 min beforehand) an $NK_2$ receptor-selective tachykinin antagonist. Vertical scale indicates maximal response to KCl (160 mM). Asterisks mark the 'off' contractions.

consisted of the following phases: (1) initial twitch-like contraction, starting 1–2 s after the beginning of stimulation; (2) tonic contraction with superimposed phasic contractions; this phase could be clearly distinguished from the initial response and was present until stimulation was stopped; (3) 'Off' contraction upon cessation of stimulation, followed by a variable number of phasic contractions while the intercontraction tone was returning to baseline. All these components of the motor response were abolished by tetrodotoxin (Figure 1).

For quantitative evaluation, the peak of the tonic component (phase 2) of the contractile response was used (see Figure 1) because of its highest sensitivity to TK antagonists. Phase 3 was considerably less affected by TK antagonists while phase 1, which was depressed similarly to the tonic contraction, was more variable during repeated control stimulations.

For the rest of the study of atropine-resistant contraction, a stimulation frequency of 5 Hz was selected in order to produce a tonic primary contraction averaging 30–40% of maximal response to 160 mM KCl.

The effect of GR 71,251, MEN 10,376 and L 659,877 on the response to electrical stimulation (5 Hz for 30 s) was tested in the presence of atropine and apamin. GR 71,251 (10 μM) produced 52% inhibition of the evoked response (Figure 3). MEN 10,376 caused 47 and 75% inhibition of the evoked response at 3 and 10 μM, respectively (Figure 3, see also Figure 2). L 659,877 failed to elicit a significant change at all concentrations tested (Figure 3).

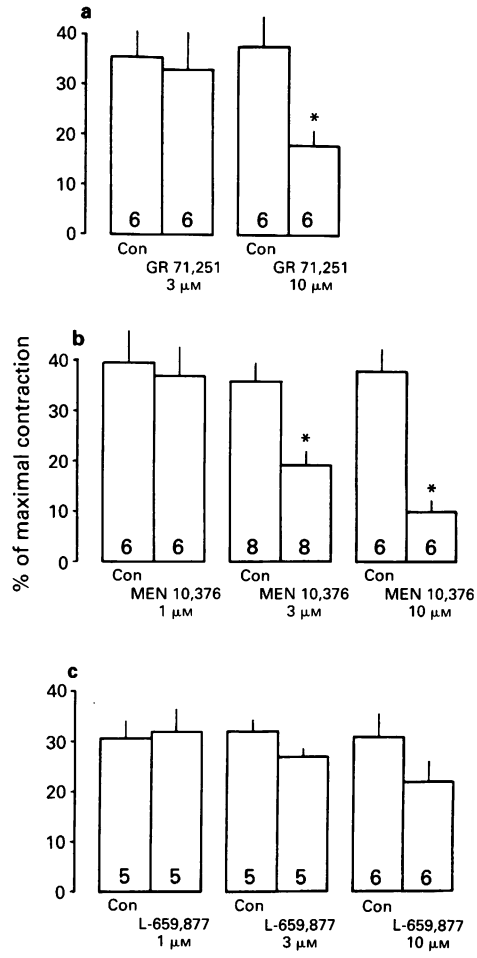


**Figure 3** Effect of (a) GR 71,251 (b) MEN 10,376 or (c) L659,877 on the primary tonic contraction produced by electrical stimulation of circular muscle strips from the guinea-pig ileum in the presence of atropine (2 μM) and apamin (0.1 μM). For each panel, the control (Con) response is compared to that obtained in the presence of the antagonist. Numbers in the columns indicate the number of replicates of each experiment. Vertical scale indicates maximal response to KCl (160 mM). *Significantly different from controls: $P<0.05$.

### *Circular muscle strips: effect of antagonists on the response to receptor selective tachykinin agonists*

From the above experiments, GR 71,251 and MEN 10,376 were selected for further evaluation of selectivity in blocking tachykinin receptors.

To check the selectivity of antagonists for $NK_1$ vs $NK_2$ TK receptors, circular muscle strips were challenged at 20 min intervals with the $NK_1$ receptor-selective agonist [$Sar^9$] substance P sulphone or the $NK_2$ receptor-selective agonist [$\beta Ala^8$] neurokinin A (4–10) (300 nM for each agonist) which resulted in contractions of similar size (30–50% of the maximum) to those evoked by electrical stimulation at 5 Hz. Here again, the responses consisted of a tonic contraction and superimposed phasic contractions. The amplitude of tonic contractions was measured to quantitate the effect of antagonists. These experiments were performed in the presence of atropine and apamin.

Data shown in Figure 4 confirmed the selectivity of GR 71,251 and MEN 10,376: GR 71,251 (10 μM) reduced (61% inhibition) the response to the $NK_1$ receptor-selective agonist, while leaving the response to [$\beta Ala^8$]- neurokinin A(4–10) unaffected. By contrast, MEN 10,376 did not affect the response to [$Sar^9$] substance P sulphone while reducing (80% inhibition) that to the $NK_2$ receptor-selective agonist.

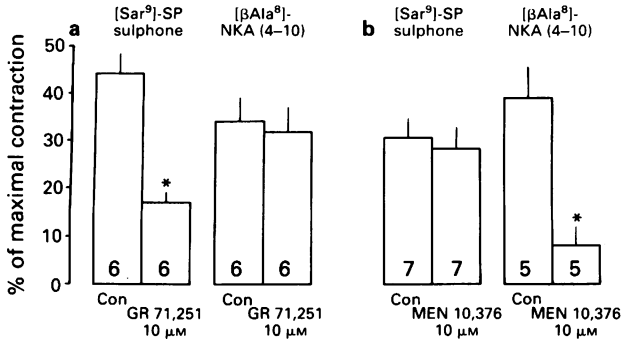


**Figure 4** Effect of (a) GR 71,251 or (b) MEN 10,376 on the tonic response of circular muscle strips from the guinea-pig ileum to the $NK_1$ tachykinin receptor-selective agonist [$Sar^9$]-SP sulphone or the $NK_2$ receptor-selective agonist [$\beta Ala^8$]-NKA(4–10) in the presence of atropine and apamin. For each panel, the control (Con) response is compared to that obtained in the presence of the antagonist. Numbers in the columns indicate the number of replicates of each experiment. Vertical scale indicates maximal response to KCl (160 mM). *Significantly different from controls: $P<0.05$.

### *Effect of antagonists on the response to field stimulation in the absence of atropine and apamin*

The effect of GR 71,251 and MEN 10,376 was also investigated on the primary contraction produced by electrical stimulation in the absence of atropine and apamin. A frequency of 10 Hz (other parameters as described above for the atropine-resistant response) was selected to obtain consistently a tonic contraction comparable to that produced by 5 Hz in the presence of atropine and apamin. Data shown in Figure 5 indicate that GR 71,251 (10 μM) or MEN 10,376 (3 μM) had no effect, while 10 μM MEN 10,376 produced a significant reduction of the evoked response.

### *Ascending enteric reflex*

Distension of the balloon (0.1 ml) in untreated preparations produced a rapid contraction of the ileum (84 ± 5% of maximal response to KCl, $n = 15$) which was largely or totally atropine-sensitive (reduction 85% or more), confirming previous observations from Costa *et al.* (1985b) and Holzer (1989). In the presence of atropine (2 μM) an atropine-resistant response to distension developed in about 30 min, the amplitude of which averaged 61 ± 5% ($n = 16$) of the maximal contraction to KCl. The atropine-resistant reflex required larger distension of the balloon (0.2–0.3 ml) than that produced in the absence of atropine to give a maximal response. In confirmation of previous observations (Holzer *et al.*, 1989), apamin (0.1 μM) enhanced the amplitude of the atropine-resistant response to distension, which averaged 81 ± 4% of the KCl response ($n = 19$).

The effect of GR 71,251 (10 μM), MEN 10,376 (3 and 10 μM) and L 659,877 (10 μM) was first studied in the presence of atropine and apamin, in order to match experiments performed in circular strips. In these experiments, MEN 10,376 had a limited and statistically not significant effect at 3 μM, while producing a significant inhibition at 10 μM (about 50% reduction as compared to control, Figure 6). GR 71,251 or L 659,877 (10 μM, $n = 4$ for each peptide) failed to affect the ascending enteric reflex in the presence of atropine and apamin (Figure 6).

The effect of MEN 10,376 (3–10 μM) was also investigated in the absence and presence of atropine (balloon distension with 0.1 and 0.3 ml, respectively, Figures 7 and 8). Apamin was not present in these experiments. Data in Figure 8 show that 3 μM MEN 10,376 had no effect in the absence of atropine while 10 μM significantly reduced the evoked response by about 30%. In the presence of atropine, both

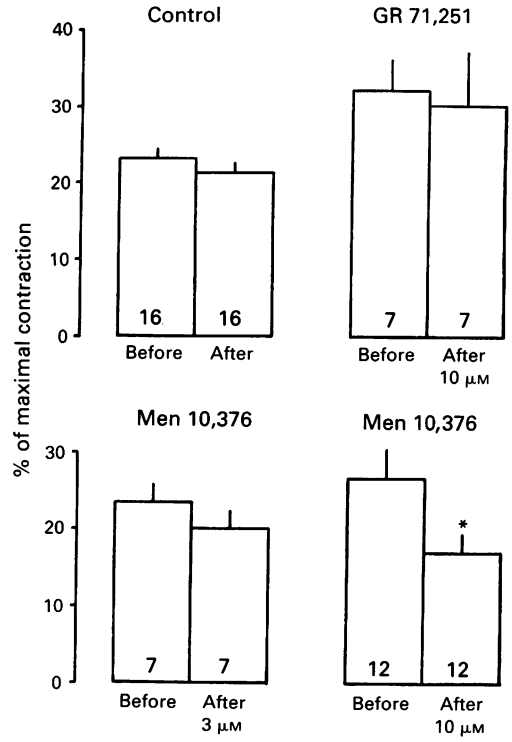


**Figure 5** Effect of GR 71,251 or MEN 10,376 on the tonic contractile response of circular muscle strips from the guinea-pig ileum to electrical field stimulation (10 Hz) in the absence of atropine. Numbers in the columns indicate the number of replicates of each experiment. Vertical scale indicates maximal response to KCl (160 mM). *Significantly different from controls: $P<0.05$.

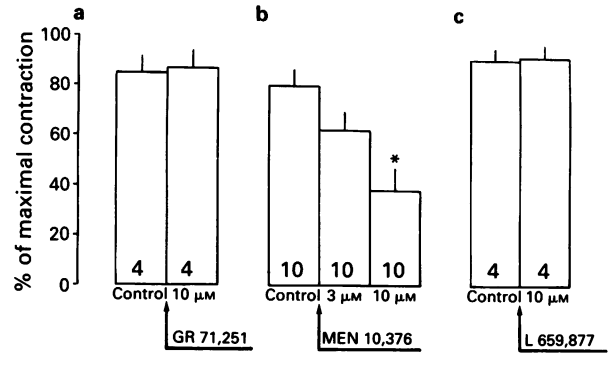


**Figure 6** Effect of (a) GR 71,251, (b) MEN 10,376 or (c) L 659,877 on the ascending excitatory reflex in the guinea-pig isolated ileum elicited by balloon distension in the presence of atropine and apamin. Numbers in the columns show the number of replicates of each experiment. Vertical scale indicates maximal response to KCl (80 mM). *Significantly different from controls: $P<0.05$.

concentrations of MEN 10,376 were effective and the response was inhibited by 39 and 70% at 3 and 10 μM, respectively. In the presence of atropine, complete inhibition of the evoked response by 10 μM MEN 10,376 was observed in 3 out of 10 cases tested.

## Discussion

Previous studies have implicated TKs as transmitters in the atropine-resistant contraction of the circular muscle of the guinea-pig ileum produced by electrical field stimulation or stimulation of the ascending enteric reflex (Costa *et al.*, 1985a,b; Holzer, 1989). The aim of this study was to gain

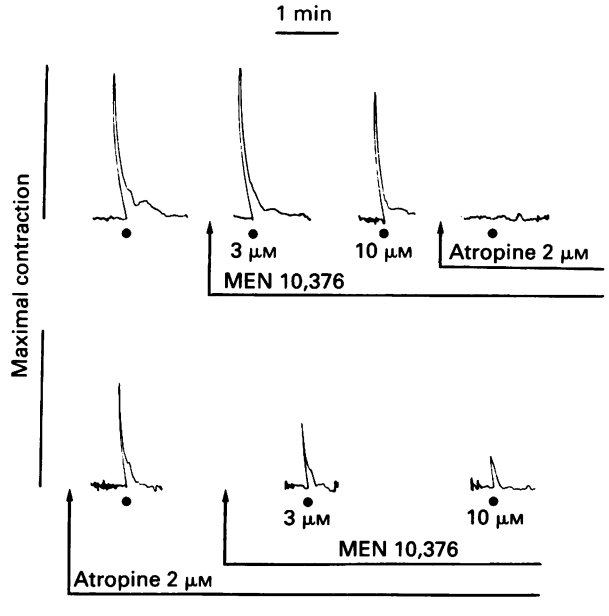


**Figure 7** Typical tracing showing the effect of MEN 10,376 (contact time 15 min) on the ascending enteric reflex of the guinea-pig isolated ileum in the absence (upper panel) or presence (lower panel) of atropine. Vertical scale indicates maximal response to KCl (80 mM).

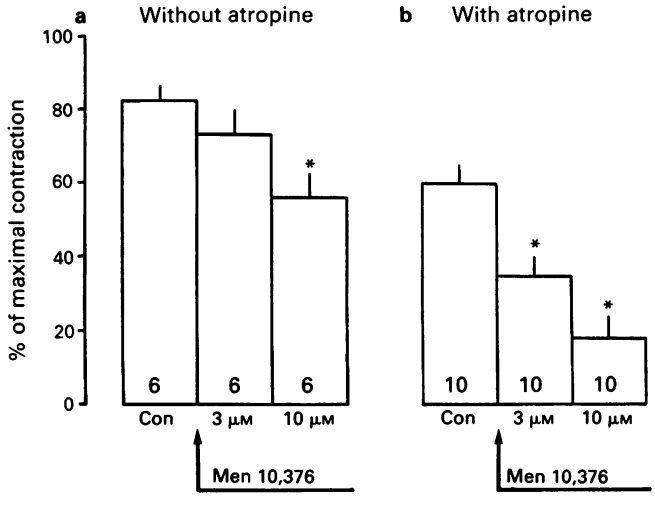


**Figure 8** Effect of MEN 10,376 on the ascending enteric reflex of the guinea-pig isolated ileum in the absence (a) or presence (b) of atropine. Numbers in the columns show the number of replicates of each experiment. Vertical scale indicates maximal response to KCl (80 mM). Con = control. *Significantly different from controls: $P<0.05$.

information about the relative role of $NK_1$ and $NK_2$ tachykinin receptors in mediating circular muscle contraction produced by activation of noncholinergic nerves in the ileum. Assuming a concomitant release of SP and NKA from tachykininergic nerves of the ileum and, owing to the preferential activity of SP at $NK_1$ and NKA at $NK_2$ receptors, respectively, the use of receptor-selective TK antagonists should theoretically yield information about the relative contributions of different TKs to the overall response (cf. Maggi *et al.*, 1991b,c). The conclusions of this study are strictly dependent on the selectivity of the antagonists used.

GR 71,251 is a newly developed derivative of substance P which acts as a competitive $NK_1$ receptor antagonist having at least 100 fold selectivity over $NK_2$ or $NK_3$ receptors. In the longitudinal muscle of the ileum, and with substance P methylester as an agonist, GR 71,251 reportedly blocks $NK_1$ receptor activation with a $pA_2$ value of 7.7–7.8 (Hagan *et al.*, 1990; Hall & Morton, 1991).

MEN 10,376 and L 659,877 have been characterized as $NK_2$ receptor selective antagonists having at least 100 fold selectivity over $NK_1$ or $NK_3$ receptors (Williams *et al.*, 1988; Maggi *et al.*, 1991c). For both peptides, however, affinity at $NK_2$ receptors varies in different preparations and this has been taken as evidence for the existence of $NK_2$ receptor subtypes (Maggi *et al.*, 1990c; 1991d; Van Giersbergen *et al.*, 1991). In the present study, GR 71,251 and MEN 10,376 behaved as selective $NK_1$ and $NK_2$ receptor antagonists, respectively. L 659,877 failed to affect significantly the atropine-resistant response to nerve stimulation. This may indicate the presence, in the circular muscle of guinea-pig ileum, of an $NK_2$ receptor subtype for which L 659,877 has low affinity (or anyway lower affinity than that of MEN 10,376).

The reason(s) why a TK-mediated response became evident only in the presence of apamin cannot be conclusively determined on the basis of present findings. Whatever the mechanisms involved, an atropine-resistant circular muscle contraction sensitive to TK antagonists is easily detected by use of the ascending reflex arrangement, even without addition of apamin. It is evident that in the latter case, a discrete intramural neuronal circuitry is activated, leading to excitation of circular muscle at the recording site (Tonini & Costa, 1990), while electrical field stimulation is likely to activate all intramural neurones.

As far as the participation of $NK_1$ receptors is concerned, GR 71,251, at a concentration that selectively blocks $NK_1$ receptors in the ileum, reduced the noncholinergic response of circular strips to field stimulation while it had almost no effect on the atropine-resistant ascending enteric reflex. In both cases, experiments were performed in the presence of apamin. The available data indicate that although $NK_1$ receptors are present in the circular muscle of the ileum they play only a minor role during its physiological activation. Owing to the limited supply of GR 71,251 we were unable to extend the analysis of its action to other experimental conditions (e.g. studying the atropine-resistant ascending reflex in the absence of apamin).

The inhibitory actions of MEN 10,376 (3–10 μM) on the ascending enteric reflex and on the electrically-evoked circular muscle contractions provide strong experimental evidence for an involvement of $NK_2$ receptors both in the physiological and in electrically-evoked activation of the circular muscle of the ileum by endogenous TKs. MEN 10,376, at concentrations (3–10 μM) that selectively block $NK_2$ receptors in the circular muscle, inhibited the atropine-resistant ascending enteric reflex both in the absence and presence of apamin, as well as the primary contraction of circular muscle strips to electrical stimulation. MEN 10,376 also reduced the ascending enteric reflex and the response to electrical field stimulation in the absence of atropine, by about 30%. A similar effect has been reported previously when a high concentration (30 μM) of Spantide was used (Holzer, 1989) and may indicate a partial involvement of TKs in the ascending enteric reflex evoked in the absence of atropine. Confirming previous observations (Holzer, 1989) larger volumes of balloon distension were required to evoke maximal noncholinergic circular muscle contractions than those effective without atropine. This agrees with the hypothesis (Costa *et al.*, 1985a; Grider, 1989) that the tachykininergic component of the ascending reflex comes into action at larger degrees of radial stretch than the cholinergic one.

In conclusion, a major role for NKA, or anyway for endogenous ligands with high affinity for $NK_2$ TK receptors, can be postulated in the excitatory response of the circular muscle of the guinea-pig ileum to nerve stimulation.

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# Sodium-dependence and ouabain-sensitivity of the synthesis of dopamine in renal tissues of the rat

[1]P. Soares-da-Silva & [2]M. Helena Fernandes

Department of Pharmacology and Therapeutics, Faculty of Medicine, 4200 Porto, Portugal

**1** The present study has examined the influence of sodium chloride (0–160 mM) and ouabain (100 and 500 μM), an inhibitor of the enzyme $Na^+$–$K^+$ ATPase, on the synthesis of dopamine in slices of rat renal cortex loaded with exogenous L-dihydroxyphenylalanine (L-DOPA). The deamination of newly-formed dopamine into 3,4-dihydroxyphenylacetic acid (DOPAC) was also examined. The assay of L-DOPA, dopamine and DOPAC in kidney slices was performed by high performance liquid chromatography (h.p.l.c.)· with electrochemical detection.

**2** The accumulation of newly-formed dopamine and DOPAC in kidney slices loaded with L-DOPA (50 and 100 μM) was found to be dependent on the concentration of NaCl in the medium. A similar picture could be observed for DOPAC. The fractional rate of accumulation ($k$; mM $NaCl^{-1}$) was at 50 and 100 μM L-DOPA, respectively, 0.00305 ± 0.00036 and 0.00328 ± 0.00029 for dopamine and 0.00672 ± 0.00072 and 0.00641 ± 0.00069 for DOPAC. The sodium-dependent formation of dopamine was completely abolished when the experiments were performed in the absence of oxygen.

**3** In experiments performed in the presence of 120 mM NaCl, but not in conditions of low sodium (20 mM NaCl in the medium), ouabain (100 and 500 μM) was found to inhibit the accumulation of newly-formed dopamine and DOPAC (14–57% reduction; $P<0.05$); this effect was more marked at 50 and 100 μM L-DOPA. When the experiments were performed in the absence of oxygen the renal production of dopamine and DOPAC became markedly decreased (respectively, 40% and 77% reduction; $P<0.05$) and ouabain (100 and 500 μM) was no longer able to reduce the accumulation of both newly-formed dopamine and DOPAC.

**4** The formation of dopamine and its deamination to DOPAC in kidney homogenates closely depended on the concentration of L-DOPA added to the medium; ouabain (100, 500 and 1000 μM) was found to affect neither the formation of dopamine nor its deamination to DOPAC when homogenates were used instead of kidney slices.

**5** In conclusion, the results presented here show that the formation of dopamine in kidney slices loaded with L-DOPA is sodium-dependent and ouabain-sensitive suggesting the involvement of a co-transport system of sodium and DOPA into the tubular epithelial cell.

**Keywords:** Dopamine; DOPAC; L-DOPA; kidney; sodium; ouabain

## Introduction

There is evidence suggesting that most of the dopamine appearing in urine is produced in the kidney. Urinary excretion of dopamine is believed to reflect tubular amine production, as denervation has been found not to affect dopamine levels in urine (Baines, 1982; Suzuki *et al.*, 1984). Dietary sodium appears to be a determinant factor for dopamine production as evidenced by a close relationship between the concentration of urinary sodium and urinary dopamine (Cuche *et al.*, 1972) and the result that sodium loading is accompanied by an increased excretion of dopamine in the urine of both man and laboratory animals (Alexander *et al.*, 1974; Ball *et al.*, 1978; Baines, 1982; Goldstein *et al.*, 1989; Bass & Murphy, 1990; Lee *et al.*, 1990; Young, 1990). By contrast, a low sodium diet is accompanied by a decrease in the urinary excretion of dopamine (Baines, 1982; Goldstein *et al.*, 1989; Hayashi *et al.*, 1990). It has been reported that tubular transport of some aromatic amino acids is sodium-dependent (Ulrich *et al.*, 1974), and, therefore, it might be hypothesized that changes in the renal production of dopamine during modifications in the renal delivery of sodium would be related to modifications in the membrane transport of DOPA and subsequent effects on the intracellular availability of the amino acid. There is also evidence that the renal production of dopamine under *in vitro* conditions depends on the sodium chloride concentration in the medium (Hagege & Richet, 1985; Fernandes & Soares-da-Silva, 1990). Furthermore, it has also been suggested that α-human atrial natriuretic peptide and guanosine 3′:5′-cyclic monophosphate (cyclic GMP), which are effective inhibitors of the tubular transport of sodium (Zeidel *et al.*, 1987), may decrease the intracellular availability of L-DOPA in rat kidney slices and reduce the renal formation of dopamine (Soares-da-Silva & Fernandes, 1990a,b; 1991).

The aim of the present work was to characterize the role of extracellular sodium in the production of dopamine in rat renal tissues under *in vitro* conditions and to seek further evidence of the involvement of mechanisms regulating the tubular transport of sodium on the intracellular availability of L-DOPA and the synthesis of dopamine.

## Methods

Male Wistar rats (Biotério do Instituto Gulbenkian de Ciência, Oeiras, Portugal) aged 45–60 days and weighing 200–280 g were used in the experiments. Animals were kept two per cage under controlled environmental conditions (12 h light/dark cycle and room temperature 24°C). Food and tap water were allowed *ad libitum*. The experiments were all carried out during day time. The rats were killed by decapitation under ether anaesthesia and both kidneys removed and rinsed free from blood with saline (0.9% NaCl). The kidneys were

[1] Author for correspondence.
[2] Permanent address: Faculty of Medical Dentistry, 4200 Porto, Portugal.

placed on an ice cold glass plate, the kidney poles removed and slices of the renal cortex approximately 1.5 m thick and weighing about 40 mg wet weight were prepared with a scalpel. Thereafter, renal slices were preincubated for 60 min in 2 ml warmed (37°C) and gassed (95% $O_2$ and 5% $CO_2$) Krebs solution. The Krebs solution had the following composition (mM): NaCl 120, KCl 4.7, $CaCl_2$ 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 25, $KH_2PO_4$ 1.2, EDTA 0.4, ascorbic acid 0.57 and glucose 11; (−)-α-methyl-*p*-tyrosine (50 μM), tropolone (50 μM) and copper sulphate (10 μM) were also added to the Krebs solution in order to inhibit the enzymes tyrosine hydroxylase and catechol-*O*-methyltransferase and inhibit the endogenous inhibitors of dopamine β-hydroxylase, respectively. After preincubation, renal slices were incubated for 15 min in Krebs solution with added L-DOPA. The preincubation and incubation were carried out in glass test tubes, continuously shaken throughout the experiment. In experiments performed in order to test the influence of sodium on the renal production of dopamine, slices of renal cortex were incubated in Krebs solution with increasing concentrations (0–160 mM) of sodium chloride in the medium in the presence of 50 and 100 μM L-DOPA; the osmolarity of the incubation medium was kept constant by the addition of choline chloride. The other constituents of the Krebs solution were as described above. In some experiments, oxygenation was stopped during the incubation with L-DOPA (100 μM). In experiments in which the effects of ouabain (100 and 500 μM) were tested, the $Na^+$–$K^+$ ATPase inhibitor was present during the preincubation and incubation periods. Some of the experiments in which the effects of ouabain (100 and 500 μM) were tested have been performed in the absence of oxygen during the incubation period, i.e. after the addition of L-DOPA. In another group of experiments, kidney slices were incubated in the presence of 20 mM NaCl, with the osmolarity kept constant by the addition of 100 mM choline chloride, and the effect of 500 μM ouabain tested. After incubation, renal slices were collected, washed in ice cold saline, blotted with filter paper, weighed and stored in 2 ml of 0.2 M perchloric acid.

In some experiments kidney homogenates, instead of tissue slices, were used. Whole kidneys were homogenized in a modified Krebs solution with Duall-Kontes homogenizers and kept continuously on ice. A modified Krebs solution was also prepared, which was similar to that described above except that the NaCl concentration was reduced to 50 mM; the osmolarity of the medium was kept constant by the addition of 68 mM choline chloride. Aliquots of 1.0 ml of kidney homogenates plus 1.0 ml Krebs solution were placed in glass test tubes and incubated for 60 min with increasing concentrations of ouabain (100, 500 and 1000 μM). Thereafter, L-DOPA (10.0 μM) or dopamine (0.5, 2.5 and 5.0 μM) was added to the medium for further 15 min. During incubation, kidney homogenates were continuously shaken and gassed (95% $O_2$ and 5% $CO_2$) and maintained at 37°C. The reaction was stopped by the addition of 250 μl of 2 M perchloric acid and the preparations kept at 4°C for 60 min. The kidney homogenates were then centrifuged (2000 r.p.m., 2 min, 4°C) and 1.5 ml aliquots of the supernatant used for the assay of L-DOPA, dopamine and DOPAC.

The assay of L-DOPA, dopamine, noradrenaline and DOPAC in renal tissues and kidney homogenates was performed by means of h.p.l.c. with electrochemical detection, as previously described (Soares-da-Silva & Fernandes, 1991). In brief, aliquots of 1.5 ml of perchloric acid in which tissues have been kept or 1.5 ml of supernatant of kidney homogenates were placed in 5 ml conical-based glass vials with 50 mg alumina and the pH of the samples immediately adjusted to pH 8.6 by the addition of Tris buffer. The adsorbed catecholamines were then eluted from the alumina with 200 μl of 0.2 M perchloric acid on Millipore microfilters (MF1); 50 μl of the eluate was injected into a high pressure liquid chromatograph (Gilson Medical Electronics, Villiers le Bel, France). The lower limits for detection of L-DOPA, dopamine, noradrenaline and DOPAC were 1.0, 1.4, 0.9 and 2.0 pmol $g^{-1}$, respectively.

The protein content of the homogenates (mg of protein per g of tissues) was determined by the method of Lowry *et al.* (1951), with human serum albumin as a standard.

### *Statistics*

The accumulation of dopamine and DOPAC in kidney slices as a function of the concentration of sodium chloride in the medium was calculated from a semilog plot of the concentration of the amine and of its deaminated metabolite vs concentration of sodium chloride in the medium; the slope of accumulation was calculated by linear regression. The fractional rate constant of the accumulation of dopamine and DOPAC ($k$) was then obtained from the expression: $k$ = slope value/0.434 (Brodie *et al.*, 1966) and the sodium-dependent rates of amine and amine metabolite accumulation calculated by multiplying the tissue levels at 0 mM sodium chloride by the rate constant of accumulation.

Mean values ± s.e.mean of $n$ experiments are given. Significance of differences between one control and several experimental groups was evaluated by Tuckey-Kramer method (Sokal & Rohlf, 1981). A $P$ value less than 0.05 was assumed to denote a significant difference.

### *Drugs*

3,4-Dihydroxyphenylacetic acid (DOPAC), L-dihydroxyphenylalanine (L-DOPA), dopamine hydrochloride, (−)-α-methyl-*p*-tyrosine, ouabain, noradrenaline bitartrate and tropolone hydrochloride were purchased from Sigma Chemical Company (St. Louis, Mo, U.S.A.).

## Results

Figure 1 shows the semilog plots of accumulation of newly-formed dopamine and of its deaminated metabolite DOPAC in kidney slices loaded with 50 and 100 μM L-DOPA and incubated with increasing concentrations of sodium chloride in the medium. In the presence of either 50 or 100 μM L-DOPA, the accumulation of newly-formed dopamine and DOPAC was found to be exponential and dependent on the concentration of sodium chloride in the medium. The accumulation of newly-formed dopamine was greater in kidney slices loaded with the highest concentration of L-DOPA (100 μM), with the rate constant of accumulation of newly-formed dopamine being similar to that obtained when the preparations were loaded with a lower concentration of L-DOPA (50 μM). A similar picture could be observed for DOPAC; i.e., the tissue levels of DOPAC were greater in kidney slices incubated with 100 μM L-DOPA, but the rate constant of DOPAC accumulation was about the same with either 50 or 100 μM L-DOPA. This can be shown by the analysis of $k$ values shown in Table 1; the fractional rate of accumulation ($k$) was at 50 and 100 μM L-DOPA, respectively, 0.0031 and 0.0033 for dopamine and 0.0067 and 0.0064 for DOPAC.

It has been suggested that the transport of water and electrolytes in tubular epithelial cells is dependent on a high consumption of oxygen (Burg & Orloff, 1962) and the experiments described next were performed in order to test the sensitivity to oxygen of the sodium-dependent accumulation of newly-formed dopamine in kidney slices loaded with L-DOPA. In this set of experiments, tissues were incubated with L-DOPA (100 μM) with increasing concentrations of sodium chloride (0–160 mM) in the medium; only during the incubation period (15 min) with L-DOPA, but not during preincubation (60 min), was the oxygenation of tissues stopped. As can be observed in Figure 2, the sodium-dependent accumulation of newly-formed dopamine and DOPAC was completely abolished in experiments performed in the absence of oxygen. Also, as shown in Table 2, the constant rates of accumulation ($k$) of newly-formed dopamine and DOPAC were, respectively,

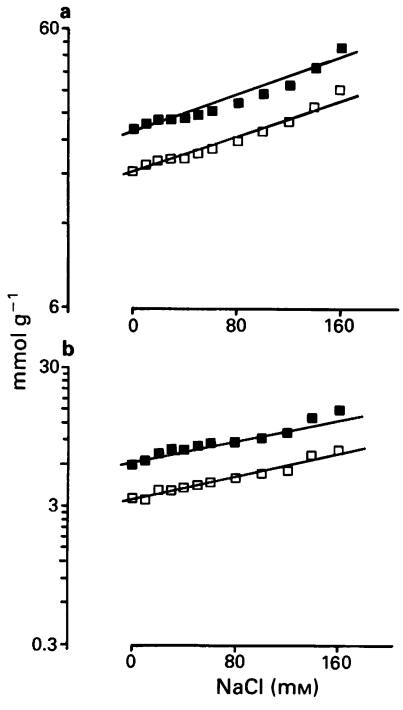


**Figure 1** Accumulation of (a) newly-formed dopamine and of (b) its deaminated metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in slices of rat renal cortex incubated for 15 min with L-dihydroxyphenylalanine (L-DOPA) in the presence of increasing concentrations (0–160 mM) of sodium chloride in the medium. Results are shown for observations of the accumulation of dopamine and DOPAC in the presence of 100 μM (■) and 50 μM (□) L-DOPA. Each point represents the mean of 5 to 6 determinations; s.e. of mean values were less than 10% of the corresponding means. Linear coefficient values were in the presence of 50 μM L-DOPA for dopamine, $r = 0.8954$, $n = 78$; DOPAC, $r = 0.7956$, $n = 78$; and in the presence of 100 μM L-DOPA for dopamine, $r = 0.9457$, $n = 60$; DOPAC, $r = 0.9135$, $n = 60$.

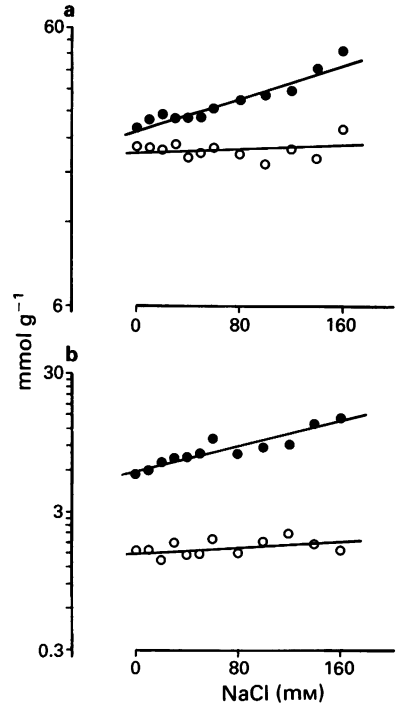


**Figure 2** Accumulation of (a) newly-formed dopamine and of (b) its deaminated metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in slices of rat renal cortex incubated for 15 min with 100 μM L-dihydroxyphenylalanine (L-DOPA) in the presence of increasing concentrations (0–160 mM) of sodium chloride in the medium. Results are shown for observations of the accumulation of dopamine and DOPAC in the presence (●) and absence of oxygenation (○) during incubation with L-DOPA. Each point represents the mean of 5 determinations; s.e. of mean values were less than 10% of the corresponding means. Linear coefficient values were in the presence of oxygenation of dopamine, $r = 0.9743$, $n = 58$; DOPAC, $r = 0.9569$, $n = 58$; and in the absence of oxygenation for dopamine, $r = 0.0978$, $n = 60$; DOPAC, $r = 0.1952$, $n = 59$.

**Table 1** Constant rates of accumulation ($k$) and turnover rates of dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) (in nmol $g^{-1}$ 15 $min^{-1}$ mM NaCl) in slices of rat renal cortex incubated with 50 and 100 μM L-dihydroxyphenylalanine (L-DOPA) for 15 min in the presence of increasing concentrations (0–160 mM) of sodium chloride in the medium

| | *Rate constant of accumulation* k *(mM $NaCl^{-1}$)* | *Turnover rate (nmol $g^{-1}$ 15 $min^{-1}$)* |
|---|---|---|
| *Dopamine* | | |
| 50 μM L-DOPA | 0.00305 ± 0.00036 | 0.0842 ± 0.0079* |
| 100 μM L-DOPA | 0.00328 ± 0.00029 | 0.0361 ± 0.0041 |
| *DOPAC* | | |
| 50 μM L-DOPA | 0.00672 ± 0.00072 | 0.0349 ± 0.0046* |
| 100 μM L-DOPA | 0.00641 ± 0.00069 | 0.0186 ± 0.0016 |

Results are shown for observations of the accumulation of dopamine and DOPAC during incubation with L-DOPA. Values are means ± s.e. mean of five to six experiments per group.
Significantly different from corresponding values for DOPAC: *$P<0.01$.

**Table 2** Constant rates of accumulation ($k$) and turnover rates of dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) (in nmol $g^{-1}$ 15 $min^{-1}$ mM NaCl) in slices of rat renal cortex incubated with L-dihydroxyphenylalanine (L-DOPA) (100 μM) for 15 min in the presence of increasing concentrations (0–160 mM) of sodium chloride in the medium

| | *Rate constant of accumulation* k *(mM $NaCl^{-1}$)* | *Turnover rate (nmol $g^{-1}$ 15 $min^{-1}$)* |
|---|---|---|
| *Dopamine* | | |
| With Oxygen | 0.00325 ± 0.00036* | 0.0836 ± 0.0089* |
| Without Oxygen | 0.00075 ± 0.00029 | 0.0151 ± 0.0060 |
| *DOPAC* | | |
| With Oxygen | 0.00684 ± 0.00105*,† | 0.0368 ± 0.0056*,† |
| Without Oxygen | 0.00055 ± 0.00009 | 0.0086 ± 0.0016 |

Results are shown for observations of the accumulation of dopamine and DOPAC in the presence and absence of oxygenation during incubation with L-DOPA. Values are means ± s.e. mean of five experiments per group.
Significantly different from corresponding values in the absence of oxygen: *$P<0.01$.
Significantly different for corresponding values for dopamine; †$P<0.01$.

four and ten fold lower when compared with experiments performed in the presence of oxygen. Another interesting result is that the tissue levels of newly-formed dopamine at 160 mM sodium chloride in the absence of oxygen were slightly lower than those at 0 mM sodium chloride in the presence of oxygen. The formation of DOPAC was also markedly reduced in experiments performed in the absence of oxygen and the accumulation of this deaminated metabolite of dopamine was no longer dependent on the concentration of sodium chloride in the medium.

The ultimate mechanism intervening in the process of

sodium transport across the tubular cells is that governed by the enzyme $Na^+$–$K^+$ ATPase and it is possible through the inhibition of this enzyme with ouabain to decrease the net tubular transport of sodium. The experiments in which the effect of ouabain was tested were performed in the presence of 120 mM sodium chloride in the medium; in some experiments oxygenation of tissues was stopped immediately before the addition of L-DOPA. As shown in Figure 3, the incubation of kidney slices with increasing concentrations of L-DOPA (10–100 μM) resulted in a concentration-dependent accumulation of newly-formed dopamine and DOPAC. The addition of ouabain (100 and 500 μM) to the incubation medium resulted in a concentration-dependent inhibition (14–57% reduction) in the accumulation of newly-formed dopamine and DOPAC; this effect was, however, more marked at 50 and 100 μM L-DOPA. In the set of experiments performed in the absence of oxygen during the incubation with L-DOPA, the renal production of dopamine and DOPAC became markedly decreased (respectively, 40% and 77% reduction) and ouabain (100 and 500 μM) was no longer able to reduce the accumulation of either newly-formed dopamine or DOPAC (Figure 4).

Figure 5 shows the results of experiments in which kidney slices were incubated with increasing concentrations of L-DOPA (10–1000 μM) and the concentration of sodium chloride in the medium was lowered to 20 mM. In these experimental conditions and in the presence of oxygen during the incubation with L-DOPA, 500 μM ouabain failed to reduce the accumulation of newly-formed dopamine and DOPAC.

As shown in Figure 6, the synthesis of dopamine and its deamination to DOPAC in kidney homogenates closely depended on the concentration of L-DOPA added to the medium. In contrast to that observed in kidney slices, ouabain (100, 500 and 1000 μM) was found to affect neither the formation of dopamine nor its deamination to DOPAC in homogenates of the rat kidney. Incubation of kidney homogenates with exogenous dopamine (0.5, 2.5 and 5.0 μM) was found to result in a concentration-dependent formation of DOPAC (Table 3). In this set of experiments, as has been found to occur in experiments in which kidney homogenates were incubated with L-DOPA, the addition of ouabain (100 and 500 μM) to the incubation medium was found not to affect the formation of DOPAC.

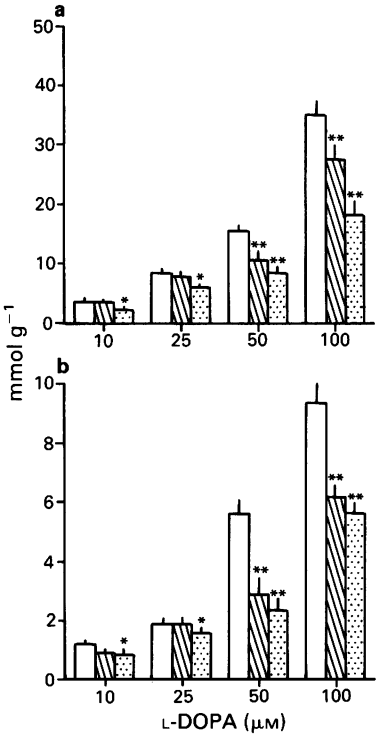


**Figure 3** Tissue levels of (a) dopamine and (b) 3,4-dihydroxyphenylacetic acid (DOPAC) (in nmol $g^{-1}$) in slices of the rat renal cortex loaded with increasing concentrations of L-dihydroxyphenylalanine (10–100 μM) in the presence of 120 mM sodium chloride and in the presence of oxygenation during incubation. Results are shown for observations of the accumulation of dopamine and DOPAC in control conditions and in the presence of ouabain (100 and 500 μM). Each column represents the mean of five experiments per group; vertical lines show s.e.mean. Significantly different from corresponding control values using Student's *t* test (*$P<0.05$; **$P<0.01$). Control, open columns; ouabain 100 μM, hatched columns; ouabain 500 μM, stippled columns.

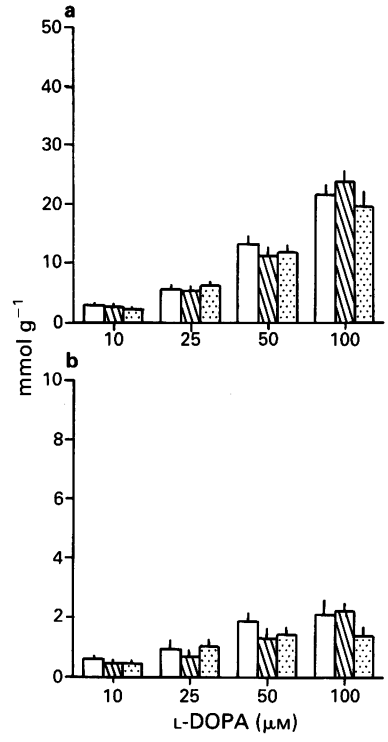


**Figure 4** Tissue levels of (a) dopamine and (b) 3,4-dihydroxyphenylacetic acid (DOPAC) (in nmol $g^{-1}$) in slices of the rat renal cortex loaded with increasing concentrations of L-dihydroxyphenylalanine (10–100 μM) in the presence of 120 mM sodium chloride and in the absence of oxygenation during incubation. Results are shown for observations of the accumulation of dopamine and DOPAC in control conditions and in the presence of ouabain (100 and 500 μM). Each column represents the mean of five experiments per group; vertical lines show s.e.mean. Control, open columns; ouabain 100 μM, hatched columns; ouabain 500 μM, stippled columns.

## Discussion

The present study shows that the renal formation of dopamine in kidney slices loaded with exogenous L-DOPA is dependent on the concentration of sodium chloride in the medium and sensitive to inhibition of the tubular transport of sodium. These results agree well with the earlier evidence of a decreased intracellular availability of L-DOPA in rat renal slices and reduced renal formation of dopamine as induced by α-human atrial natriuretic peptide and cyclic GMP, two effective inhibitors of the tubular transport of sodium (Soares-da-Silva & Fernandes, 1990a,b; 1991).

The sensitivity of the renal formation of dopamine in kidney slices loaded with L-DOPA is clearly shown in experiments in which renal tissues were incubated in the presence of increasing concentrations of sodium chloride. The accumulation of newly-formed dopamine in these experimental conditions as a function of the concentration of sodium in the medium was

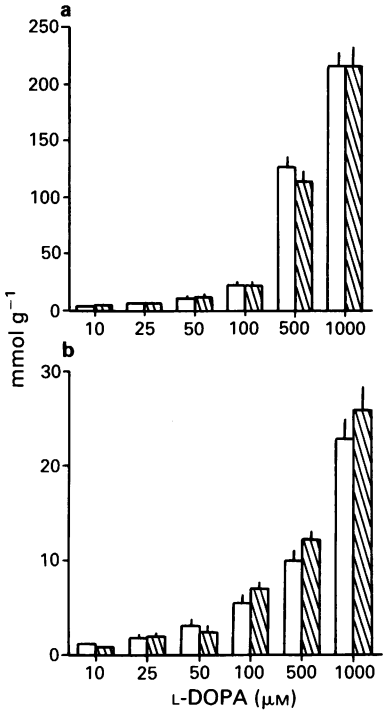


**Figure 5** Tissue levels of (a) dopamine and (b) 3,4-dihydroxyphenylacetic acid (DOPAC) (in nmol $g^{-1}$) in slices of the rat renal cortex loaded with increasing concentrations of L-dihydroxyphenylalanine (10–1000 μM) in the presence of 20 mM sodium chloride and in the presence of oxygenation during incubation. Results are shown for observations of the accumulation of dopamine and DOPAC in control conditions and in the presence of ouabain (500 μM). Each column represents the mean of five experiments per group; vertical lines show s.e.mean. Control, open columns; ouabain 500 μM, hatched columns.

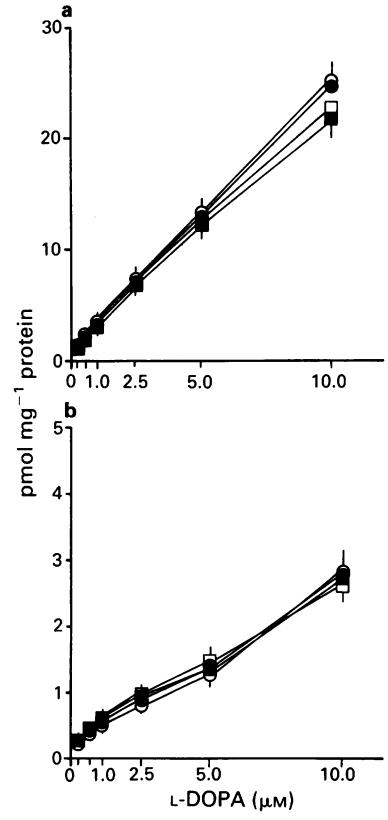


**Figure 6** Levels (in pmol $mg^{-1}$ protein) of (a) dopamine and (b) 3,4-dihydroxyphenylacetic acid (DOPAC) in whole kidney homogenates incubated with increasing concentrations of L-DOPA (0.1–10.0 μM) for 15 min in control conditions and in the presence of ouabain (100, 500 and 1000 μM). Each point represents the mean of five to six experiments per group; vertical lines show s.e.mean. Control (○); ouabain 100 μM, (●); ouabain 500 μM, (□); ouabain 1000 μM (■).

found to be monoexponential. The finding that the sodium-dependent monoexponential accumulation of newly-formed dopamine was completely abolished when tissues were incubated in the absence of oxygenation, strongly suggests that the mechanism responsible involves the facilitation of the tubular co-transport of sodium and L-DOPA. Another argument which favours this view is that concerning the result that the rate constant of accumulation of newly-formed dopamine was found to be similar with either 50 or 100 μM L-DOPA. The results presented here, however, also show that the tubular transport of L-DOPA coupled to sodium might not be the only one in operation, since in the absence of sodium chloride in the incubation medium and in the absence of oxygenation, kidney slices loaded with L-DOPA are still able to accumulate substantial amounts of newly-formed dopamine.

The experiments in which the effect of ouabain on the renal formation of dopamine was tested give further support to the view that the synthesis of dopamine in the kidney is dependent on the tubular transport of sodium. Ouabain is a well known inhibitor of $Na^+$–$K^+$ ATPase, the enzyme controlling the net trans-tubular transport of sodium in proximal renal tubules, and the concentration-dependent inhibitory effect of ouabain on the formation of renal dopamine fits well with the evidence that the tubular transport of L-DOPA is sodium-dependent. Another argument along this line is that the inhibitory effect of ouabain on the renal synthesis of dopamine is completely abolished when the experiments are performed in the absence of oxygen or when the concentration of sodium chloride in the medium is reduced to 20 mM; the specific activity of sodium inside the tubular epithelial cell has been reported to be higher than 20 mM (Lang *et al.*, 1986). The inhibitor effect of ouabain on the formation of dopamine and DOPAC appears, however, not to be the result of a direct action upon the enzymes

**Table 3** Levels (in pmol $mg^{-1}$ protein) of 3,4-dihydroxyphenylacetic acid (DOPAC) in whole kidney homogenates incubated with increasing concentrations of dopamine (0.5–5.0 μM) for 15 min in control conditions and in the presence of ouabain (100 and 500 μM)

| | *Control* | *Ouabain (100 nM)* | *Ouabain (500 μM)* |
|---|---|---|---|
| Dopamine | | | |
| 0.5 μM | 0.64 ± 0.03 | 0.46 ± 0.06 | 0.59 ± 0.07 |
| 2.5 μM | 1.67 ± 0.07 | 1.59 ± 0.16 | 1.44 ± 0.24 |
| 5.0 μM | 3.35 ± 0.23 | 4.15 ± 0.27 | 3.87 ± 0.47 |

Values are means ± s.e. mean of four experiments per group.

aromatic L-amino acid decarboxylase (AAAD) and monoamine oxidase (MAO), since the formation of these two compounds was found not to be affected by ouabain when kidney homogenates are used. This result also supports the view that ouabain, in order to be active, requires the integrity of tubular epithelial cell membranes.

The formation of DOPAC in kidney slices loaded with L-DOPA has been found to parallel the accumulation of newly-formed dopamine; this is most evident when concentrations of L-DOPA up to 100 μM are employed, but not with higher concentrations. This suggests that in renal tissues MAO has a limited capacity to deaminate dopamine (Fernandes *et al.*, 1991). It is interesting, therefore, to observe that the rate constant of accumulation of DOPAC in kidney slices, as a

function of the concentration of sodium chloride in medium, is almost twice the corresponding value for dopamine. This appears to suggest that this second event, the deamination of dopamine into DOPAC, might also be sensitive to sodium, namely as a result of interference with the intracellular dynamics of newly-formed dopamine.

The results presented here on the sodium-dependent formation of dopamine bring into discussion the question of the relative unresponsiveness of salt-sensitive essential hypertensive subjects in excreting larger amounts of dopamine in urine in conditions of increased renal delivery of sodium (Gill *et al.*, 1988). In this subgroup of essential hypertensive patients the renal excretion of dopamine is not influenced by sodium load and the amount of dopamine appearing in urine during sodium loading is as low as that observed in salt resistant essential hypertensive patients during a low sodium diet (Gill *et al.*, 1988; Williams *et al.*, 1990). In salt-sensitive hypertensive patients urinary DOPA has, however, been found greater than in salt-resistant hypertensive and normotensive subjects during low or high sodium intake; the mean ratio of dopamine to DOPA has also been found to be subnormal during low sodium intake and remained subnormal in conditions of increased sodium intake (Gill *et al.*, 1989). This led to the suggestion that the decreased ability to excrete sodium in response to an increased renal delivery of sodium in salt-sensitive hypertensives might be due to a reduced capacity to synthesize dopamine (Gill *et al.*, 1988; 1989; William *et al.*, 1990). However, one should also take into consideration the possibility of a deficient coupled transport of DOPA and sodium into the tubular epithelial cell where its conversion into dopamine takes place.

In conclusion, the results presented here show that the formation of dopamine in kidney slices loaded with L-DOPA is dependent on the concentration of sodium chloride in the medium and sensitive to the inhibition of the enzyme $Na^+$–$K^+$ ATPase by ouabain. This sensitivity of dopamine formation to sodium and ouabain appears to involve the activation of tubular transport systems, as oxygen and the integrity of cell membranes are required for the effects of both sodium and ouabain to be observed.

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# Investigation of the prostaglandin E (EP-) receptor subtype mediating relaxation of the rabbit jugular vein

Ruth A. Lawrence & [1]R.L. Jones

Department of Pharmacology, University of Edinburgh, 1 George Square, Edinburgh EH8 9JZ

**1** Prostaglandin $E_2$ ($PGE_2$) relaxes circular smooth muscle of the rabbit isolated jugular vein at very low concentrations (mean $pIC_{50}$ against histamine-induced contraction = 9.34). This effect is not blocked by the $EP_1$-receptor antagonist, AH 6809 (2 μM).

**2** From a group of prostaglandin E analogues examined, 16,16-dimethyl $PGE_2$, misoprostol, 11-deoxy $PGE_2$-1-alcohol and 11-deoxy $PGE_1$ were highly potent relaxant agents, whereas 17-phenyl-ω-trinor $PGE_2$, MB 28767 and butaprost had low potency and sulprostone and oxoprostol were virtually inactive.

**3** Comparison of the jugular vein data with published data for inhibitory agonist potencies on the cat trachea ($EP_2$ preparation) and the field-stimulated guinea-pig vas deferens ($EP_3$) indicates that the EP-receptor in the rabbit jugular vein is closest to the $EP_2$ subtype. However, the correlation is not entirely convincing. For example, butaprost, 16,16-dimethyl $PGE_2$ and 11-deoxy $PGE_1$ are of similar potency on the cat trachea, whereas butaprost is about 300 times less potent than the other two analogues on the jugular vein. The existence of more than one $EP_2$-receptor appears possible.

**4** It was felt that the activity of butaprost required further investigation in view of the claim that it is a specific $EP_2$-receptor agonist. We have shown that butaprost has very low inhibitory activity on the guinea-pig vas deferens, a very sensitive $EP_3$-receptor containing preparation. However, on the chick ileum, the original $EP_3$ preparation, butaprost showed potent contractile activity ($pEC_{25}$ ~8.0). In addition, its maximum response was lower than that of $PGE_2$; lower maxima were also found for sulprostone, MB 28767 and oxoprostol, but not for ICI 80205, 16,16-dimethyl $PGE_2$ and 17-phenyl-ω-trinor $PGE_2$. The maximal response to a combination of either sulprostone and butaprost or sulprostone and $PGE_2$ was similar to that achieved by $PGE_2$ alone. Analysis of the interaction between sulprostone and $PGE_2$ appears to exclude a partial agonist action for sulprostone. Furthermore neither sulprostone nor butaprost appear to have inhibitory activity on the ileum. AH 6809 at 2 μM produced only a small shift of the $PGE_2$ log concentration-response curve.

**5** It is likely that contraction of the longitudinal smooth muscle of the chick ileum is mediated by (at least) two EP-receptor subtypes; activation of only one receptor system does not induce the maximum response (i.e. the acetylcholine maximum) of the preparation. One receptor could be an $EP_3$ subtype, at which sulprostone exerts a selective agonist action. The other receptor is unlikely to be an $EP_1$ subtype, because of the high agonist potency of butaprost, the low agonist potency of iloprost, and the low antagonist potency of AH 6809. An alternative hypothesis is that the chick ileum contains a novel EP-receptor subtype in addition to an $EP_3$-receptor.

**Keywords:** Synthetic prostaglandins E; EP-receptors; prostaglandin receptor antagonists; rabbit jugular vein; chick ileum; smooth muscle relaxation

## Introduction

Prostaglandin E (EP-) receptors which are susceptible to block by SC 19220 and AH 6809 have been designated $EP_1$-receptors (Kennedy *et al.*, 1982). EP-receptors resistant to block by these agents have been divided into $EP_2$ and $EP_3$ subtypes on the basis of the relative agonist potencies of prostaglandin E (PGE) analogues on isolated smooth muscle preparations (Coleman *et al.*, 1987c). Thus sulprostone is a potent agonist on the chick ileum (contraction; $EP_3$ preparation), but has very low potency on the cat trachea (relaxation; $EP_2$ preparation). In contrast AY 23626 (*rac* 11-deoxy $PGE_0$) is of similar potency on both preparations.

We wished to study in detail the $EP_2$ agonist activities of a range of PGE analogues, but a regular supply of cat trachea was not available to us. In addition, the relaxant actions of PGE analogues on the guinea-pig trachea (presumed to be $EP_2$-receptor-mediated) are difficult to study owing to the presence of a very sensitive $EP_1$-receptor system mediating contraction (Dong *et al.*, 1986). We therefore investigated the suitability of other isolated smooth muscle preparations. One of these, the rabbit jugular vein, proved to be highly sensitive to the relaxant action of $PGE_2$ and the relative potencies of a range of prostanoid analogues are described here.

The PGE analogue butaprost was of particular interest to us since it is claimed to be a specific $EP_2$-receptor agonist (Gardiner, 1986). During studies to confirm its specificity we found it had potent contractile activity on the chick ileum, nominally an $EP_3$ preparation. Further investigations, described here, suggest that more than one EP-receptor subtype may be present in the preparation.

## Methods

### *Rabbit jugular vein*

Male New Zealand White rabbits (2–4 kg) were injected with heparin (1000 u) via a marginal ear vein prior to stunning and exsanguination. The external jugular veins were removed, cleared of fat and adherent connective tissue and cut into rings 4 mm wide. Each ring was suspended under a

[1]Author for correspondence at present address: Department of Pharmacology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong.

tension of 0.75 g between two Z-shaped hooks in a 10 ml organ bath containing Krebs solution (composition, mM: NaCl 118, KCl 5.4, $CaCl_2$ 2.5, $MgSO_4$ 1.0, $NaH_2PO_4$ 1.1, $NaHCO_3$ 25, glucose 10). The bathing solution was aerated with 95% $O_2$/5% $CO_2$, maintained at 37°C, and contained indomethacin, 1 μM. Changes in tension were measured by means of Grass FT03 isometric transducers connected to a Grass Polygraph recorder. Preparations were allowed 1 h to equilibrate during which time the resting tension was re-adjusted to 0.75 g. Each preparation was initially contracted with histamine, 10 μM, to establish its maximum contractile response.

For investigation of the relaxant actions of prostanoids, the TP-receptor antagonist GR 32191 (10 μM) was added 5 min before the addition of a dose of histamine (usually 1 μM) sufficient to induce a stable level of tone 50–60% of the maximum. Larger histamine responses were subject to a considerable degree of fade. Each preparation was exposed to two series of cumulative doses of $PGE_2$ (standard agonist) before the testing of a prostaglandin analogue. The preparations were washed frequently during the 30 min period between cumulative additions. Log concentration-response curves were plotted, taking response as the percentage of the histamine-induced tone remaining. $IC_{50}$ values, defined as the concentration required to reduce the histamine response by 50%, were read by eye from the graph and converted into $pIC_{50}$ values ($-\log IC_{50}$). Values from single preparations from 5 different animals were averaged and s.e.mean calculated. Equi-effective molar ratios (e.m.r.) were calculated in the following way: $IC_{50}$ for the analogue/$IC_{50}$ for $PGE_2$ (second curve) in the same preparation. A mean e.m.r. from the 5 preparations was calculated.

The antagonism of the contractile action of U-46619 and MB 28767 by GR 32191 was measured as described previously (Jones *et al.*, 1982). The antagonist was added 20 min before the first agonist dose.

### *Chick ileum*

Chicks (5–20 days old) were killed by decapitation. The abdomen was opened, the ileum removed and adherent mesenteric tissue cut away. Segments about 20 mm long were mounted vertically in 10 ml organ baths under 0.5–0.75 g tension and tension changes were recorded as described above. The bathing solution was Tyrode solution of the following composition (mM): NaCl 136, KCl 2.7, $CaCl_2$ 1.4, $MgCl_2$ 0.49, $NaH_2PO_4$ 0.32, $NaHCO_3$ 12 and glucose 5; it was bubbled with 95% $O_2$/5% $CO_2$ and maintained at 37°C. All preparations were exposed to several, almost maximal, doses of acetylcholine (bath concentration = 2 μM) during the first 1 h after setting up, followed by a single dose of $PGE_2$ (14 nM). Following one of the procedures described below, the maximal response to acetylcholine was obtained. Responses were calculated as a percentage of the acetylcholine maximum.

Concentration-response relationships for $PGE_2$ (one preparation) and for test prostanoids (3 preparations from the same animal) were obtained by use of non-cumulative (1, 3, 10, 30 or 1, 5, 10, 50) sequences. The drug contact time was 30–90 s and a minimum of 15 min was allowed between additions. For each analogue, a mean $pEC_{25}$ value ($-\log$ of concentration producing 25% of the acetylcholine maximum response) was calculated from results obtained on single preparations from 5 animals.

To study the interaction between two agonists, a cumulative concentration-response curve to the first agonist was obtained on one preparation. A single dose of the second agonist was added to another preparation, followed 2–3 min later by cumulative doses of the first agonist.

To study the effect of potential inhibitors, cumulative concentration-response curves for $PGE_2$ were obtained on two preparations. Following wash-out, AH 6809 or the 'inhibitor cocktail' was added to one (test) preparation and 15 min later a second concentration-response curve to $PGE_2$ was obtained. A control curve to $PGE_2$ was obtained on the second preparation. A dose-ratio was calculated from the two $EC_{50}$ values obtained on the test preparation. Dose-ratios from 5 preparations were averaged and the s.e.mean calculated.

### *Compounds*

11-Deoxy $PGE_2$-1-alcohol was prepared in our laboratory from *nat* $PGA_2$. The following compounds were gifts: sulprostone, iloprost, $PGI_2$ sodium salt, carbacyclin and cicaprost from Prof. H. Vorbruggen, Schering AG, Berlin; ICI 80205 (*rac* 16-*p*-chlorophenoxy-ω-tetranor $PGE_2$) from Dr K. Gibson, ICI Pharmaceuticals, U.K.; MB 28767 (15S-hydroxy-9-oxo-16-phenoxy-ω-tetranorprost-13E-enoic acid) and oxoprostol (both racemic) from Dr M. Caton, Rhone-Poulenc, U.K.; misoprostol and enisoprost from Dr P. Collins, G.D. Searle, U.S.A.; butaprost from Dr P. Gardiner, Bayer, U.K.; AH 6809 (6-isopropoxy-9-oxoxanthen-2-carboxylic acid) and GR 32191 (9α-(biphenylyl)methoxy-17β-hydroxy-12β-(N-piperidinyl)-ω-octanorprost-4Z-enoic acid) from Dr R.A. Coleman, Glaxo, U.K. $PGE_2$, 16,16-dimethyl $PGE_2$, 17-phenyl-ω-trinor $PGE_2$ and 11-deoxy $PGE_1$ were purchased from Cayman Chemicals, U.S.A. The structural formulae of these analogues may be found in a previous publication (Lawrence *et al.*, 1991).

Ethanolic stock solutions of the prostanoids ($10^{-2} - 3 \times 10^{-2}$ M) were stored at −20°C and diluted with 0.9% NaCl solution for use. Because of the unexpected low potency of butaprost on the jugular vein and the known ease of dehydration of the β-ketol system in the PGE ring, the butaprost stock solution was chemically analysed at one month intervals. Thin layer chromatography showed a single spot with a mobility similar to the structurally similar misoprostol (both prostanoids are methyl esters). U.v. spectroscopy showed no evidence of a PGA chromophore (expected $\lambda_{max}$ = 220 nm) and alkali conversion (0.1 M NaOH, 25°C, 30 min) to the corresponding PGB derivative ($\lambda_{max}$ = 280 nm) confirmed the concentration of butaprost in the stock solution.

## Results

### *Rabbit jugular vein*

$PGE_2$, the standard agonist, produced complete relaxation of rabbit jugular vein preparations contracted by histamine, with $pIC_{50}$ values falling between 8.82 and 9.96 (third cumulative sequence on 13 preparations). AH 6809 at a concentration of 2 μM did not block the relaxant action of $PGE_2$ (Figure 1a), the dose-ratio being 0.87 ± 0.03 (s.e.mean, $n$ = 5). The relaxant action of $PGE_2$ was also unaffected by the TP-receptor antagonist GR 32191 at a concentration of 10 μM; control $pIC_{50}$ = 9.21 ± 0.11, GR 32191-treatment $pIC_{50}$ = 9.24 ± 0.08 (s.e.mean, $n$ = 5).

*Relaxant potencies of prostanoids* Eleven PGE analogues were tested for relaxant activity in the presence of 10 μM GR 32191. With the exception of sulprostone, oxoprostol and MB 28767, log concentration-response curves for the analogues were parallel to that of $PGE_2$ and at least 90% relaxation was produced. $pIC_{50}$ values and equi-effective molar ratios (e.m.r.) are given in Table 1. Sulprostone and oxoprostol were of very low potency: at the highest concentrations tested of 3.6 and 1.4 μM, the relaxations were 22 ± 8% and 14 ± 5% (s.e.mean, $n$ = 5) respectively.

The three stable PGI analogues, cicaprost, iloprost and carbacyclin, also relaxed the jugular vein with log concentration-response curves parallel to that of $PGE_2$. However they were considerably less potent than $PGE_2$ (Table 1).

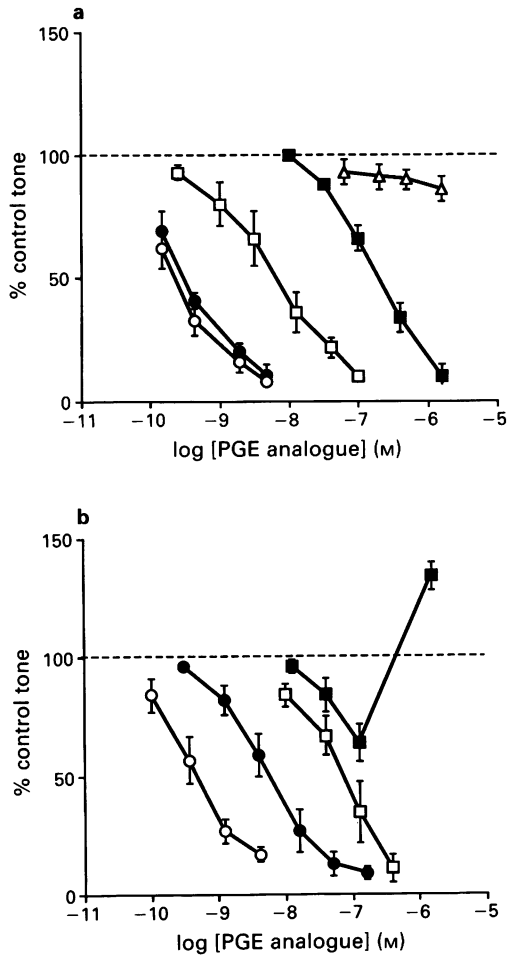


**Figure 1** Log concentration-response curves for relaxation of the histamine-contracted rabbit jugular vein by prostaglandin E (PGE) analogues. (a) $PGE_2$ (●); $PGE_2$ in the presence of 2 μM AH 6809 (○); misoprostol (□); butosprost (■) and oxoprostol (△); (b) *nat* 11-deoxy $PGE_1$ (○); 11-deoxy $PGE_2$-1-alcohol (●); 17-phenyl-ω-trinor $PGE_2$ (□) and MB 28767 (■). The TP-receptor antagonist GR 32191 (10 μM) was present in all tests. Means for 5 experiments are shown; s.e.mean indicated by vertical bars.

*Contractile/relaxant actions of MB 28767* In the presence of 10 μM GR 32191, MB 28767 relaxed the histamine-contracted jugular vein preparation, but the concentration-response curve was bell-shaped (Figure 1) with distinct contractile responses being seen at concentrations in excess of 100nM. The $pIC_{25}$ for MB 28767 was 7.12, giving an e.m.r. of about 400 (Table 1).

In the absence of GR 32191 (and histamine), MB 28767 contracted the vessel rings and its log concentration-response curve was parallel to that of the TP-receptor agonist, U-46619 (Figure 2). The e.m.r. for MB 28767 relative to U-46619 was about 5.5. In the presence of 10 μM GR 32191, a large parallel rightward shift of the U-46619 curve was obtained; $pA_2 = 7.2 \pm 0.1$ (s.e.mean, $n = 5$). The contractile action of MB 28767 was blocked to a similar extent by 10 μM GR 32191, but a $pA_2$ value was not calculated since the highest concentration of MB 28767 tested (4.3 μM) produced only a 28% response.

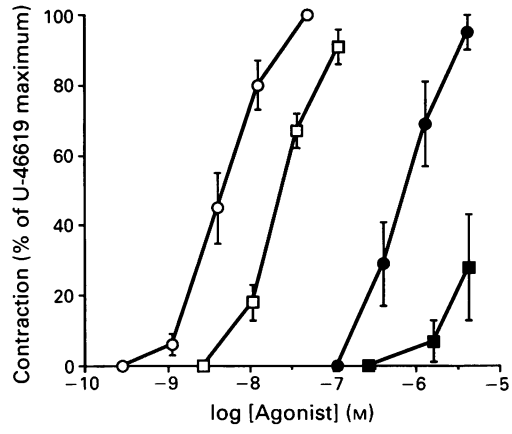


**Figure 2** Log concentration-response curves for contraction of the rabbit jugular vein by U-46619 in the absence (○) and presence (●) of 10 μM GR 32191 and by MB 28767 in the absence (□) and presence (■) of 10 μM GR 32191. Each point is the mean of 5 experiments; vertical bars show s.e.mean.

**Table 1** Potencies of prostanoids for relaxation of rabbit jugular vein and cat trachea

| | *Rabbit jugular vein* | | *Cat trachea* |
|---|---|---|---|
| *Prostanoid* | $pIC_{50}$ | *e.m.r.* | *e.m.r.* |
| *PGE analogues* | | | |
| ICI 80205 | 7.25 ± 0.17 | 83 | 70* |
| 16,16-Dimethyl $PGE_2$ (16,16 PGE) | 9.21 ± 0.19 | 2.1 | 9.4*, 20** |
| $PGE_2$ | 9.34 ± 0.11 | 1.0 | 1.0 |
| 17-Phenyl-ω-trinor $PGE_2$ (17-Phe PGE) | 7.21 ± 0.11 | 200 | — |
| Sulprostone (Sul) | < 5.44 | > 3000 | > 7000** |
| MB 28767 | see text | ~400§ | — |
| Oxoprostol (Oxo) | < 5.85 | > 2000 | — |
| Misoprostol (Miso) | 8.21 ± 0.21 | 8.3 | 3.7** |
| 11-Deoxy $PGE_2$-1-alcohol (PGE-1-alc) | 8.27 ± 0.16 | 4.6 | — |
| *nat* 11-Deoxy $PGE_1$ | 9.35 ± 0.12 | 1.4 | — |
| *rac* 11-Deoxy $PGE_1$ | 9.22 ± 0.14 | 2.1 | 13* |
| Butaprost (Buta) | 6.70 ± 0.10 | 685 | 17*** |
| *PGI analogues* | | | |
| Cicaprost (Cica) | 8.02 ± 0.19 | — | > 300* |
| Iloprost (Ilo) | 7.90 ± 0.08 | — | > 270* |
| Carbacyclin | 7.26 ± 0.19 | — | — |

Rabbit jugular vein: $pIC_{50}$ valus are means ± s.e.mean of 5 experiments; prostaglandin $E_2$ ($PGE_2$) is the standard agonist. §Bell-shaped log concentration-response curve; e.m.r. calculated at $IC_{25}$ level.
Cat trachea: published data, *Dong *et al.*, 1986 ($PGE_2$ is the standard agonist, $pIC_{30} = 7.4$); **Coleman *et al.*, 1988 ($pIC_{50} = 7.7$); ***Gardiner, 1986 ($pIC_{50} = 7.7$).
Abbreviation in parentheses used in Figure 5.

*Chick ileum*

*Concentration-response curves* All the PGE analogues tested contracted the chick ileum. However not all of them elicited the same maximum response (Figure 3). Using non-cumulative addition of doses, $PGE_2$, ICI 80205, 16,16-dimethyl $PGE_2$ (not shown), and 17-phenyl-ω-trinor $PGE_2$ elicited responses of at least 80–95% of the acetylcholine (ACh) maximum, whereas the maxima for sulprostone, butaprost and MB 28767 were 40 ± 4, 47 ± 3 and 56 ± 4% (s.e.mean, $n = 5$) respectively. Misoprostol and oxoprostol also showed a tendency towards a lower maximum. Iloprost showed weak contractile activity (Figure 3a) and cicaprost was even less active (0 and 20% of the ACh maximum at 0.13 and 1.3 μM respectively).

*Interactions of agonists* Cumulative log concentration-response curves to $PGE_2$ and butaprost in the presence of a supramaximally effective concentration of sulprostone (220 nM) were obtained (Figure 4a,b). In both cases the maximum response to the combination of agonists was very similar to that of $PGE_2$ alone. $EC_{50}$ values (own maximum) for butaprost alone and in the presence of sulprostone were 38 and 51 nM respectively. The maximum response to butaprost by cumulative addition is higher than that obtained with ascending non-cumulative addition (Figures 3b and 4b). This appears to be related to the slow decay of butaprost contractions on washout, resulting in some desensitization by the non-cumulative technique.

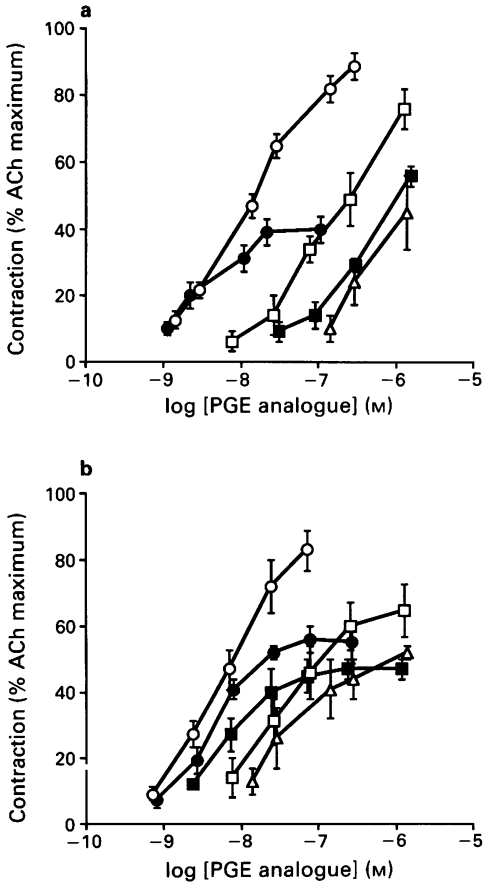


**Figure 3** Non-cumulative log concentration-response curves for contraction of the chick ileum by prostanoids. (a) Prostaglandin $E_2$ ($PGE_2$) (○); sulprostone (●); 17-phenyl-ω-trinor $PGE_2$ (□); 11-deoxy $PGE_2$-1-alcohol (■) and iloprost (△). (b) ICI 80205 (○); MB 28767 (●); misoprostol (□); butaprost (■) and oxoprostol (△). Each point is the mean of 5 experiments; vertical bars show s.e.mean.

A maximally effective concentration of butaprost (500 nM) did not oppose the contractile action of ACh (Figure 4c); $EC_{50}$ values (own maximum) for ACh alone and in the presence of butaprost were 165 and 140 nM respectively.

*Effects of AH 6809 and the 'inhibitor cocktail'* AH 6809 at 2 μM produced a parallel rightward shift in the log concentration-response curve of $PGE_2$ (Figure 4b). The respective $pEC_{50}$ values show a statistically significant difference ($P < 0.05$, unpaired Student's $t$ test); dose-ratio = 2.7 ± 0.8 (s.e.mean, $n = 5$), $pA_2 = 5.9$. However the cocktail of inhibitors (hyoscine 0.1, mepyramine 0.1, phenoxybenzamine 0.1, propranolol 3, methysergide 0.2 μg $ml^{-1}$ and indomethacin 3 μM) used by Gardiner (1986) had no effect on the log concentration-response curves of either $PGE_2$ or butaprost.

## Discussion

The results we have obtained for the PGE analogues on the rabbit jugular vein would suggest that their relaxant actions are mediated through $EP_2$-receptors. Thus the relaxant action of $PGE_2$ was not blocked by the $EP_1$-receptor antagonist AH 6809 ($pA_2$ for block of $EP_1$-receptors = 6.8–7.5, Coleman *et al.*, 1987c; Eglen & Whiting, 1988; Lawrence *et al.*, 1991). Furthermore, the highly potent $EP_3$-receptor agonist, sulprostone (agonist potencies: $EP_3 > EP_1 >> EP_2$) (Coleman *et al.*, 1987a,b) was only a very weak relaxant agent on the jugular vein. $EP_3$-receptors appear to mediate contraction in some vascular preparations, for example the rabbit renal artery (Ahluwalia *et al.*, 1988). In the present studies MB 28767, which also has potent $EP_3$ agonist activity (Jones & Wilson, 1990), contracted the rabbit jugular vein. However, MB 28767 is also a potent TP-receptor agonist (Banerjee *et al.*, 1985) and the present experiments with the TP-receptor antagonist GR 32191 (Lumley *et al.*, 1989) indicate that the contractile action of MB 28767 on the rabbit jugular vein is due to activation of TP-receptors.

Although we have used GR 32191 in the rabbit jugular vein experiments to suppress potential TP-receptor agonist actions of the PGE analogues, its affinity on the vein is lower than its affinities on other preparations (see Lumley *et al.*, 1989) and a concentration of 10 μM is barely sufficient when the PGE analogue has high TP agonist potency and low $EP_2$ agonist potency (e.g. MB 28767). We have recently reported low affinities for several TP-receptor antagonists (e.g. EP 092, EP 169 and ONO 11120) on two other rabbit isolated preparations, the thoracic aorta ring and blood platelets (Tymkewycz *et al.*, 1991). In early experiments on the jugular vein it was found that none of the three antagonists showed greater blocking potency than GR 32191 (unpublished observations).

Before discussing the potencies of the other PGE analogues as $EP_2$-receptor agonists on the jugular vein, it is necessary to consider possible interference from other prostanoid receptors mediating relaxation. Using the potent and specific DP-receptor antagonist BW A868C, Giles and coworkers (1989) showed that the rabbit jugular vein contains a DP-receptor which mediates relaxation. Low concentrations of BW A868C shifted the log concentration-response curves to $PGD_2$ and the DP-receptor agonist, BW 245C, to the right. However little further shift was seen with higher concentrations of BW A868C, since both agonists activate a second receptor (presumably the $EP_2$-receptor) which is not blocked by the antagonist. We do not know whether any of our PGE analogues activate the DP-receptor in the jugular vein since BW A868C was not available to us at the time of the experiments. However, by comparing concentration-response relationships in the Giles study with those presented here, it would appear that the DP-receptor relaxant system is considerably less sensitive than the $EP_2$-receptor system.

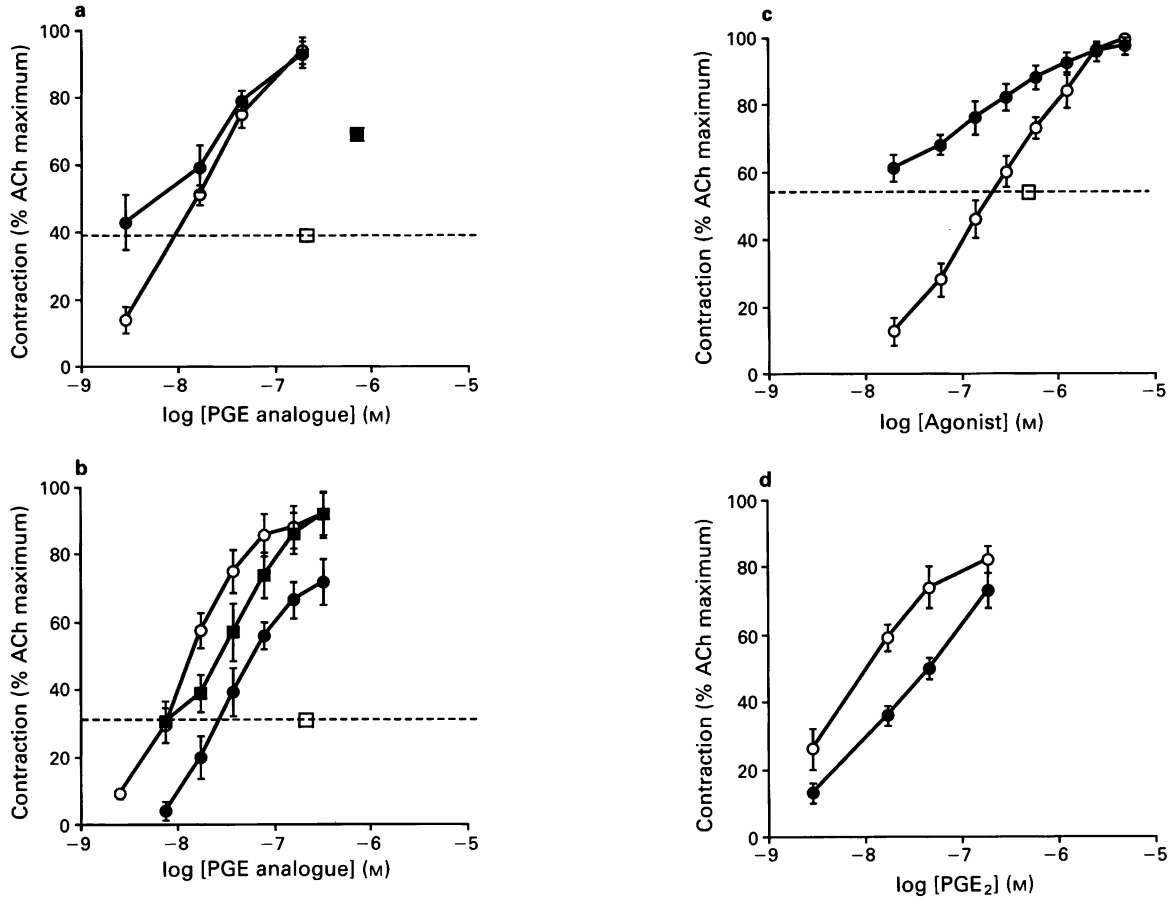


**Figure 4** Log concentration-response curves for contraction of chick ileum. (a) Prostaglandin $E_2$ ($PGE_2$) alone (○) and $PGE_2$ in the presence of 220 nM sulprostone (●); this concentration of sulprostone elicits its own maximum response (□). The predicted $EC_{50}$ for the $PGE_2$ curve assuming sulprostone is a partial agonist with a $K_d$ of 5 nM is also shown (■); the $PGE_2$ maximum is assumed to equal the acetylcholine maximum. (b) $PGE_2$ alone (○); butaprost alone (●) and in the presence of 220 nM sulprostone (■); the response to sulprostone is also shown (□). (c) Acetylcholine alone (○) and in the presence of 500 nM butaprost (●); the response to butaprost is also shown (□). (d) $PGE_2$ alone (○) and in the presence of 2 μM AH 6809, an $EP_1$-receptor antagonist. (●). Each point is the mean of 5 experiments; vertical bars show s.e.mean.

In a preliminary report Giles *et al.* (1990) have also shown that prostacyclin, iloprost and carbacyclin relax the rabbit jugular vein, with $pIC_{50}$ values of 7.36, 7.42 and 6.57 respectively. Our preparations were slightly more sensitive to the relaxant actions of iloprost and carbacyclin. In addition cicaprost, which shows high specificity for IP-receptors (Dong *et al.*, 1986) was found to be marginally more potent than iloprost. However, $PGE_2$ is at least 20 times more potent than these $PGI_2$-mimetics in relaxing the jugular vein and since none of the PGE analogues examined here are potent agonists on the very sensitive IP-receptor system of the human platelet, we feel that their relaxant activities are unlikely to be due to activation of IP-receptors.

The relaxant activity of butaprost is unexpectedly low on the rabbit jugular vein ($pIC_{50} = 6.70$, e.m.r. = 685, Table 1). Gardiner (1986) reported that butaprost induces relaxation of guinea-pig and cat trachea with $pIC_{50}$ values of about 6.5, whereas higher concentrations are required on the cat lung strip ($pIC_{50} = 5.4$) and human bronchiole (5.1). We have confirmed the absolute potency of butaprost on the guinea-pig trachea ($pIC_{50} = 6.5-7.0$, unpublished observations), and hence in combination with physico-chemical tests (see Methods) have established the integrity of our sample of butaprost. However an accurate estimate of the potency of butaprost relative to $PGE_2$ can be obtained only on the cat trachea because of the dual contractile/relaxant activity of $PGE_2$ on the other preparations. Table 1 shows that there appears to be a marked difference between the e.m.rs for butaprost on the rabbit jugular vein and cat trachea. The discrepancy is even greater if the potencies of butaprost relative to either 16,16-dimethyl $PGE_2$ or 11-deoxy $PGE_1$ are compared. On the cat trachea the three PGE analogues have similar potencies whereas on the rabbit jugular vein butaprost is about 300 times less potent. The argument (see Kenakin, 1984) that, in preparations containing similar receptors, agonist efficacies influence relative potencies through differences in 'post-receptor gains' between the preparations is unlikely to explain our data. The rabbit jugular vein, on which butaprost has a low relative potency, is by far the more sensitive preparation; $pIC_{50}$s for $PGE_2$ on rabbit jugular vein and cat trachea are 9.34 and 7.7 respectively. If butaprost were a low efficacy $EP_2$ agonist, we would expect it to be much weaker on the cat trachea and also to act as a partial agonist; this is not evident in the concentration-response curves presented by Gardiner (1986). We conclude therefore that the EP-receptors mediating relaxation in the rabbit jugular vein and the cat trachea appear to be different; further studies are obviously required.

Eglen & Whiting (1988) reported that misoprostol at 300 nM contracted the guinea-pig trachea and there was no evidence of relaxation; from this and other data on the guinea-pig ileum and oesophageal muscularis mucosae, they proposed that misoprostol is a selective $EP_1$-receptor agonist. In our experience (Jones, unpublished observations) misoprostol (10–1000 nM) does not contract the indomethacin-treated guinea-pig trachea, whereas $PGE_1$ and $PGE_2$ show

contractile ($EP_1$) effects which reverse to relaxant ($EP_2$) effects as the concentration is raised. Furthermore when the tone of the trachea is raised by a TP-receptor agonist (e.g. U-46619, EP 171, Jones *et al.*, 1989), or an $EP_1$-receptor agonist (17-phenyl-ω-trinor $PGE_2$, iloprost) or histamine, misoprostol demonstrates potent relaxant activity. These latter results would agree with the potent relaxant action of misoprostol on the rabbit jugular vein reported here and on the cat trachea as reported by Coleman *et al.* (1988) (Table 1). Misoprostol is thus a potent agonist at $EP_2$-receptors. It is also a highly potent agonist at $EP_3$-receptors mediating inhibition of transmitter release (guinea-pig vas deferens, Lawrence *et al.*, 1991; guinea-pig atria, Mantelli *et al.*, 1991). Finally both $EP_3$ (initial contractile) and $EP_2$ (secondary relaxant) actions are seen on the non-pregnant human myometrium *in vitro* (Senior *et al.*, 1991). Although misoprostol undoubtably does have some agonist activity at $EP_1$-receptors (see Lawrence *et al.*, 1991), it is incorrect to label this prostanoid as a selective $EP_1$-receptor agonist.

11-Deoxy $PGE_2$-1-alcohol also has high relaxant potency on the rabbit jugular vein and is of particular interest because of the presence of a 1-alcohol group. It would appear that an ionised carboxyl function at the α-chain terminus is not a prerequisite for high $EP_2$ agonist potency. This cannot be implied from the high activity of methyl esters such as misoprostol, since one can never be certain that enzymatic de-esterification has not occurred within the tissue. 1-Alcohol PGE analogues are worthy of further investigation since they may lead to highly selective $EP_2$-receptor agonists. For example, 11–deoxy $PGE_2$-1-alcohol has low $EP_1$ agonist potency and only moderate $EP_3$ agonist potency (Lawrence *et al.*, 1991). Another 1-alcohol PGE analogue which has undergone considerable investigation is rioprostil (Kluender & Woessner, 1979). It has high $EP_2$ (cat trachea) and $EP_3$ (guinea-pig vas deferens) agonist potencies, but very low $EP_1$-agonist potency (guinea-pig fundus) (Coleman *et al.*, 1988; Reeves *et al.*, 1988). Finally the 1-alcohol PGE analogue oxoprostol has very low relaxant activity on the rabbit jugular vein. This is almost certainly related to the combination of 15-oxo and 16-phenoxy-17,18,19,20-tetranor groups in its ω-chain. Firstly, a 15(S)-15-hydroxyl group has been shown to be an important determinant of dilator potency in the dog hind limb (Nakano, 1972). Secondly a 16-phenoxy substituent accentuates $EP_1$ whilst reducing $EP_2$ agonist activity (Dong *et al.*, 1986). It is of interest that oxoprostol still retains considerable $EP_3$ agonist potency; e.m.r. on guinea-pig vas deferens = 3.1 (Lawrence *et al.*, 1991).

Our studies show that 17-phenyl-ω-trinor $PGE_2$ has only weak relaxant activity on the rabbit jugular vein (e.m.r. = 200). However it is a potent $EP_1$-receptor agonist on the guinea-pig ileum in the presence of morphine (e.m.r. relative to $PGE_2$ = 1.8). It is also a potent $EP_1$ agonist on the guinea-pig trachea, but its e.m.r. relative to $PGE_2$ is difficult to estimate since the latter has relaxant actions which oppose $EP_1$-receptor mediated contractions; its e.m.r. with respect to sulprostone (which lacks relaxant activity) = 0.32. 17-Phenyl-ω-trinor $PGE_2$ also has moderate $EP_3$ agonist potency on guinea-pig vas deferens; its e.m.r. relative to $PGE_2$ = 6.3 and relative to sulprostone = 49 (Lawrence *et al.*, 1991). Thus in combination with sulprostone it could be usefully employed to distinguish between $EP_1$- and $EP_3$-receptors. It may also be a lead to more selective $EP_1$-receptor agonists.

During our corroborative studies on butaprost, we observed that it had potent contractile activity on the chick ileum, but very little inhibitory activity on the guinea-pig vas deferens; both preparations are designated as $EP_3$-receptor containing preparations. The chick ileum finding is surprising, since Gardiner (1986) reported that butaprost was inactive on this preparation over a wide concentration range (2 nM–200 μM). In Gardiner's experiments the chick ileum was simultaneously exposed to several receptor antagonists (hyoscine, mepyramine, phenoxybenzamine, propranolol, methysergide) and the cyclo-oxygenase inhibitor indomethacin. We have found that this combination of agents does not affect the contractile activity of either butaprost or $PGE_2$.

Further investigations on the chick ileum have revealed that several PGE analogues, including sulprostone and butaprost, give smaller maximum responses than $PGE_2$. We first considered the possibility of partial agonism at an $EP_3$-receptor system in the chick ileum, particularly since the chick ileum is less sensitive than the guinea-pig vas deferens to $PGE_2$ ($pEC_{50}$ = 7.8 and $pIC_{50}$ = 8.8 respectively). If sulprostone is a partial agonist on a single receptor system in the chick ileum, it should shift the log concentration-response curve for $PGE_2$ (full agonist) to the right and it should be possible to predict the $EC_{50}$ of this curve for any single concentration of sulprostone in the following manner. First the dissociation constant ($K_d$) of the partial agonist is estimated by comparison of its log concentration-response curve with that of the full agonist acting on the same receptors (Roberts, 1984). Applied to the data for sulprostone and $PGE_2$ in Figure 3, this method gives a $K_d$ of about 5 nM for sulprostone. In the interaction experiments shown in Figure 4, sulprostone was used at a concentration of 220 nM. This is some 44 times its estimated $K_d$; therefore from van Rossum (1963) (also see Jenkinson, 1979) the concentration of $PGE_2$ required to produce a 70% maximum response (i.e. corresponding to half of the increment between the sulprostone response and the tissue maximum) is calculated to be 770 nM. The actual value in our experiments was 28 nM (Figure 4a). Clearly sulprostone does not antagonize the contractile action of $PGE_2$, as would be expected for a partial agonist present at a concentration in which it occupies a large proportion of the receptor pool. Furthermore, it is unlikely that the lower maximum of sulprostone is due to a second action of sulprostone opposing its contractile action (for example an agonist action on a separate receptor mediating smooth muscle relaxation), since inhibition of $PGE_2$ action would also be expected. In addition, sulprostone does not appear to oppose the contractile action of butaprost. Finally, there is no inhibition by butaprost of the contractile action of ACh as would be expected if it activated EP-receptors to produce an inhibitory response.

We feel that the most likely explanation of our data at this stage is that the chick ileum contains at least two EP-receptor subtypes which mediate contraction, and that maximal activation of only one receptor system cannot produce a maximum response of the preparation. One of these may be similar to the $EP_3$-receptor found in the guinea-pig vas deferens. A comparison of $IC_{50}$ values for prostanoids on the vas deferens (Lawrence *et al.*, 1991) with $pEC_{25}$ values on chick ileum from the present study is shown in Figure 5. We suggest that certain analogues (sulprostone, misoprostol and oxoprostol) elicit responses of the chick ileum in the 0–50% response range solely by activating $EP_3$-receptors; they lie close to the broken line in Figure 5. Prostanoids with points to the right of the line have potencies on the chick ileum which are greater than would be predicted from their $EP_3$ agonist potencies on the vas deferens and could therefore act on a second EP-receptor. In the case of $PGE_2$, ICI 80205, 16,16-dimethyl $PGE_2$, 17-phenyl-ω-trinor $PGE_2$ and perhaps 11-deoxy $PGE_2$-1-alcohol, both EP-receptors are activated giving rise to higher maxima, but the $EC_{50}$ values for the two log concentration-response curves are not sufficiently different to give rise to biphasic curves. Butaprost lies well to the right of the broken line and probably acts solely on a second receptor. An estimate of the relative potencies of $PGE_2$ and butaprost on the second receptor system can be obtained from the log concentration-response curves for the two prostanoids in the presence of the supramaximally effective concentration of sulprostone; $PGE_2$ is about twice as potent as butaprost.

Since $PGE_2$, ICI 80205, 16,16-dimethyl $PGE_2$ and 17-phenyl-ω-trinor $PGE_2$ all have potent $EP_1$ agonist activity,

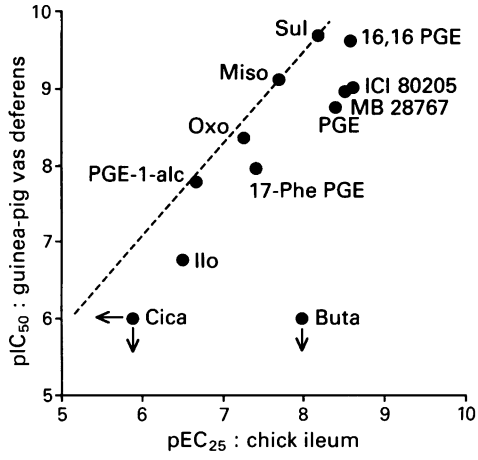


**Figure 5** Correlation of agonist potencies of prostanoids on the guinea-pig vas deferens and the chick ileum (the full names of the analogues are given in Table 1). The broken line indicates a hypothetical relationship between $EP_3$-receptor agonist potencies on the vas deferens and the ileum. Prostanoids situated to the right of this line have greater agonist potency (in the 0–50% response range) on the ileum than would be expected from their $EP_3$ potency on the vas deferens; they may be agonists at another EP-receptor subtype in the ileum. The arrows by the cicaprost and butaprost points indicate that potency is less than that represented by the position of the point.

the second receptor may be an $EP_1$-receptor. However, selective block of $EP_1$-receptors by AH 6809 would be expected to give a biphasic log concentration-response curve for $PGE_2$ with a dose ratio of about 20 for the $EP_1$ component. This profile was not observed in our experiments. However we have observed this type of behaviour for AH 6809 on the guinea-pig ileum where sulprostone acts on both $EP_1$- and $EP_3$(?)-receptors to produce contraction (Lawrence *et al.*, 1991). Secondly, the potency of iloprost, which has potent $EP_1$-agonist activity (Dong & Jones, 1982; Dong *et al.*, 1986), is rather low on the chick ileum and this does not support the presence of an $EP_1$-receptor (the low potency of cicaprost also excludes IP-receptor-mediated contraction). However at this stage we cannot entirely rule out the presence of $EP_1$-receptors in the chick ileum.

The presence of both $EP_1$- and $EP_3$-receptors is unlikely to explain the quite potent contractile activity of butaprost, since this analogue has no detectable $EP_1$-agonist action (guinea-pig ileum) and only minimal $EP_3$ agonist action (guinea-pig vas deferens) (Lawrence *et al.*, 1991). Butaprost could be acting as an $EP_2$-agonist on the chick ileum. However this does not correlate with the low $EP_2$ agonist potency of ICI 80205 (Dong *et al.*, 1986; this study). An alternative explanation for our findings is that the chick ileum contains an $EP_3$-receptor and a novel EP-receptor, the latter being activated by $PGE_2$, ICI 80205, 16,16-dimethyl $PGE_2$, 17-phenyl-ω-trinor $PGE_2$ and butaprost. Further studies are in progress to investigate this possibility.

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# ATP overflow from the mouse isolated vas deferens

[1]M. Eliane Drake & S.A. Petersen

Department of Physiology, Leicester University, P.O. Box 138, LE1 9HN Leicester

**1** A modified form of the highly specific luciferin-luciferase assay was used to measure the overflow of adenosine 5'-triphosphate (ATP) from the field-stimulated mouse vas deferens *in vitro*. Precise timing of the stimulation, superfusate collection and assay minimized ATP degradation before assay, offering the opportunity for quantitative studies.

**2** Stimulation with between 50 and 200 supramaximal pulses at 5 Hz increased ATP overflow by between 7 and 28 times over basal.

**3** ATP overflow increased steadily with increasing numbers of stimuli from 50 to 200 pulses. Increasing the frequency of stimulation initially had no effect, but above 10 Hz there was increased overflow, suggesting an interaction between facilitated release and the rate of degradation by extracellular ATPases.

**4** Contractions of the vas induced by exogenous (−)-phenylephrine produced only a small increase in ATP overflow, suggesting that the stimulation induced increase in ATP overflow is mostly pre-junctional in origin, though tetrodotoxin eliminated stimulation-induced overflow only in a proportion of preparations.

**Keywords:** Mouse vas deferens; adrenergic nerves; bioluminescence; luciferin-luciferase; co-transmission; ATP

## Introduction

The postjunctional contractile response of the rodent vas deferens seems to be produced by an interaction between electrical and chemical transmission processes mediated by different transmitters: adenosine 5'-triphosphate (ATP) and noradrenaline (NA) (Sneddon & Westfall, 1984; Allcorn *et al.*, 1986; Cunnane *et al.*, 1987; Dunn & Blakeley, 1988; von Kügelgen *et al.*, 1989). Exogenous NA produces a mechanical response, through $\alpha_1$-adrenoceptors, without an intervening electrical event. By contrast, ATP seems to act on $P_2$-purinoceptors to initiate contraction of electromechanical coupling via voltage-dependent $Ca^{2+}$ channels (Sneddon & Burnstock, 1984; Sneddon & Westfall, 1984).

Overflow techniques have been widely used for measurement of the stimulation induced 'overflow' of NA (Brown *et al.*, 1961) and this technique is potentially applicable to ATP using the highly specific and very sensitive luciferin-luciferase assay. As ATP degrades very rapidly after release, some workers have bathed preparations in a solution containing luciferin-luciferase (White *et al.*, 1981; Lew & White, 1987) but it is possible to detect ATP in aliquots of superfusate (Kirkpatrick & Burnstock, 1987; Kasakov *et al.*, 1988) provided they are assayed quickly. This avoids potential problems of contact between tissue and assay system. The precise temporal pattern of overflow, however, has yet to be studied, particularly in relationship to the overflow of NA, so, in this study we have examined the temporal pattern of ATP and NA overflow from the isolated vas deferens of the mouse following various patterns of field stimulation.

## Methods

### *Animals and dissection*

Male MF1 mice weighing between 19–40 g and aged between 28–64 days were used. All mice were killed by cervical dislocation and decapitation followed by exsanguination. Both vasa deferentia were rapidly dissected and then allowed to recover from dissection trauma for 1 h in Krebs solution of the following composition (mM), maintained at 37°C: sodium chloride 118.4, potassium chloride 4.7, sodium hydrogen carbonate 25, sodium dihydrogen orthophosphate 1.13, calcium chloride 2.7, magnesium chloride 1.2, glucose 11.1 saturated with 95% oxygen and 5% carbon dioxide by gassing for at least 1 h before use and then continuously gassed during the course of the experiment. The vasa were mounted vertically in perspex cylindrical organ baths which were designed and made in the laboratory since a small internal volume (175 µl) was required. Parallel platinum electrodes ran on either side of the organ bath. A resting tension of 0.5 g was applied. Krebs solution was pumped from a reservoir towards the organ bath with a Watson-Marlow H.R. flow inducer at a rate of $0.8 \pm 0.05$ ml $min^{-1}$.

### *ATP overflow*

*Luciferase preparation* A buffered solution containing 25 mM of Tricine buffer, 2 mM EDTA and 20 mM $MgSO_4$, pH 7.45 corrected with KOH, was first prepared. Crude firefly lantern extract (FLE-50, Sigma Co.) was then dissolved in 5 ml of distilled water and added to 15 ml of the buffered solution. This mixture was then centrifuged at 27,000 *g* for 15 min and the supernatant placed in 10 ml glass exelco tubes and refrigerated at 4°C. It was used between 24 h and 7 days after its preparation. During the assay, the enzyme solution was maintained at room temperature.

*Luciferin preparation* 1 mg quantities of Synthetic D-luciferin crystalline (L-9504, Sigma Co.) were dissolved in 5 ml of deoxygenated methanol, divided into 5 equal aliquots and the methanol then evaporated off in the dark by a stream of oxygen free nitrogen. Aliquots were used within 10 days. Thirty min before use a dried aliquot (200 µg) was dissolved into 7 ml of a buffer solution (25 mM Tricine, 2 mM EDTA, pH 7.75 corrected with KOH) and kept in a dark-chamber gassed with oxygen-free nitrogen and allowed to equilibrate to room temperature for 30 min.

*Adenosine triphosphate preparation* ATP (Sigma Co.) was prepared in a cold buffer solution containing 25 mM Tricine, 2 mM EDTA at pH 7.75 corrected with KOH and maintained on ice during the course of the experiment.

[1] Author for correspondence at: Laboratoire de Biochimie Médicale, 28 place Henri Dunant, 63001 Clermont-Ferrand, France.

*Collection of superfusates* Aliquots were collected over 1 min each, for 5–8 min spanning stimulation (0.5 ms, supramaximal voltage, 5 Hz, number of pulses from 50 to 200). Field stimulation was applied for 5, 30 or 40 s during one of the collection periods (see below). The assay volume was 0.95 ± 0.05 ml (perfusion rate: 0.8 ± 0.05 ml $min^{-1}$; enzyme: 100 µl; luciferin: 50 µl). The assay procedure used was slightly modified from the one used by Stanley & Williams (1969). The experiment was scheduled into a 'just-in-time' collection-detection cycle in which no dead time occurred i.e., by the time one vial finished being counted, the next one was ready. Aliquots caps containing 100 µl enzyme and 50 µl luciferin were prepared and maintained in the dark under oxygen-free nitrogen only minutes before the assay. Aliquots were shaken manually but steadily at a rate of 1 agitation $s^{-1}$, for 5 s. The time interval from the initiation of the reaction to the beginning of quantum counting was as short as possible and constant from one vial to another. Each aliquot of superfusate was assayed for ATP contents before completion of the following superfusate collection. Two time intervals must remain constant during the assay: the interval between the end of superfusate collection and the beginning of the reaction and the interval between the initiation of the reaction and the beginning of light output integration.

*Quantum counting* A Packard scintillation counter model 3330 was used for the determination of ATP by measuring light output in the presence of luciferin-luciferase. The coincidence gate was switch off (as recommended by Stanley & Williams, 1969), the gain was set at 100% since the light energy is low. The wavelength window was wide open, from 50 to its maximum i.e., 1000. The counting period was automatically ended after 6 s.

### *[$^3$H]-noradrenaline overflow*

Incubation of the tissue with [$^3$H]-noradrenaline ([$^3$H]-NA) was carried out whilst allowing the vas deferens to recover from the trauma of dissection. Both vasa deferentia were totally submerged in 1 ml of Krebs solution. [$^3$H]-NA (20 µl, representing 20 µCi) was injected into the Krebs solution. Throughout the incubation period (1 h) the Krebs solution was kept oxygenated with a fine glass tube delivering bubbles of 95% $O_2$ and 5% $CO_2$. Each sample of [$^3$H]-NA collected was mixed with 4 ml of scintillation counting solution (NE-265, New England Nuclear) in order to determine radioactivity. The vial inserts were then placed in polythene vials and finally left in the scintillation counter (Packard, model 3330) for at least 1 h in order to equilibrate to counter temperature (4°C). Each set of vials was then read for a period of 5 min each. Optimal reading of tritium was achieved by setting the pulse-height discriminator window at 160–400 and the gain at 60% with the gate on coincidence.

### *Recording of the evoked mechanical response*

The recording of the evoked mechanical response following sympathetic stimulation was achieved by attaching the thread from the epididymal end to an isometric force transducer. The transducer was connected to an Ultra Violet sensitive paper (Kodak).

Vasa deferentia were suspended in the organ bath under an initial resting tension of 0.5 g. Perfusion of the bath with Krebs solution was immediately initiated. After a period of 45 min, the vasa had equilibrated and the resting tension was approximately 0.2 g. No further adjustment of the tension was performed.

### *Statistical analysis*

All results show the mean ± s.e.mean. Statistical significance were calculated by Student's $t$ test for paired or unpaired observations and by a one way or two way analysis of variance (ANOVA), as specified.

## Results

### *The ATP assay system*

Several standards curves were prepared during one experiment and in order to check the assay itself, 9 control curves over 7 h were analysed. Standard ATP-response curves were generated by adding a known amount of ATP to aliquots of Krebs solution which had been superfused over a resting vas deferens. Standard curves were reproducible within ± 10% for ATP amounts above 0.2 pmol. The variability was significantly higher at 0.2 pmol (Student's unpaired $t$ test, $P < 0.05$) and was 13.27 ± 1.01%. At blank values the variability exceeded ± 20%, which was significantly higher than at 0.2 pmol ATP (Student's $t$ test, $P < 0.05$) and than values above 0.2 pmol ATP (Student's unpaired $t$ test, $P < 0.001$). The variability observed was not due to a variable spontaneous release of ATP from the tissue since the variability was not reduced with non-superfused aliquots (Student's $t$ test, $P > 0.05$).

During an experiment the sensitivity of the assay decays in a predictable way, though the shape of the standard curve remains constant. Data were corrected for this decay.

### *ATP overflow from the mouse vas deferens*

In a series of experiments, stimulation began 5, 30 and 40 s respectively into the minute of the first stimulated aliquot. Moreover, experiments were conducted at 22°C or 37°C. Finally the number of pulses was varied, giving the experimental groups shown in Table 1.

The stimulated-ATP period represents ATP overflow collected within the minute in which stimulation began and the two successive ones. The post-stimulated-ATP period began at the 4th aliquot after the stimulation was applied.

In response to field stimulation (5 Hz, 0.5 ms, supramaximal voltage) ATP-overflow greatly exceeded basal, non-stimulated-ATP by 6.66 and 9.51 times with 50 pulses (groups A and B, respectively) and by 14.23 and 28 times with 100 pulses (groups C and D, respectively). For statistical significance see Figure 1. Within minutes after field-stimulation, ATP-overflow returned toward previous low levels of ATP. Post-stimulated-ATP values did not differ from basal-ATP values (Student's unpaired $t$ test, $P > 0.05$), except for group D i.e., when increasing the temperature from 22°C to 37°C.

Varying the time at which stimulation began into the minute of the 1st stimulated aliquot affected the temporal pattern of ATP overflow (Figure 1) while increasing the temperature from 22°C to 37°C did not affect either stimulated-ATP (Student't unpaired $t$ test, $P > 0.5$) or the temporal pattern of ATP overflow.

In only 17 experiments out of 139, stimulated-ATP failed to exceed basal-ATP values. The criteria for failure was defined as any stimulated-ATP less than, or equal to, the mean + (s.e.mean × 1.96) of the highest basal-ATP aliquot of the corresponding experimental group. Stimulated-ATP

**Table 1** The experimental groups used in this work

| *Group* | *Temperature* | *Stimulation at:* | *No. of pulses* | n |
|---|---|---|---|---|
| A | 22°C | 5 s | 50 | 36 |
| B | 22°C | 30 s | 50 | 17 |
| C | 22°C | 40 s | 100 | 27 |
| D | 37°C | 40 s | 100 | 50 |

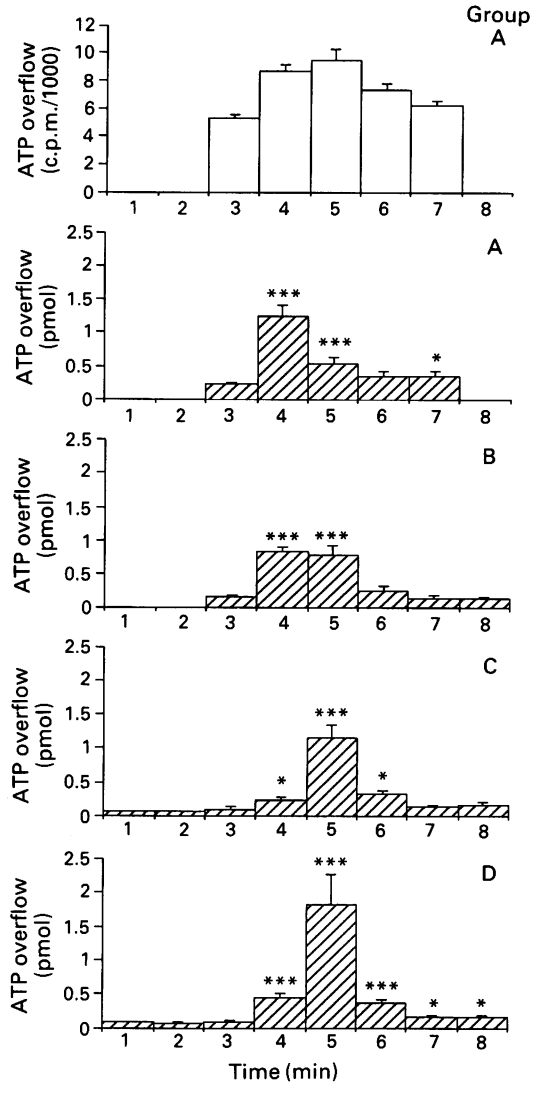


**Figure 1** The temporal pattern of ATP overflowing from the mouse isolated vas deferens during the 1st collection sequence for group A, B, C and D as defined in Table 1. Superfusate were collected in 1 min aliquots. Field stimulation was applied at the arrow. Column show means of 36, 17, 27 and 50 measurements for group A, B, C and D, respectively; vertical bars show s.e.mean. Statistically significant differences denote greater than basal-ATP within each group (Student's $t$ test: $*P<0.05$; $**P<0.01$ and $***P<0.001$). For group A the temporal pattern of [$^3$H]-noradrenaline ([$^3$H]-NA) is shown. Columns show means of 30 measurements; vertical bars show s.e.mean. There were statistically significant differences between conditions (ANOVA two way, $F = 14,6$, d.f.$_{(5,145)}$, $P<0.001$).

values were variable between experiment (Figure 2). The variability of the stimulated-ATP was comparable in all groups and, thus, was not influenced by temperature, number of pulses or position of stimulation into the minute.

### *Noradrenaline overflow*

For group A, the mouse isolated vas deferens was preloaded with [$^3$H]-NA. Following stimulation [$^3$H]-NA levels were significantly higher than basal NA (ANOVA, two way, $F = 14.11$, d.f.$_{(5,145)}$, $P<0.001$) (see Figure 1). The highest level of NA was detected in the second aliquot of the stimulation period while for stimulated-ATP the highest level of ATP in this group was detected in the first aliquot of the stimulation period.

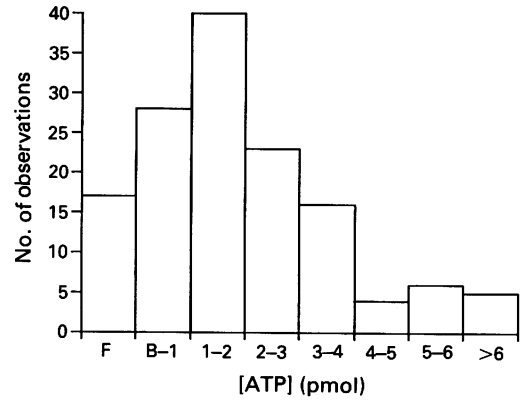


**Figure 2** The distribution of stimulated-ATP overflowing from the mouse isolated vas deferens after the 1st stimulation. Ordinate scale shows number of observations and abscissa scale shows the class in pmol. The observations in each class were greater than the lower limit but less than or equal to the upper limit. 'F' denotes failure to observe stimulated-ATP greater than basal-ATP. The criteria for failure were determined as follows: stimulation induced-ATP less than, or equal to, the mean + (s.e.mean × 1.96) of the aliquot collected just prior to stimulation which is multiplied by 3 since stimulated-ATP was collected over a 3 min period.

### *The effects of number of pulses on stimulated-ATP overflow*

Increasing the number of stimulation pulses increased mean total stimulated-ATP while the per pulse stimulated-ATP did not vary. Mean total stimulated-ATP ($n = 8$) was $1.6 \pm 0.3$ pmol ATP at 50 pulses, $3.76 \pm 0.87$ pmol ATP at 100 pulses, $5.57 \pm 0.98$ pmol ATP at 150 pulses and $6.74 \pm 1.66$ pmol ATP at 200 pulses. For statistical differences see Figure 3. The difference between 150 and 200 pulses was not significant mainly because for 4 experiments stimulated-ATP plateaued after 150 pulses.

### *The effects of frequency on stimulated-ATP overflow*

Increasing the frequency of stimulation significantly increased the per pulse stimulated-ATP from $0.31 \pm 0.02$ pmol ($n = 45$) at low (2.5, 5, 6.6 and 10 Hz) frequencies to $0.63 \pm 0.10$ pmol ($n = 15$) at high (16.6, 25 and 50 Hz) frequencies (ANOVA, one way, d.f.$_{(1,58)}$, $F = 24.71$, $P<0.001$) (Figure 4).

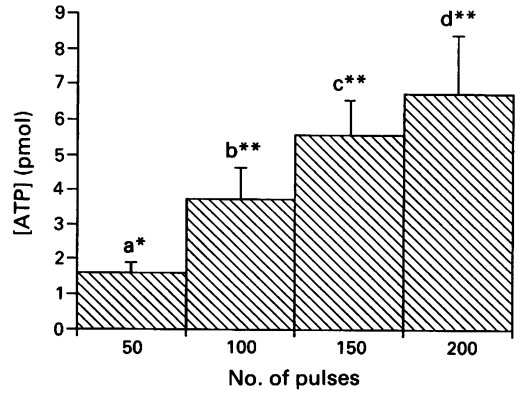


**Figure 3** The effects of number of pulses on the release of stimulated-ATP from the mouse isolated vas deferens. Columns show means expressed as percentage of stimulated-ATP at 200 pulses of 8 measurements and vertical bars show standard errors. Statistically significant differences between 50 and 100 (a), 100 and 150 (b), 50 and 150 (c) 100 and 200 (d) were observed (Student's $t$ test: $*P<0.05$; $**P<0.01$ and $***P<0.001$).

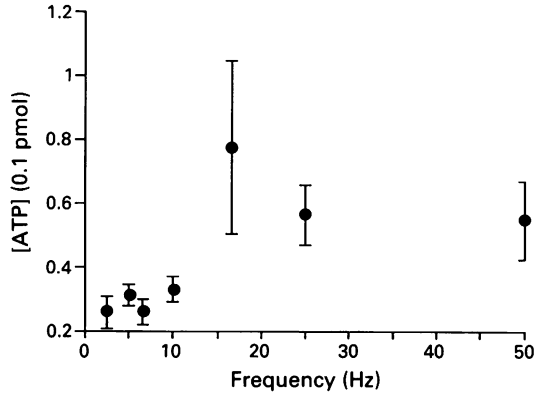


**Figure 4** The effects of frequency on the release of stimulated-ATP from the mouse isolated vas deferens. Points show means expressed as per pulse stimulated-ATP of 5 to 15 measurements and bars show standard errors. Statistically significant differences between low (2.5 to 10 Hz, $n = 15$) and high (16.7 to 50 Hz, $n = 45$) frequencies were observed (ANOVA, $d.f._{(1,58)}$, $F = 24.71$, $P < 0.001$).

### *The effects of chemical stimulation*

Mouse vasa deferentia ($n = 8$) were field-stimulated (C) and chemically contracted by directly injecting $5 \times 10^{-5}$ M (−)-phenylephrine (PE) into the inflow tubing. For half of the vasa deferentia, field-stimulation was applied before PE. The mechanical contractions evoked by field-stimulation at 5 Hz (pulse width 0.5 ms, supramaximal voltage, 200 pulses) were biphasic. The twitch component obtained had a maximum tension of $1.05 \pm 0.06$ g and the hump $0.66 \pm 0.07$ g. The mechanical responses obtained with PE were monophasic and had a maximum tension of $0.61 \pm 0.06$ g lasting $102.6 \pm 5.4$ s.

Stimulated-ATP evoked by the 1st and 2nd chemical stimulation was $0.80 \pm 0.14$ and $0.72 \pm 0.15$ pmol ATP respectively and, thus, represents only a minor fraction of the stimulated-ATP evoked by the 1st and 2nd field stimulation which was $3.77 \pm 0.74$ and $2.66 \pm 0.73$ pmol ATP, respectively. For statistical significance see Figure 5.

### *The effects of tetrodotoxin*

Mouse vasa deferentia ($n = 8$) were superfused with tetrodotoxin (TTX) (3 μM) for 30 min before stimulation and sampling. Data were expressed as percentage inhibition ± s.e.mean. In 5 experiments, TTX significantly reduced stimulated-ATP by $79.55 \pm 8.02\%$ (Student's paired $t$ test, $P < 0.001$). The residual ATP i.e., $20.45 \pm 8.02\%$, was accompanied by a residual contraction due, probably, to direct stimulation of the vas deferens (Figure 6).

In the presence of TTX and for the remaining 3 experiments, disturbances were observed characterized by the presence of massive amounts of ATP i.e., much higher than the stimulated-ATP normally observed. These massive ATP levels were not related to the stimulation since both basal-ATP and post-stimulated-ATP were affected by TTX-exposure.

## Discussion

This work presents the first measurements of ATP overflow from the mouse vas deferens. ATP was reliably detected in superfusate samples from the field-stimulated mouse vas deferens by use of a refined and inexpensive version of the highly specific luciferase-catalysed reaction for the determination of ATP which can be used concomitantly with the detection of [$^3$H]-NA. The limits of the assay were estimated from the reproducibility of the ATP-standard curves and it can be concluded that for any amount of ATP above 0.2 pmol, any variability above 10% is very likely to be due to change in ATP-overflow.

In the present work, stimulated-ATP increased by 6.66 and 9.51 times above spontaneous levels (groups A and B) following stimulation at 5 Hz (0.5 ms pulse width, supramaximal voltage) with 50 pulses. When 100 pulses were applied, stimulated-ATP increased by 14.23 and 28 times above spontaneous levels (groups C and D). In the guinea-pig vas deferens, Ellis & Burnstock (1989) reported levels of ATP following stimulation of $1.95 \pm 1.17$ ($n = 6$) times above spontaneous levels at 2 Hz and $6.30 \pm 1.11$ ($n = 6$) times at 20 Hz, even so, the number of pulses used (240 pulses) was much higher. Kirkpatrick & Burnstock (1987) reported at 16 Hz a 50 fold increase of ATP efflux above spontaneous levels of release. However, at a similar frequency (4 Hz) to the one used here (5 Hz) they obtained an increase from 0.004 nmol min$^{-1}$ g$^{-1}$ to $0.042 \pm 0.01$ nmol min$^{-1}$ g$^{-1}$ i.e., an increase by 10 times above spontaneous levels, though the number of pulses was much higher, 480 pulses against 50 pulses used here.

The per pulse ATP overflowing from the mouse vas deferens seems to be higher than that reported for the guinea-pig vas deferens. In the present work, following stimulation at 5 Hz with up to 200 pulses, the mean per pulse stimulated-ATP was $0.3517 \pm 0.0074$ pmol ($n = 32$). For a typical sized vas, $18.36 \pm 0.78$ mg ($n = 32$), this corresponds

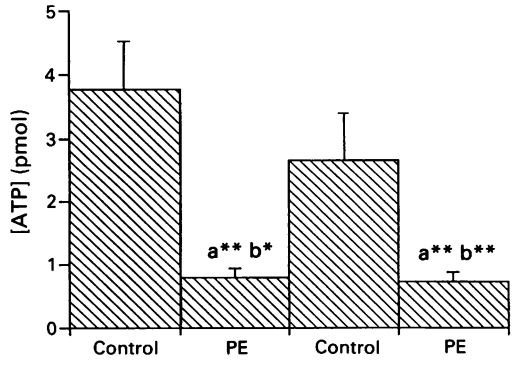


**Figure 5** Stimulated-ATP following field stimulation (Control) and chemical stimulation ((−)-phenylephrine, PE; $5 \times 10^{-5}$ M) from the mouse isolated vas deferens. Columns show means of 8 measurements and bars show standard errors. Statistically significant differences between PE and first control (a) and PE and second control (b) are significant to $*P < 0.05$ and $**P < 0.01$ (Student's paired $t$ test).

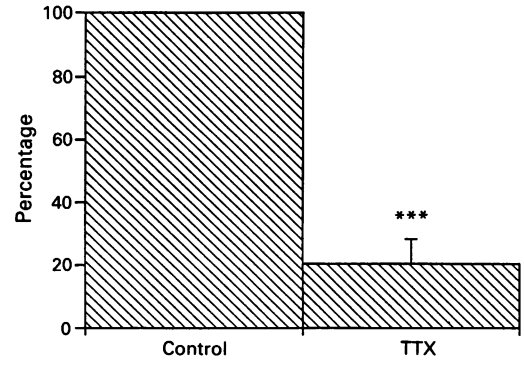


**Figure 6** Comparative ATP overflow from field-stimulated (Control) and tetrodotoxin-exposed (TTX) mouse isolated vas deferens. Columns show means of 5 measurements and bars show standard errors. Statistically significant differences between control and TTX-exposed tissues were observed (Student's $t$ test: $***P < 0.001$).

to 19.13 pmol pulse$^{-1}$ g$^{-1}$. For the guinea-pig vas deferens, Kirkpatrick & Burnstock (1987) reported overflow of 180 pmol min$^{-1}$ g$^{-1}$ at 16 Hz corresponding, thus, to an efflux of 0.1875 pmol pulse$^{-1}$ g$^{-1}$ and Levitt *et al.* (1984) reported 0.0249 ± 0.002 pmol pulse$^{-1}$ following stimulation at 0.5 ms duration, 1 Hz, with 240 pulses.

The reasons for such differences might be explained in terms of the timing of the assay used. Levitt *et al.* (1984) reported that the pump for the superfusing medium was turned off at the onset of transmural electrical stimulation and remained off for a total of 20 min. It is possible that during this 20 min period the amount of ATP decreased. In the present work, experiments were scheduled into a 'just-in-time' collection-detection cycle in which no dead time occurred i.e., by the time one vial finished being counted, the next one was ready. ATP-content after collection of the superfusate decreases dramatically with time. We found that if aliquots were first collected and then immediately assayed after the last aliquot was collected, stimulated-ATP did not exceed basal, non-stimulated ATP. Using this 'just-in-time' collection-detection cycle, there was no need to bathe the preparation with the reagents as reported by Lew & White (1987).

ATP determination is complicated by ecto-enzymatic breakdown. However, when the precise temporal pattern of ATP overflow was characterized, it was shown that it was influenced by the position of the stimulation into the minute of the aliquot in which stimulation began but was not influenced by number of pulses or temperature. This suggests that precise timing of the stimulation, superfusate collection and assay, minimized ATP degradation prior to assay, offering the opportunity for quantitative studies.

ATP may be released from pre-junctional sites, smooth muscle cells or from both. Vizi & Burnstock (1988) observed that in the rat vas deferens, a significant amount of ATP was released secondarily as a result of NA action via $\alpha_1$-adrenoceptors. Similar findings were recently reported by Katsuragi *et al.* (1990) working with guinea-pig vas deferens. In the present study, chemical stimulation led to only a small increase in ATP overflow, showing that a large part of the stimulated-ATP observed was not due to muscle contraction or to $\alpha_1$-adrenoceptor activation and thus, providing evidence for neurally-released ATP. Nevertheless, as found for the rat (Vizi & Burnstock, 1988) and for the guinea-pig (Katsuragi *et al.*, 1990) vas deferens a small portion of the stimulated-ATP was release as a consequence of $\alpha_1$-adrenoceptor activation. TTX usually reduced stimulation-induced overflow of ATP to low levels suggesting, again, a predominantly pre-junctional origin. In a few preparations, however, it had quite different effects, promoting massive ATP release independent of stimulation. This may reflect some TTX-induced disruption of smooth muscle cells, leading to uncontrolled release of ATP but it is unclear why some preparations should be affected more than others.

Stimulated-ATP following different patterns of stimulation behaved in a physiological way. Over the whole range studied (50 to 200 pulses), the mean per pulse stimulated-ATP was independent of stimulation duration implying that, at the stimulation duration range used (10 to 40 s), ATP overflow reflects accurately ATP release despite the known process of ATP removal which might have taken place after the release of ATP.

Thus, as previously shown for the efflux of NA from the guinea-pig vas deferens (Kalsner, 1980) stimulated-ATP in the mouse vas deferens is independent of train length; even so, tissues were not pretreated in order to eliminate the complications of either neuronal or extraneuronal uptake of neurally released transmitter, as normally done when studying the efflux [$^3$H]-NA.

Facilitation might be observed when increasing the frequency of stimulation. The per pulse efflux of [$^3$H]-NA has been shown to increase with increasing frequency of stimulation in rodent vas deferens (Kalsner, 1979, 1980). It was of interest to see if similar results could be observed on the per pulse stimulated-ATP. Increasing the frequency of stimulation over the range 2.5 to 50 Hz did not increase progressively the per pulse stimulated-ATP, but a significant increase was observed between high and low frequencies of stimulation. It is possible that the lack of a progressive increase in stimulated-ATP in response to increasing the frequency of stimulation, as observed for the efflux of [$^3$H]-NA (Kalsner, 1979, 1980), reflects the fact that ATP determination is complicated by ecto-enzymatic breakdown while the determination of NA is performed on pretreated tissue and, thus, is not complicated by either neuronal or extraneuronal uptake of neurally released NA. At low frequencies of stimulation ecto ATPase activity may not be saturated and thus, any increase in the per pulse ATP-overflow will be degraded; however, at high frequencies of stimulation, saturation of the ectoATPase activity might occur, enabling the increase per pulse of stimulated-ATP to be observed.

In conclusion, the present study provides a useful way of systematically and quantitatively detecting release of endogenous ATP from field-stimulated mouse vas deferens at a relatively low frequency and number of pulses. Moreover, such methods allow concomitant measurement of [$^3$H]-NA. The observed physiological variations in ATP-overflow support the case that this technique may have further application for the study of prejunctional factors.

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# Inhibition by salmeterol of increased vascular permeability and granulocyte accumulation in guinea-pig lung and skin

[1]C.J. Whelan & M. Johnson

Department of Peripheral Pharmacology, Glaxo Group Research Limited, Ware, Hertfordshire, SG12 0DP

**1.** The long-acting $\beta_2$-adrenoceptor agonist, salmeterol has been evaluated for its anti-inflammatory effects in the guinea-pig lung and skin.

**2.** Salmeterol, administered in bronchodilator doses to conscious guinea-pigs by both oral (0.01–1.0 mg kg$^{-1}$) and inhaled (nebulizer concentration, 0.001–1.0 mg ml$^{-1}$) routes, inhibited histamine-induced plasma protein extravasation (PPE) into the airway lumen.

**3.** Inhibition of PPE by salmeterol was long-lasting (>6 h) and was inhibited by prior administration of propranolol (1 mg kg$^{-1}$, s.c.), indicating an effect mediated by β-adrenoceptors.

**4.** Inhaled salbutamol (nebulizer concentration, 0.001– 1.0 mg ml$^{-1}$) also inhibited PPE in guinea-pig lung but, in contrast to salmeterol, this effect was short-lived with substantial loss of activity 2 h after administration.

**5.** Inhaled salmeterol (0.1 mg ml$^{-1}$) and salbutamol (1.0 mg ml$^{-1}$) inhibited the accumulation of neutrophils in guinea-pig lung in response to lipopolysaccharide (100 μg ml$^{-1}$). Salmeterol, but not salbutamol, inhibited the infiltration of eosinophils into the airway lumen in response to platelet activating factor (100 μg ml$^{-1}$). These effects of salmeterol were blocked by prior administration of propranolol (5 mg kg$^{-1}$, s.c.), indicating that they were also β-adrenoceptor-mediated.

**6.** Oral salmeterol (10 mg kg$^{-1}$, p.o.), but not salbutamol (10 and 100 mg kg$^{-1}$, p.o.), inhibited zymosan-induced granulocyte accumulation and PPE in guinea-pig skin. Lower doses of salmeterol (0.1 and 1 mg kg$^{-1}$) inhibited PPE, but not granulocyte accumulation. The effects of salmeterol were blocked by prior administration of propranolol (1 mg kg$^{-1}$, s.c.). Both salmeterol and salbutamol inhibited histamine-induced PPE in guinea-pig skin.

**7.** Intradermal salmeterol ($10^{-8}$ mol per site), but not salbutamol, was also effective in inhibiting zymosan-induced granulocyte accumulation and PPE in guinea-pig skin.

**8.** It is concluded that salmeterol, at bronchodilator doses in the guinea-pig, inhibits granulocyte accumulation and PPE, possibly by an action on the vasculature. As this profile of activity is not shared by the shorter-acting compound, salbutamol, it would seem that anti-inflammatory activity is associated with β-adrenoceptor agonism of long duration. The implications of these findings for the use of salmeterol in the treatment of bronchial asthma are discussed.

**Keywords:** Salmeterol; salbutamol; anti-inflammatory; β-adrenoceptor agonist; vascular permeability; neutrophil; eosinophil; lung; skin; granulocyte

## Introduction

The anti-inflammatory properties of β-adrenoceptor agonists in experimental animals are well documented (Spector & Willoughby, 1960; Green, 1972; Persson *et al.*, 1978). Adrenaline inhibits oedema formation following thermal injury to rats (Spector & Willoughby, 1960), while salbutamol has been shown to inhibit carrageenin-induced oedema in the mouse paw following local and intraperitoneal injection (Green, 1972), and PAF-induced oedema in the rat following oral administration (Bonnet, 1981). These anti-inflammatory actions were due to stimulation of β-adrenoceptors, as they were inhibited by β-adrenoceptor blocking agents (Green, 1972; Arntzen & Briseid, 1973; Persson *et al.*, 1979). Moreover, the anti-inflammatory effects of β-agonists are not restricted to skin and subcutaneous tissue, since terbutaline has been shown to inhibit plasma protein extravasation (PPE) and the increase in lung weight induced by histamine in the guinea-pig (Persson *et al.*, 1979).

One explanation for the inhibitory actions of β-adrenoceptor agonists in some of the studies described above is via inhibition of the release of inflammatory mediators (Butchers *et al.*, 1980). However, this mechanism is unlikely to account for all the effects seen, since in many experiments the direct actions of inflammatory mediators are inhibited (Svensjo *et al.*, 1976).

β-Adrenoceptor agonists inhibit capillary permeability by a mechanism which does not appear to be secondary to vasodilatation. Rippe & Grega (1978) demonstrated that isoprenaline reduced capillary filtration in a maximally vasodilated, rat perfused hindquarter preparation. Further studies showed that isoprenaline and terbutaline inhibited PPE, whereas equi-vasodilator doses of acetylcholine or papaverine were ineffective (Svensjo *et al.*, 1976; Grega *et al.*, 1980; Raymond *et al.*, 1980; Persson *et al.*, 1982; Prasad *et al.*, 1982). Joyner *et al.* (1979) and Svensjo & Grega (1986) suggested that β-agonists modulate PPE by an action on β-adrenoceptors on post-capillary venules.

This hypothesis was supported by Gudgeon & Martin (1989) who demonstrated that isoprenaline inhibits phorbol myristate acetate-induced transit of albumin across porcine endothelial monolayers in culture, by a mechanism which was blocked by propranolol.

To our knowledge, few studies have examined the effect of β-adrenoceptor agonists on other aspects of acute-inflammation, although Spicer *et al.* (1990) demonstrated that a number of β-adrenoceptor agonists prevent sephadex-induced blood eosinophilia in rats while subcutaneous isoprenaline also inhibited sephadex-induced eosinophil accumulation in rat lung. Furthermore, in spite of the experimental evidence,

[1] Author for correspondence.

currently available $\beta_2$-adrenoceptor agonists with a short duration of action, such as salbutamol, do not appear to have any significant anti-inflammatory activity in therapeutic situations such as atopic dermatitis or the late phase response in asthmatics following antigen challenge (Cockroft & Murdock, 1987; Archer & Macdonald, 1987; Green *et al.*, 1988). The duration of action of the $\beta$-agonist may be an important factor, since many inflammatory processes both develop and resolve slowly.

The present paper compares the anti-inflammatory properties of the new long-acting $\beta_2$-adrenoceptor agonist, salmeterol (Bradshaw *et al.*, 1987), with those of salbutamol in guinea-pig lung and skin. Preliminary accounts of part of this work have been published in abstract form (Whelan & Johnson, 1990; 1991a; 1991b).

## Methods

### *Lung inflammation*

*Histamine-induced plasma protein extravasation (PPE)* Male guinea-pigs (300–400 g) were given an intra-cardiac injection of iodinated ($^{125}$I) human serum albumin (0.5 $\mu$Ci, 0.3 ml) in heparinised saline (10 u ml$^{-1}$) under light isoflurane anaesthesia. Following recovery, animals were placed in a plexiglas chamber and exposed to an aerosol of histamine (0.5 mg ml$^{-1}$) generated by a Devilbiss nebuliser for 30 s, followed by a further 30 s exposure to the atmosphere in the chamber.

Thirty minutes after histamine challenge, guinea-pigs were killed with an overdose of pentobarbitone sodium (Expiral) administrated intraperitoneally and a blood sample was taken by cardiac puncture. The trachea of each animal was cannulated, and the lungs lavaged twice with 10 ml heparinised (10 u ml$^{-1}$), phosphate-buffered saline at 37°C. The radioactivity in both an aliquot of plasma and a 5 ml sample of the pooled bronchoalveolar lavage fluid (BALF) was measured in a scintillation counter (LKB Compugamma). From these data, plasma protein extravasation (expressed as $\mu$l plasma ml$^{-1}$ BALF) was calculated.

Where potency was estimated, drugs were administered to the animals by the oral or aerosol route 30 min before histamine challenge. In duration of action studies, the time interval between $\beta$-agonist administration and histamine exposure was extended for periods of up to 8 h. In experiments where salmeterol, salbutamol or prostaglandin $E_2$ ($PGE_2$) was administered by inhalation, guinea-pigs were placed in a plexiglas chamber and exposed to aerosols of these agents as described by Ball *et al.* (1991), in that solutions of these agents were nebulised into the chamber for 1 min and the animals breathed the atmosphere within the chamber for a further 2 min.

*Lipopolysaccharide (LPS) and platelet activating factor (PAF)-induced granulocyte accumulation* Male guinea-pigs (300–400 g) were placed in plexiglas chambers and exposed to aerosols of LPS (100 $\mu$g ml$^{-1}$) or PAF (100 $\mu$g ml$^{-1}$) for 10 min as described by Folkerts *et al.* (1988) and Aoki *et al.* (1987) respectively. Four hours after LPS, or 24 h after PAF, guinea-pigs were killed and the lungs lavaged with heparinised, phosphate-buffered saline as described above.

A total leucocyte count was performed, and a cytospin preparation was made from the BALF recovered. Cytospin preparations were fixed with methanol, stained with Wright's stain and differential leucocyte counts carried out by an operator who was unaware of the treatments given to the animals during the experiment.

### *Dermal inflammation*

*Granulocyte accumulation and granulocyte-dependent vascular permeability:- preparation and labelling of guinea-pig granulocytes* Guinea-pig peritoneal granulocytes, elicited by intraperitoneal injection of glycogen (20 ml, 0.1% w/v), were harvested from exudates (18 h after injection of glycogen) by centrifugation on histopaque 1077 (20 min, 200 *g*). Granulocytes recovered in the pellet were washed twice with heparinised Tyrode solution. Cells were then resuspended in 2 ml of heparinised Tyrode solution, mixed with $^{111}$indium oxine (150 $\mu$Ci: 5.55 MBq), and incubated at room temperature for 15 min. Labelled granulocytes (>85% pure) were washed twice with heparinised Tyrode solution and suspended in an appropriate volume of heparinised Tyrode solution for reinjection (usually 2–5 ml). In experiments where vascular permeability was measured, ($^{125}$I) iodinated human serum albumin (10 $\mu$Ci: 0.37 MBq) was added to the labelled-granulocyte suspension.

*Experimental protocol* Male guinea-pigs (300–400 g) were anaesthetized with ketamine (40 mg kg$^{-1}$, i.m.)/xylazine (8 mg kg$^{-1}$, i.m.) and the ventral surface of the abdomen shaved. Each guinea-pig received an intra-cardiac injection of indium-labelled granulocytes (usually 0.3 ml) and six intradermal injections of either sterile saline (100 $\mu$l) or a suspension of zymosan (0.06–2 mg) in saline. The animals were then allowed to recover from the anaesthetic. After 4 h, guinea-pigs were killed by the intraperitoneal administration of pentobarbitone sodium. A blood sample (2 ml) was taken into heparin (50 u ml$^{-1}$ final conc.) and a full thickness biopsy of the injection site taken with an 18 mm hollow punch.

An aliquot (500 $\mu$l) of the blood sample was removed for counting, whilst the remainder was centrifuged. An aliquot of the resulting plasma (200 $\mu$l) was then also taken for counting.

The radioactivity of the blood, plasma and skin biopsies was determined. From these data, the granulocyte content of each skin site was calculated according to equation (1), given a granulocyte count of $2.25 \times 10^3$ cells $\mu$l$^{-1}$ of blood (determined from differential counts of the blood from 6 control guinea-pigs).

$$\text{Tissue granulocytes} = 2.25 \times 10^3 \times \text{tissue } ^{111}\text{In count} \div \frac{\text{blood } ^{111}\text{In count}}{\text{vol blood counted } (\mu\text{l})} \quad (1)$$

Similarly, plasma protein extravasation, expressed as $\mu$l plasma equivalents, was calculated according to equation (2).

$$\text{Plasma protein } (\mu\text{l}) = \text{Tissue } ^{125}\text{I count} \div \frac{\text{plasma } ^{125}\text{I count}}{\text{vol plasma counted } (\mu\text{l})} \quad (2)$$

In the experiments described, 83.4 ± 1.5% of $^{111}$In was incorporated into the granulocytes. Approximately $10^7$ cells were injected into the circulation of each guinea-pig and 4 h after injection <20% of $^{111}$In in the blood was recovered from the plasma, indicating that >80% of the $^{111}$In administered remained associated with the blood cells. However less than 20% of the injected neutrophils were circulating in the blood 4 h after injection. All values quoted for granulocyte accumulation and plasma protein extravasation (vascular permeability) are corrected for the increases induced by an intradermal injection of sterile saline.

*Granulocyte-independent vascular permeability* Male guinea-pigs (250–350 g) were anaesthetised with ketamine/xylazine as described above. Each guinea-pig was given an intracardiac injection of iodinated human serum albumin (≃0.5 $\mu$Ci in 0.3 ml heparinised saline) followed by a series of intradermal injections of sterile saline or histamine in sterile saline. After 30 min, the animals were killed by an injection of pentobarbitone sodium. A blood sample and full thickness biopsies of the injection sites were taken. The radioactivity of a sample of plasma (200 $\mu$l) and each biopsy was determined and the

plasma protein extravasation, expressed as μl plasma, calculated as described in equation (2).

### *Statistical analysis*

In lung, histamine-induced increases in plasma protein extravasation were log normally distributed. These data are expressed as geometric means and 95% confidence limits. $EC_{50}$ values were calculated from a regression analysis. Plasma protein extravasation in skin, and granulocyte accumulation in skin and lung were normally distributed and are expressed as arithmetic means ± s.e.mean. Where appropriate, levels of statistical significance were calculated, for normally distributed data, by Student's *t* test.

### *Drugs and reagents*

Iodinated human serum albumin (specific activity 2.5 μCi $mg^{-1}$ albumin) and indium oxine were purchased from Amersham International. Zymosan, histamine, propranolol, platelet activating factor (PAF) and LPS (*E. coli* 026:B6) were obtained from Sigma Ltd and $PGE_2$ (Prostin $E_2$) from Upjohn Ltd. Salmeterol and salbutamol (Glaxo Group Research Ltd) were dissolved in distilled water to an initial concentration of 10 mg $ml^{-1}$ with the addition of a few drops of glacial acetic acid. This solution was diluted further in distilled water prior to oral or inhaled administration. In experiments where compounds were administered intradermally, they were dissolved in dimethyl sulphoxide (DMSO, final concentration 1%) and co-injected with histamine or zymosan.

## Results

### *Lung inflammation*

*Histamine-induced plasma protein extravasation in the guinea-pig lung* Thirty minutes after inhalation of histamine (0.5 mg $ml^{-1}$) the plasma protein content of BALF increased (Figure 1) from 0.80 μl $ml^{-1}$ (geometric mean, 95% confidence limits 0.6–1.08 μl $ml^{-1}$, $n = 10$) to 5.26 μl $ml^{-1}$ (4.82–5.72 μl $ml^{-1}$, $n = 13$). Although bronchoconstriction was not measured in these experiments, this level of histamine challenge did not provoke respiratory distress, and was well tolerated by all animals.

*Effect of inhaled salmeterol and salbutamol on histamine-induced PPE* When salmeterol (nebulizer concentration, 0.001–1.0 mg $ml^{-1}$) was administered to guinea-pigs by aerosol, 30 min prior to challenge with histamine, a concentration-related inhibition of PPE was observed. At a dose of 0.1 mg $ml^{-1}$ salmeterol, total inhibition of histamine-induced PPE was achieved (Figure 1). The aerosol concentration of salmeterol required to reduce histamine-induced PPE by 50% ($ED_{50}$) was found by regression analysis to be 0.015 mg $ml^{-1}$. The vehicle for salmeterol (acetic acid/water) had no effect on histamine-induced PPE.

Like salmeterol, inhaled salbutamol (0.001–1.0 mg $ml^{-1}$) also inhibited histamine-induced PPE (Figure 1). An $ED_{50}$ of 0.038 mg $ml^{-1}$ was obtained for salbutamol which was not significantly different from that of salmeterol. Pretreatment of guinea-pigs with a β-adrenoceptor blocking dose of propranolol (1 mg $kg^{-1}$ s.c.; Daly *et al.*, 1975), prevented the inhibition of histamine-induced PPE by salmeterol (Figure 1).

*Effect of inhaled prostaglandin $E_2$ on histamine-induced PPE* In contrast to the $\beta_2$-adrenoceptor agonists, administration of an equi-effective bronchodilator dose of $PGE_2$ (0.1 mg $ml^{-1}$; Ball *et al.*, 1987), 30 min prior to histamine, did not inhibit PPE in guinea-pig lung. When administered at shorter time intervals before histamine challenge, $PGE_2$ also had no effect on PPE.

*Effect of oral salmeterol on histamine-induced PPE* Salmeterol (0.01–1.0 mg $kg^{-1}$, p.o.), 30 min prior to histamine, also inhibited PPE in a dose-related manner, with the highest dose producing total inhibition (Table 1). Analysis of the

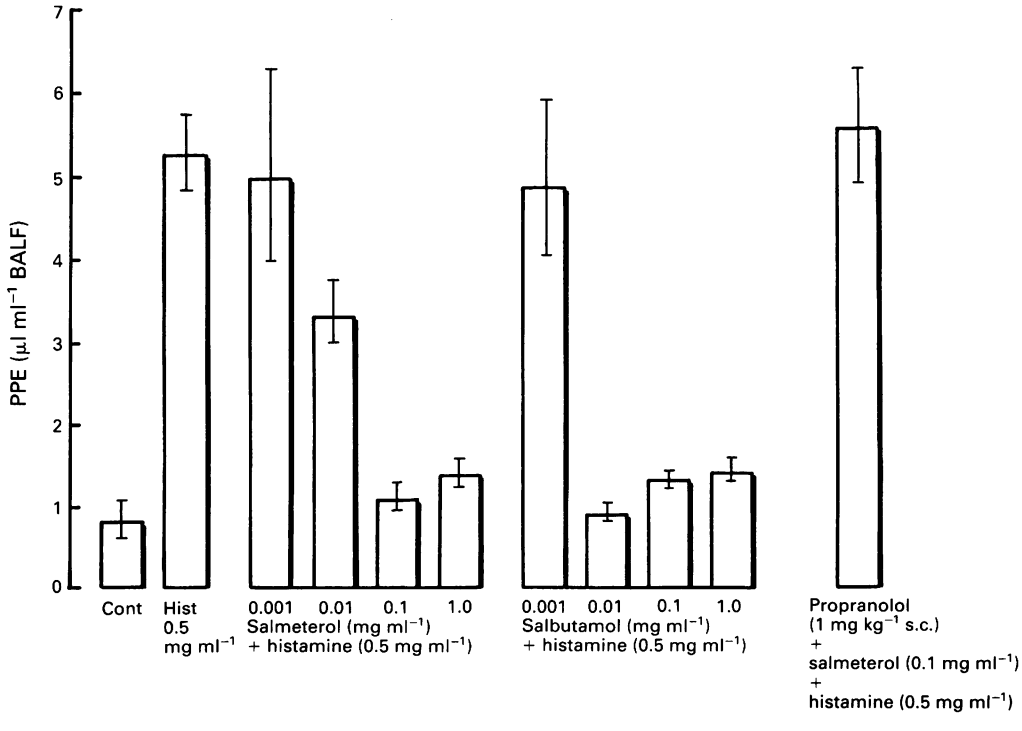


**Figure 1** Inhibition of histamine-induced plasma protein extravasation (PPE) in guinea-pig lung by inhaled salmeterol (0.001–1 mg $ml^{-1}$) and salbutamol (0.001–1 mg $ml^{-1}$) and blockade of the effects of salmeterol by propranolol (1 mg $kg^{-1}$, s.c.). Each column is the geometric mean and 95% confidence limits of at least 6 determinations. Concentrations refer to the concentration of the β-agonists in the nebuliser solution.

**Table 1** Inhibition of histamine (0.5 mg ml$^{-1}$)-induced plasma protein extravasation (PPE) in guinea-pig lung by orally administered salmeterol (0.01–1 mg kg$^{-1}$)

| Treatment | Dose (mg kg$^{-1}$) | PPE* (µl plasma ml$^{-1}$ BALF) | n |
|---|---|---|---|
| Control | – | 1.18 (1.10–1.28) | 21 |
| Vehicle | – | 4.38 (4.02–4.78) | 25 |
| Salmeterol | 0.01 | 5.40 (4.36–6.70) | 6 |
| Salmeterol | 0.1 | 1.52 (1.32–1.74) | 6 |
| Salmeterol | 1.0 | 0.62 (0.58–0.66) | 6 |

*Values shown are geometric means and 95% confidence limits, in parentheses, of *n* determinations.
BALF = bronchoalveolar lavage fluid.

data obtained revealed that the $ED_{50}$ oral dose of salmeterol was 0.02 mg kg$^{-1}$. The effect of orally administered salbutamol on histamine-induced PPE was not determined.

*The duration of inhibition of PPE by salmeterol and salbutamol* Inhibition of histamine-induced PPE by inhaled salmeterol (0.1 mg ml$^{-1}$) was long-lasting, being still evident for at least 6 h after administration (Figure 2). In contrast, salbutamol (0.1 mg ml$^{-1}$) had a shorter duration of action, with substantial loss of activity within 2 h (Figure 2).

*Inhibition of lipopolysaccharide and PAF-induced granulocyte accumulation by salmeterol and salbutamol* Exposure of guinea-pigs to a sub-maximal, aerosol dose of LPS (100 µg ml$^{-1}$) for 10 min resulted in an increase in the BALF neutrophil count from 3.8 ± 1.2 × 10$^3$ cells ml$^{-1}$ (mean ± s.e.mean) to 141.3 ± 33.3 × 10$^3$ cells ml$^{-1}$, 4 h after challenge. No significant ($P > 0.05$) change in any other cell type was observed. Pretreatment of guinea-pigs with an aerosol of salmeterol (0.1 mg ml$^{-1}$), 30 min before challenge with LPS, significantly ($P < 0.05$) reduced the BALF neutrophil count to 46.8 ± 9.1 × 10$^3$ cells ml$^{-1}$ (Figure 3), a reduction of 69%. Lower aerosol concentrations of salmeterol (0.01 mg ml$^{-1}$) were without effect.

In a parallel series of experiments, a ten fold higher dose of salbutamol (aerosol concentration 1.0 mg ml$^{-1}$), also significantly reduced LPS-induced BALF neutrophilia by 67% ($n = 6$), ($P < 0.05$). Lower aerosol concentrations of salbutamol (0.1 mg ml$^{-1}$) were without effect. When guinea-pigs were pretreated with propranolol (5.0 mg kg$^{-1}$, s.c., 30 min prior to salmeterol; Kellet, 1966), salmeterol had no significant inhibitory effect on LPS-induced neutrophil accumulation ($P > 0.05$).

Similarly, exposure of guinea-pigs to an aerosol of PAF (100 µg ml$^{-1}$; Aoki *et al.*, 1987) for 10 min resulted in a significant increase in the number of eosinophils in BALF from 53.3 ± 12.5 × 10$^3$ cells ml$^{-1}$ to 97.7 ± 18.8 × 10$^3$ cells ml$^{-1}$, 24 h after challenge ($P < 0.05$). Pretreatment of guinea-pigs with salmeterol (aerosol concentration, 0.1 mg ml$^{-1}$), 30 min prior to challenge with PAF, completely abolished PAF-induced eosinophil accumulation (eosinophil count 59.2 ± 19.8 × 10$^3$ cells ml$^{-1}$; Figure 4). A lower dose of salmeterol (0.01 mg ml$^{-1}$) also had an inhibitory effect.

In contrast to salmeterol, salbutamol (aerosol concentration 1.0 mg ml$^{-1}$) did not inhibit PAF-induced infiltration of eosinophils in guinea-pig lung. Inhibition of PAF-induced eosinophilia by salmeterol was abolished when guinea-pigs were pretreated with propranolol (Figure 5).

## *Dermal inflammation*

*Zymosan-induced granulocyte accumulation and plasma protein extravasation* Skin samples which had been injected with sterile saline contained 34.5 ± 6.0 × 10$^3$ ($n = 20$) granulocytes, although this value varied from experiment to experiment. Histological examination of these samples revealed no granulocytes in the extravascular compartment, therefore this value presumably reflects the granulocyte content of the vasculature within the biopsy. Similarly skin samples injected with saline had a plasma volume of 9.65 ± 0.86 µl ($n = 20$).

Four hours after the intradermal administration of zymosan (0.06–2 mg per site), a dose-related increase in the number of granulocytes (measured as an accumulation of $^{111}$In) and plasma protein extravasation (measured as the accumulation of $^{125}I_2$-HSA) was observed in the injection

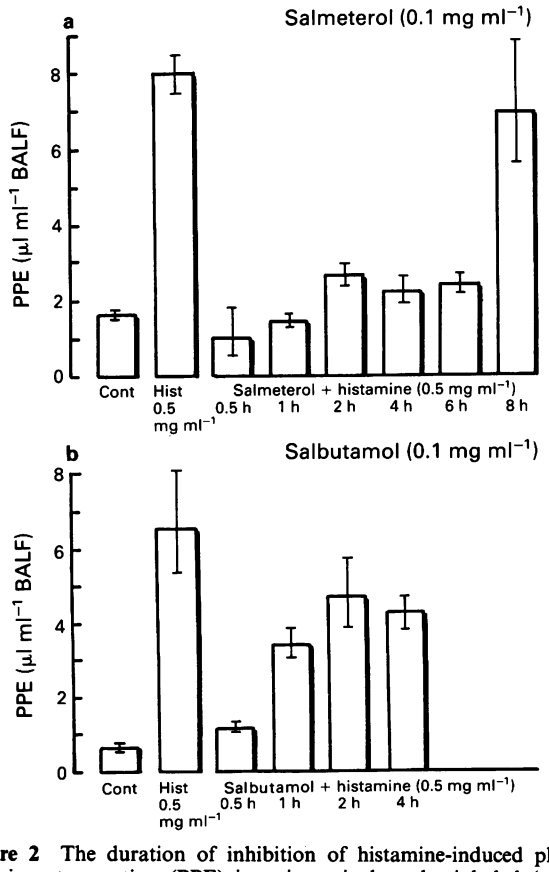


**Figure 2** The duration of inhibition of histamine-induced plasma protein extravasation (PPE) in guinea-pig lung by inhaled (a) salmeterol (0.1 mg ml$^{-1}$) and (b) salbutamol (0.1 mg ml$^{-1}$). Each column represents the geometric mean and 95% confidence limits of at least 6 determinations. Values illustrated are the plasma exudation measured following challenge with histamine (0.5 mg ml$^{-1}$) at the times indicated after administration of $\beta_2$-adrenoceptor agonists.

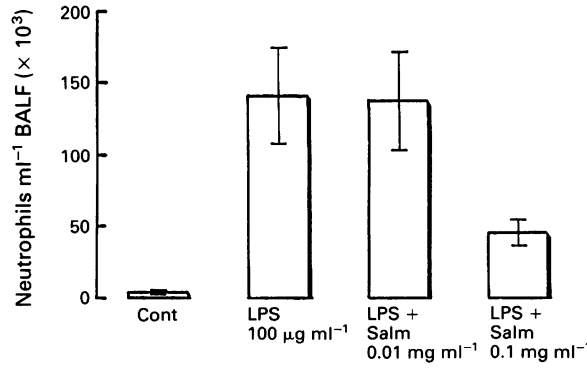


**Figure 3** Inhibition of lipopolysaccharide-induced neutrophil accumulation in guinea-pig lung by salmeterol (aerosol concentrations 0.01–0.1 mg ml$^{-1}$). Cont = control, LPS = lipopolysaccharide (100 µg ml$^{-1}$), Salm = salmeterol. Each column is the mean of 6 determinations; s.e.mean shown by vertical bars.

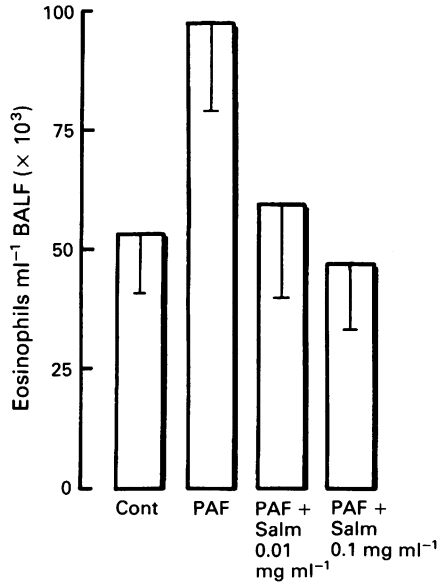


**Figure 4** Inhibition of PAF-induced eosinophil accumulation in guinea-pig lung by salmeterol (aerosol concentrations, 0.01–0.1 mg ml$^{-1}$). Cont = control, PAF = PAF (0.1 mg ml$^{-1}$), Salm = salmeterol. Each column is the mean of 6 determinations; s.e.mean shown by vertical bars.

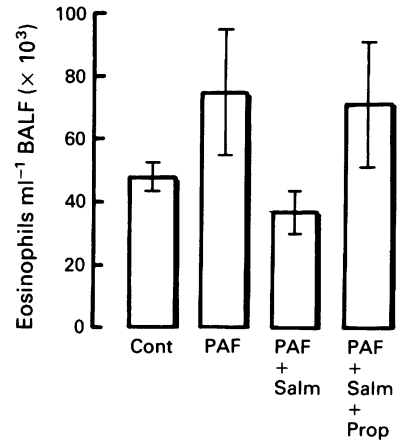


**Figure 5** Blockade by propranolol (5 mg kg$^{-1}$, s.c.) of the inhibitory actions of salmeterol (0.1 mg ml$^{-1}$) on PAF-induced eosinophil accumulation in guinea-pig lung. Cont = control, PAF = PAF (0.1 mg ml$^{-1}$), Salm = salmeterol 0.1 mg ml$^{-1}$, Prop = propranolol (5 mg kg$^{-1}$, s.c.). Each column is the mean of at least 6 determinations; s.e.mean shown by vertical bars.

sites. Granulocyte accumulation was confirmed by histology, which showed extensive cell infiltration of the dermis and subcutaneous tissue. Identification of individual cell types was not possible although the predominant cells appeared to be neutrophils. A sub-maximal dose of 0.2 mg per site zymosan was selected for further experiments. Granulocyte accumulation, but not PPE, induced by zymosan (0.2 mg per site) varied from experiment to experiment (Table 2), however, within any given experiment this response was consistent between animals.

Oral administration of salmeterol (10 mg kg$^{-1}$) significantly ($P<0.05$) decreased zymosan-induced (0.2 mg per site) granulocyte accumulation and PPE (Table 2). Lower doses of salmeterol (1.0 and 0.1 mg kg$^{-1}$, p.o) significantly ($P<0.05$) reduced PPE, but had no significant effect on granulocyte accumulation (Table 2). In contrast salbutamol (10 and 100 mg kg$^{-1}$, p.o.) had no significant inhibitory effect on zymosan-induced granulocyte accumulation or PPE. The highest dose of salmeterol used in this study (10 mg kg$^{-1}$, p.o.) had no significant ($P>0.05$) effect on circulating neutrophil numbers.

Pretreatment of guinea-pigs with propranolol (1 mg kg$^{-1}$, s.c.), 30 min prior to salmeterol (10 mg kg$^{-1}$, p.o.), prevented the inhibitory action of salmeterol on zymosan-induced granulocyte accumulation and PPE.

Salmeterol was also effective by the intradermal route. Intradermal administration of $10^{-8}$ mol per site significantly ($P<0.05$) reduced granulocyte accumulation from 79.6 ± 13.3 (mean ± s.e.mean) to 39.0 ± 10.9 × $10^3$ cells per site ($n=6$) and PPE from 15.2 ± 4.1 to 7.3 ± 1.8 ($n=6$) µl plasma per site ($n=6$). Lower doses of salmeterol ($10^{-9}$ mol per site) had no significant effect. Intradermal injection of salbutamol ($10^{-9}$–$10^{-7}$ mol per site) had no effect on zymosan-induced granulocyte accumulation or PPE.

### *Granulocyte-independent vascular permeability*

Histamine (10 ng–10 µg per site), unlike zymosan, caused plasma protein extravasation in guinea-pig skin without granulocyte accumulation. A dose of 100 ng per site histamine was selected for studies with salmeterol since this submaximal dose caused consistent increases in PPE, whereas a greater variation in response was seen with higher doses. When guinea-pigs were pretreated with salmeterol (10 mg kg$^{-1}$, p.o.) 1 h prior to injection of histamine (100 ng per site), PPE was significantly ($P<0.05$) reduced from 6.5 ± 1.0 µl plasma per site to 2.7 ± 0.7 µl plasma per site ($n=5$), a reduction of 58.5%. Lower doses of salmeterol (1 mg kg$^{-1}$, p.o.) had no inhibitory effect on histamine-induced PPE (5.9 ± 1.6 µl plasma per site) ($P>0.05$). Like salmeterol, salbutamol also inhibited histamine-induced PPE, but at a ten fold higher dose. Oral administration of 100 mg kg$^{-1}$ salbutamol significantly ($P<0.05$) reduced PPE from a control value of 11.9 ± 2.2 to 5.7 ± 1.7 ($n=4$) µl plasma per site. A lower dose of salbutamol (10 mg kg$^{-1}$) did not inhibit histamine-induced PPE (10.5 ± 2.5 to 9.8 ± 3.0 µl plasma per site ($n=8$)).

**Table 2** Effect of salmeterol and salbutamol on zymosan (0.2 mg per site)-induced inflammation in guinea-pig skin

| | | *PMN cell accumulation** (*cells per site × 10$^3$*) | | | *Plasma protein extravasation** (*µl*) | | |
|---|---|---|---|---|---|---|---|
| *Agonist* | *Dose* (*mg kg$^{-1}$, p.o.*) | *Control* | *Test* | n | *Control* | *Test* | n |
| Salmeterol | 0.1 | 141.1 ± 19.0 | 129.1 ± 30.4 | 6 | 14.6 ± 2.2 | 7.7 ± 2.5 | 6 |
| | 1.0 | 53.3 ± 13.3 | 39.8 ± 12.7 | 6 | 17.9 ± 2.1 | 8.2 ± 3.5 | 6 |
| | 10.0 | 460.0 ± 82.0 | 190.0 ± 35.0 | 8 | 12.7 ± 2.8 | 5.8 ± 1.3 | 6 |
| Salbutamol | 10 | 153.3 ± 24.1 | 135.6 ± 43.0 | 4 | 11.0 ± 2.6 | 6.4 ± 2.8 | 4 |
| | 100 | 153.3 ± 24.1 | 118.8 ± 37.7 | 6 | 11.0 ± 2.6 | 10.0 ± 3.9 | 6 |

*Values shown are arithmetic mean ± s.e.mean of $n$ determinations.
PMN = polymorphonuclear.

Pretreatment of guinea-pigs with propranolol (1 mg $kg^{-1}$, s.c.), 30 min prior to salmeterol (10 mg $kg^{-1}$, p.o.), abolished the inhibitory action of salmeterol on histamine-induced PPE in guinea-pig skin.

## Discussion

In acute inflammatory reactions, pharmacologically-active substances (kinins, histamine, $C_{5a}$ etc.) are released, and lead to increased venular permeability to plasma proteins, thus promoting oedema formation (Haddy *et al.*, 1976), and granulocyte accumulation (Hurley, 1972). Bronchial asthma is a disease increasingly regarded as being due to airway inflammation. In the asthmatic lung, the release of mast cell-derived inflammatory mediators has been detected (Wenzel *et al.*, 1990; 1991) and there is histological evidence of inflammatory cell activation and infiltration (Beasley *et al.*, 1989; Djukanovic *et al.*, 1991). Plasma proteins are also found in the bronchial lumen (Dunnill, 1960; Ryley & Brogan, 1968; Wanner, 1977; Persson, 1986) and the aqueous (non-mucus) phase of asthmatic sputum has been compared with inflammatory exudate (Ryley & Brogan, 1968). The presence of plasma protein in the bronchial secretions thickens mucus, thus impairing mucociliary clearance (Wanner, 1977), and can impair surfactant function, reducing ventilation (Seeger *et al.*, 1985). Additionally, bronchial mucosal oedema would produce a significant narrowing of the airway, thereby augmenting any bronchoconstriction (Hogg, 1985).

The inhibitory actions of β-adrenoceptor agonists on inflammatory mediator release and plasma protein extravasation are well documented, data coming from studies both in animals (Persson *et al.*, 1982) and in man (Basran *et al.*, 1982; Howarth *et al.*, 1985). However, clinical experience has not shown currently available β-adrenoceptor agonists to have significant anti-inflammatory activity (Archer & Macdonald, 1987; Cockroft & Murdock, 1987; Green *et al.*, 1988). This may be a consequence of the relatively short duration of action of such drugs. The experiments described above were therefore designed to investigate whether salmeterol, a novel long-acting $\beta_2$-agonist, would exhibit anti-inflammatory activity in addition to its long-lasting bronchodilator effects. Data published previously had shown that salmeterol inhibits inflammatory mediator release from human lung (Butchers *et al.*, 1991) and that this inhibition was sustained for up to 20 h.

The findings of this study indicate that salmeterol inhibits histamine-induced PPE in guinea-pig lung following aerosol or oral administration at bronchodilator doses (Ball *et al.*, 1991) and that these effects were mediated by β-adrenoceptors, since they were prevented by propranolol pretreatment. Furthermore, the inhibitory action of salmeterol on PPE is of long duration ($>6$ h) and is similar to that reported for the bronchodilator effects of the drug (Ball *et al.*, 1991).

Histamine increases PPE in lung by an action on the bronchial vasculature (Barnes *et al.*, 1988). Although the concentration of histamine used in these experiments did not cause overt bronchoconstriction, it is possible that histamine promotes PPE indirectly through a bronchoconstrictor action and that by inhibiting bronchoconstriction, salmeterol also inhibited PPE. This is unlikely since $PGE_2$ at bronchodilator doses did not inhibit histamine-induced PPE. In addition, salmeterol also inhibited histamine and zymosan-induced PPE in the skin of these animals. Instead, it is likely that salmeterol acts on the vasculature, probably on the endothelium of post-capillary venules (Persson *et al.*, 1982; Gudgeon & Martin, 1989), to prevent PPE.

The long duration of action of salmeterol against PPE may be of the therapeutic relevance. In animal studies, following the administration of bradykinin or histamine, the rate of PPE increases rapidly and returns to basal levels even when the stimulus persists (Adamski *et al.*, 1987). However, the resolution of this process is much slower, particularly in lung (Hurley, 1972; Sanchis *et al.*, 1972). Short-acting $\beta_2$-adrenoceptor agonists, such as salbutamol, may decrease the rate of extravasation, but only transiently. Since the rate-limiting step in clearing extravascular protein is the rate of removal (Hurley, 1972; Grega & Adamski, 1988), any transient reduction in the rate of extravasation would not result in a reduction of interstitial protein levels. In contrast, a long-acting compound, such as salmeterol, may be capable of reducing the rate of PPE for a sufficient period to allow the clearance mechanism to have an effect. This would be expected to reduce tissue oedema formation.

Salmeterol also inhibits granulocyte accumulation in the lung at bronchodilator doses and granulocyte accumulation and granulocyte-dependent PPE in the skin. These effects were again antagonized by propranolol and thus also appear to be mediated by an action on β-adrenoceptors. Eosinophil accumulation in the bronchial lumen is one of the hallmarks of asthma (Horn *et al.*, 1975; Venge, 1985), while neutrophil accumulation is characteristic of other forms of inflammation (Rylander & Haglind, 1986; Thompson *et al.*, 1989). Furthermore, eosinophil and neutrophil accumulation in the airways have been linked to nocturnal asthma (Martin *et al.*, 1991). Inhibition of granulocyte accumulation by $\beta_2$-adrenoceptor agonists is unlikely to be due to an action on the granulocyte itself, since high concentrations of $\beta_2$-adrenoceptor agonists are generally required to inhibit inflammatory cells *in vitro* (Busse & Sosman, 1984; Baker & Fuller, 1990) and these effects are not reversed by propranolol (Baker & Fuller, 1990). It is possible that, as for PPE, salmeterol inhibited granulocyte accumulation by an action on the venular endothelium. The finding that salmeterol, but not salbutamol, inhibited PAF-induced eosinophil accumulation over 24 h, and zymosan-induced granulocyte infiltration in the skin following systemic (oral) and local administration, suggests that inhibition of granulocyte accumulation is a property of long-acting $\beta_2$-adrenoceptor agonists. Thus, the finding that salbutamol albeit at ten fold higher concentrations than required to inhibit PPE, inhibited neutrophil infiltration in lung, but not in the skin, is perhaps surprising. However, the results obtained in the lung are consistent with data in the literature demonstrating that salbutamol blocked antigen-induced neutrophil, but not eosinophil, accumulation (Hutson *et al.*, 1988). This discrepancy between guinea-pig lung and skin requires further investigation, but may reflect a difference in the kinetics of neutrophil infiltration into these tissues or in the duration of action of salbutamol at higher doses.

In summary, the studies described above demonstrate that salmeterol has anti-inflammatory properties. It inhibits granulocyte accumulation, granulocyte-dependent PPE and granulocyte-independent PPE in guinea-pig lung and skin, following topical, local and oral administration. These effects occur at doses which have been shown to produce bronchodilatation in this species (Ball *et al.*, 1991). Like the effects on inflammatory mediator release (Butchers *et al.*, 1991), salmeterol has a long duration of action against both PPE and granulocyte accumulation. The duration of the anti-inflammatory effects of salmeterol is similar to that for bronchodilatation which, in man, lasts for at least 12 h (Ullman & Svedmyr, 1988), indeed it is possible to achieve a 24 h increase in lung function with twice daily dosing (Ullman *et al.*, 1990). Thus, it may be possible to achieve a 24 h anti-inflammatory effect in man, by administering salmeterol twice daily, which would give it a clear clinical advantage over the short-acting $\beta_2$-adrenoceptor agonists currently available for the treatment of asthma. Such properties may account for some of the clinical actions of salmeterol, which are not shared by the shorter-acting compounds, such as inhibition of allergen-induced late-phase responses in asthmatics (Twentyman *et al.*, 1990). The anti-inflammatory actions of salmeterol may be complementary to those of the inhaled steroids and furthermore, may be applicable to the therapy of other inflammatory disorders.

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# Involvement of $\beta_2$-adrenoceptors in the regional haemodynamic responses to bradykinin in conscious rats

[1]S.M. Gardiner, P.A. Kemp, T. Bennett, *C. Bose, *R. Foulkes & *B. Hughes

Department of Physiology and Pharmacology, University of Nottingham Medical School, Queen's Medical School, Nottingham NG7 2UH and *Celltech Ltd, Slough

**1** Bradykinin can release neuronal calcitonin gene-related peptide (CGRP) and adrenal medullary catecholamines, both of which could contribute to its cardiovascular effects *in vivo*. Therefore, in the main experiment, regional haemodynamic responses to bolus injections of bradykinin (3 nmol $kg^{-1}$, i.v.) were assessed in the same chronically-instrumented, conscious, Long Evans rats in the absence and in the presence of human α-CGRP [8-37] or ICI 118551, antagonists of $CGRP_1$-receptors and $\beta_2$-adrenoceptors, respectively. The selected doses of these antagonists caused specific inhibition of responses mediated by exogenous human α-CGRP and $\beta_2$-adrenoceptor agonists, respectively.

**2** Bradykinin administered alone as an i.v. bolus had a slight pressor effect accompanied by a marked tachycardia. There were early (at about 30 s) increases in flow and conductance in the mesenteric vascular bed, and delayed (at about 90 s), but qualitatively similar, changes in the hindquarters vascular bed. There were only slight increases in flow and conductance in the renal vascular bed.

**3** Human α-CGRP [8-37] had no statistically significant effects on the responses to bolus doses of bradykinin. However, in the presence of ICI 118551, the pressor effect of bradykinin was significantly enhanced while its tachycardic effect was significantly suppressed. The hindquarters vasodilator effect of bradykinin was converted to a vasoconstriction and there was a slight renal vasoconstriction, but the mesenteric vasodilator effect of bradykinin was unchanged by ICI 118551.

**4** In subsidiary experiments, in other animals, it was found that infusion of bradykinin (36 nmol $kg^{-1}$ $min^{-1}$) elicited a pattern of haemodynamic responses similar to that seen with bolus injections and, as in the latter case, the hindquarters hyperaemic vasodilatation was inhibited by ICI 118551. In the presence of mecamylamine (at a dose sufficient to block reflex heart rate responses to rises or falls in arterial blood pressure) bolus injection or infusion of bradykinin still elicited increases in renal, mesenteric and hindquarters blood flow. However, in additional experiments in adrenal demedullated rats ($n = 4$) the hindquarters hyperaemic effect of bradykinin was absent, although the mesenteric hyperaemic effect remained.

**5** The results indicate that the increase in hindquarters blood flow following administration of bradykinin *in vivo* is largely due to activation of $\beta_2$-adrenoceptors by catecholamines released subsequent to direct stimulation of the adrenal medulla by the peptide. However, the bradykinin-induced increase in mesenteric blood flow does not depend on this mechanism.

**Keywords:** Bradykinin; calcitonin gene-related peptide; $\beta_2$-adrenoceptors; regional haemodynamics

## Introduction

Bradykinin has complex cardiovascular effects *in vivo* (Regoli & Barabé, 1980) that may be direct or indirect. There is evidence that bradykinin causes endothelium-dependent vasorelaxation through release of prostanoids and nitric oxide from endothelial cells (Furchgott, 1983; Palmer *et al.*, 1987). However, in a recent study we found that inhibition of nitric oxide synthesis with the arginine analogue, $N^G$-nitro-L-arginine methyl ester (L-NAME), left intact a large component of the regional vasodilator responses to bradykinin in conscious rats (Gardiner *et al.*, 1990c). Hence, *in vivo*, it is likely that factors besides nitric oxide contribute to the vasodilator effects of bradykinin.

There is evidence that bradykinin can cause release of catecholamines from the adrenal medulla (Feldberg & Lewis, 1964; Warashina *et al.*, 1990) and of calcitonin gene-related peptide (CGRP) from a specific subset of neurones that are also sensitive to capsaicin (Gepetti *et al.*, 1990). In conscious rats, infusions of CGRP (Gardiner *et al.*, 1989a, b) or $\beta_2$-adrenoceptor agonists, including adrenaline (Gardiner *et al.*, 1991a, b), cause marked hyperaemia, particularly in the hindquarters vascular bed where bradykinin also elicits an increase in blood flow. Thus, release of CGRP and/or adrenaline could contribute to the hindquarters haemodynamic effects of bradykinin *in vivo*. Therefore, in the main experiment in the present work we examined the influence of human α-CGRP [8-37], an antagonist of the cardiovascular actions of CGRP (Dennis *et al.*, 1990; Han *et al.*, 1990; Gardiner *et al.*, 1990d; Maggi *et al.*, 1991), and of ICI 118551, an antagonist of $\beta_2$-adrenoceptors (Bilski *et al.*, 1983), on the regional haemodynamic effects of bolus injections of bradykinin in conscious rats. We used this protocol since our previous experiments had been concerned with the haemodynamic responses to bolus injections of bradykinin in rats with functional baroreflexes (Gardiner *et al.*, 1990c; Kiff *et al.*, 1991a). However, in subsidiary experiments we also assessed the haemodynamic effects of infusions of bradykinin in the absence and presence of ICI 118551, and the effects of bolus injections and infusions of bradykinin before and after treatment with the ganglion blocker, mecamylamine. Finally, we assessed the haemodynamic effects of bradykinin in animals that had undergone bilateral adrenal demedullation.

## Methods

Male, Long Evans rats (350–450 g) were anaesthetized (sodium methohexitone 60 mg $kg^{-1}$, i.p. supplemented as

[1] Author for correspondence.

required) and had pulsed Doppler probes (Haywood *et al.*, 1981) implanted to monitor renal, mesenteric and hindquarters blood flows. Animals were given ampicillin (Penbritin, Beecham, 7 mg $kg^{-1}$, i.m.) after surgery and returned to individual home cages with free access to food and water. At least 7 days later they were re-anaesthetized (sodium methohexitone 40 mg $kg^{-1}$, i.p.) and had intravenous catheters (3 in the right jugular vein) and an intra-arterial catheter (distal abdominal aorta via the ventral caudal artery) implanted. The probe wires and catheters passed subcutaneously and emerged at the back of the neck. A harness fitted to the rat was connected to a spring through which the catheters ran and to which the probe connector was taped. Animals were returned to their home cages and left to recover for at least 24 h before experiments were begun (Gardiner *et al.*, 1990b, c). During the experiment, animals were connected to a modified pulsed Doppler VF-1 mainframe (Crystal Biotech, Holliston, U.S.A.) (Gardiner *et al.*, 1990a).

All measurements were made in conscious, freely-moving animals, and the following experiments were carried out.

(1) *Effectiveness and selectivity of the antagonistic actions of human a-CGRP [8-37] and ICI 118551*

In previous studies we have shown that human α-CGRP [8-37] (1.5 μmol $kg^{-1}$ $min^{-1}$) inhibits the haemodynamic actions of infusions of human α-CGRP without influencing responses to isoprenaline (Gardiner *et al.*, 1990d). Furthermore, on the basis of our previous experiences, we judged that ICI 118551 (670 nmol $kg^{-1}$ bolus, 335 nmol $kg^{-1}$ $h^{-1}$ infusion) would be relatively selective for $\beta_2$-adrenoceptors (Gardiner & Bennett, 1988a, b, c). However, in order to ensure that our earlier findings with human α-CGRP [8-37] and ICI 118551 were reproducible, and hence applicable to the present studies, we carried out the following experiments.

In some animals ($n = 2$) we assessed cardiovascular responses to bolus injections of human α-CGRP (0.125 nmol $kg^{-1}$) and adrenaline (1.05 nmol $kg^{-1}$) before, during (at 20 min), and 60 min after infusion of human α-CGRP [8-37] (1.5 μmol $kg^{-1}$ $h^{-1}$).

In other animals ($n = 2$) we assessed haemodynamic responses to 3 min infusions of salbutamol (2.1 nmol $kg^{-1}$ $min^{-1}$), isoprenaline (0.24 nmol $kg^{-1}$ $min^{-1}$) and sodium nitroprusside (0.14 μmol $kg^{-1}$ $min^{-1}$) in the presence of atropine (2.7 μmol $kg^{-1}$ bolus, 1.4 μmol $kg^{-1}$ $h^{-1}$ infusion) and again in the presence of ICI 118551 (670 nmol $kg^{-1}$ bolus, 335 nmol $kg^{-1}$ $h^{-1}$ infusion) and atropine. At the end of this experiment we confirmed that the tachycardic effects of sodium nitroprusside were abolished by mecamylamine (20 min after the onset of infusion at 50 μmol $kg^{-1}$ $h^{-1}$).

(2) *Haemodynamic effects of bolus injections of bradykinin*

This protocol constituted the main experiment. In a group of 8 animals, following a baseline period of 30 min, 2 bolus injections of bradykinin (3 nmol $kg^{-1}$, i.v.) were given separated by at least 10 min. Sixty min after the second injection an infusion of human α-CGRP [8-37] (1.5 μmol $kg^{-1}$ $min^{-1}$) (Gardiner *et al.*, 1990d, and see above) was begun and the animals were re-challenged with bradykinin (3 nmol $kg^{-1}$, i.v.), 10 and 20 min into the infusion. Sixty min after the infusion of human α-CGRP [8-37] had been stopped, a fifth injection of bradykinin was given before primed infusion of ICI 118551 (670 nmol $kg^{-1}$ bolus; 335 nmol $kg^{-1}$ $h^{-1}$ infusion) (Gardiner & Bennett, 1988a, b, c and see above) was started. Bradykinin was injected 60 min after the start of the infusion of ICI 118551. A separate group of animals ($n = 4$) received both the fifth and sixth injections of bradykinin in the absence of ICI 118551 to ensure there was no desensitization to the peptide at this stage in the experiment.

After bradykinin injection, measurements were made at about 30 s (to coincide with the peaks in mean arterial blood pressure, heart rate and mesenteric blood flow), at about 90 s (to coincide with the peak in hindquarters flow) and at about 3 min (when most variables were back to baseline). At each of these time points average values for heart rate, mean arterial blood pressure, and mean renal, mesenteric and hindquarters Doppler shift signals were obtained over a 10 s period. The baseline values were measured over the 20 s preceding the injection of bradykinin. Vascular conductances were calculated from the mean arterial blood pressure and mean Doppler shift signals; % changes in the latter were taken as indices of changes in regional blood flows (Haywood *et al.*, 1981).

(3) *Haemodynamic effects of infusions of bradykinin*

In order to ensure that the cardiovascular responses to bolus injections of bradykinin were not a function of the mode of administration of the peptide we assessed the effects of 3 min infusions of bradykinin (36 nmol $kg^{-1}$ $min^{-1}$) as well as bolus injections (3 nmol $kg^{-1}$) in a separate group of animals ($n = 2$). These experiments were carried out before and 60 min after the onset of administration of ICI 118551 (670 nmol $kg^{-1}$ bolus, 335 nmol $kg^{-1}$ $h^{-1}$ infusion).

(4) *Haemodynamic effects of bolus injections and infusions of bradykinin following ganglion blockade*

In order to determine the effects of ganglion blockade on responses to bradykinin we gave bolus injections and infusions of the peptide before and 20 min after onset of an infusion of mecamylamine (50 μmol $kg^{-1}$ $h^{-1}$). This dose of mecamylamine is 10 fold higher than that used by Wright & Fozard (1990) in anaesthetized rats, but we found their dose in conscious rats did not block the reflex tachycardic effects of sodium nitroprusside (see above).

The experiments involving assessment of the effects of mecamylamine on responses to bradykinin were carried out in the same animals as those in the protocol 3, but on another day. (In pilot experiments we ensured that the effects of ICI 118551 on responses to bradykinin had disappeared within 24 h).

(5) *Haemodynamic effects of bolus injections and infusions of bradykinin in adrenal-demedullated rats*

In a further group of Long Evans rats ($n = 4$) bilateral adrenal demedullation (Gardiner & Bennett, 1988a, b) was carried out at the time the pulsed Doppler probes were implanted. Subsequently, these animals received bolus injections and infusions of bradykinin (as above). After the experiment these animals were killed with an overdose of sodium pentobarbitone (250 mg $kg^{-1}$) and their adrenal glands were removed. Microscopical examination confirmed the absence of adrenal medullae in all cases, the cores of the glands being occupied by calcified deposits and the remains of blood clots.

## *Drugs*

Human α-CGRP [8-37] was synthesized at Celltech Ltd, using routine procedures (Gardiner *et al.*, 1990d). Bradykinin was obtained from Bachem (UK), ICI 118551 (erythro-(±)-1-[7-methylindan-4-yloxy]-3-isopropyl-aminobutan-2-ol) hydrochloride was a gift from ICI Pharmaceuticals plc, and atropine methyl nitrate, (±)-adrenaline hydrochloride, (±)-isoprenaline hydrochloride, salbutamol hemisulphate, sodium nitroprusside and mecamylamine hydrochloride were obtained from Sigma. Human α-CGRP [8-37] and bradykinin were dissolved in sterile saline (157 mmol $l^{-1}$ NaCl) containing 1% bovine serum albumin (Sigma) and kept frozen (− 80°C) before use. ICI 118551 was dissolved in sterile saline by gentle warming. Atropine, adrenaline, isoprenaline, salbutamol, sodium nitroprusside and mecamylamine were dissolved in sterile saline at room temperature. In the case of

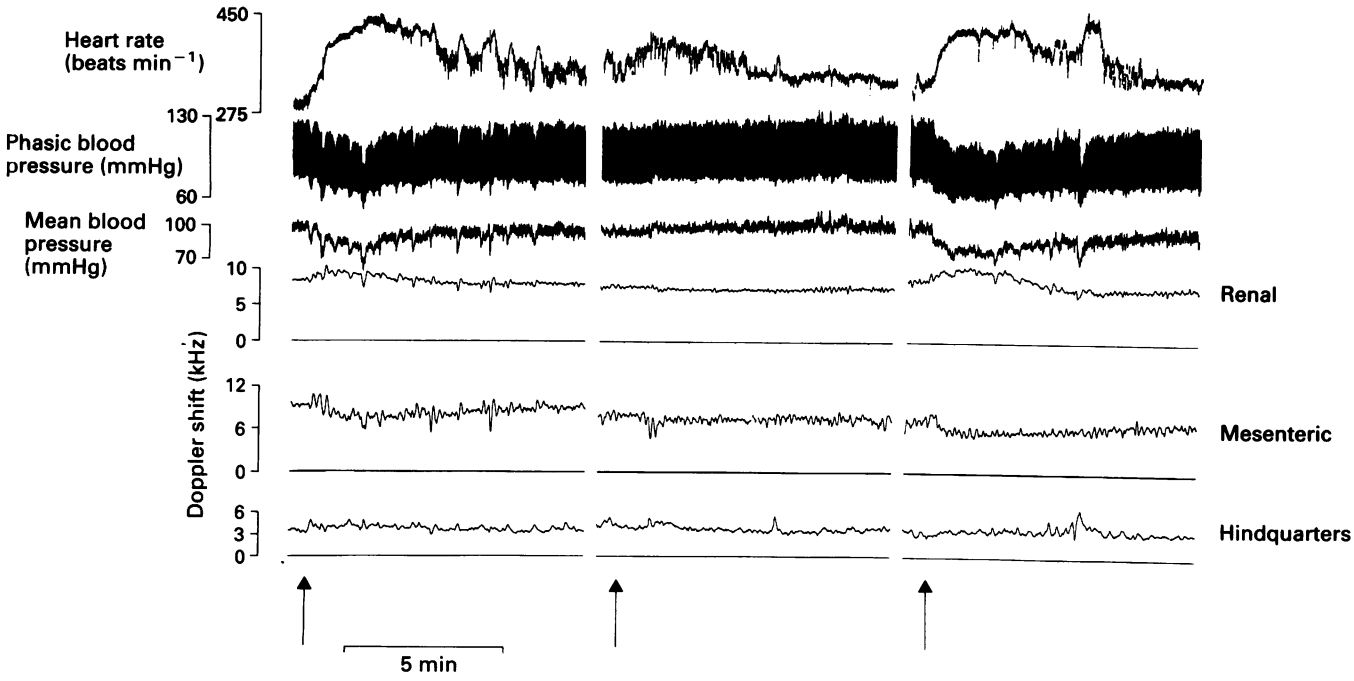


**Figure 1** Original tracings showing cardiovascular effects of bolus injections (at arrows) of human α-calcitonin gene-related peptide (α-CGRP) (0.125 nmol $kg^{-1}$) before (left-hand panel), 20 min after the start of infusion of human α-CGRP [8-37] (1.5 μmol $kg^{-1}$ $h^{-1}$) (centre panel) and 60 min after the end of infusion of human α-CGRP [8-37] (right-hand panel) in the same conscious, Long Evans rat. The results are representative of 2 experiments.

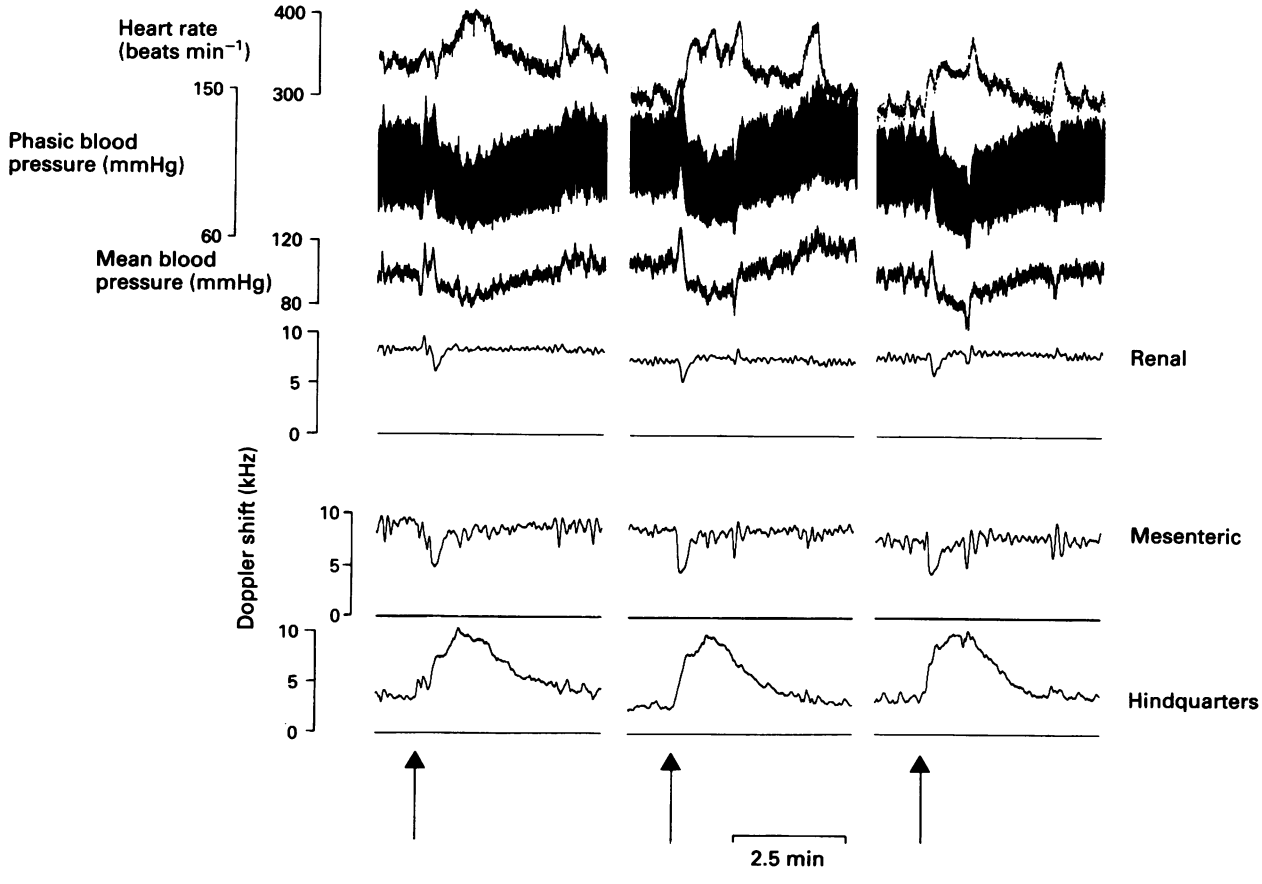


**Figure 2** Original tracings showing cardiovascular effects of bolus injections (at arrows) of adrenaline (1.05 nmol $kg^{-1}$) before (left-hand panel), 20 min after the onset of infusion of human α-calcitonin gene-related peptide [8-37] (α-CGRP [8-37]) (1.8 μmol $kg^{-1}$ $h^{-1}$) (centre panel) and 60 min after the end of infusion of human α-CGRP [8-37] (right-hand panel) in the same conscious, Long Evans rat. The results are representative of 2 experiments.

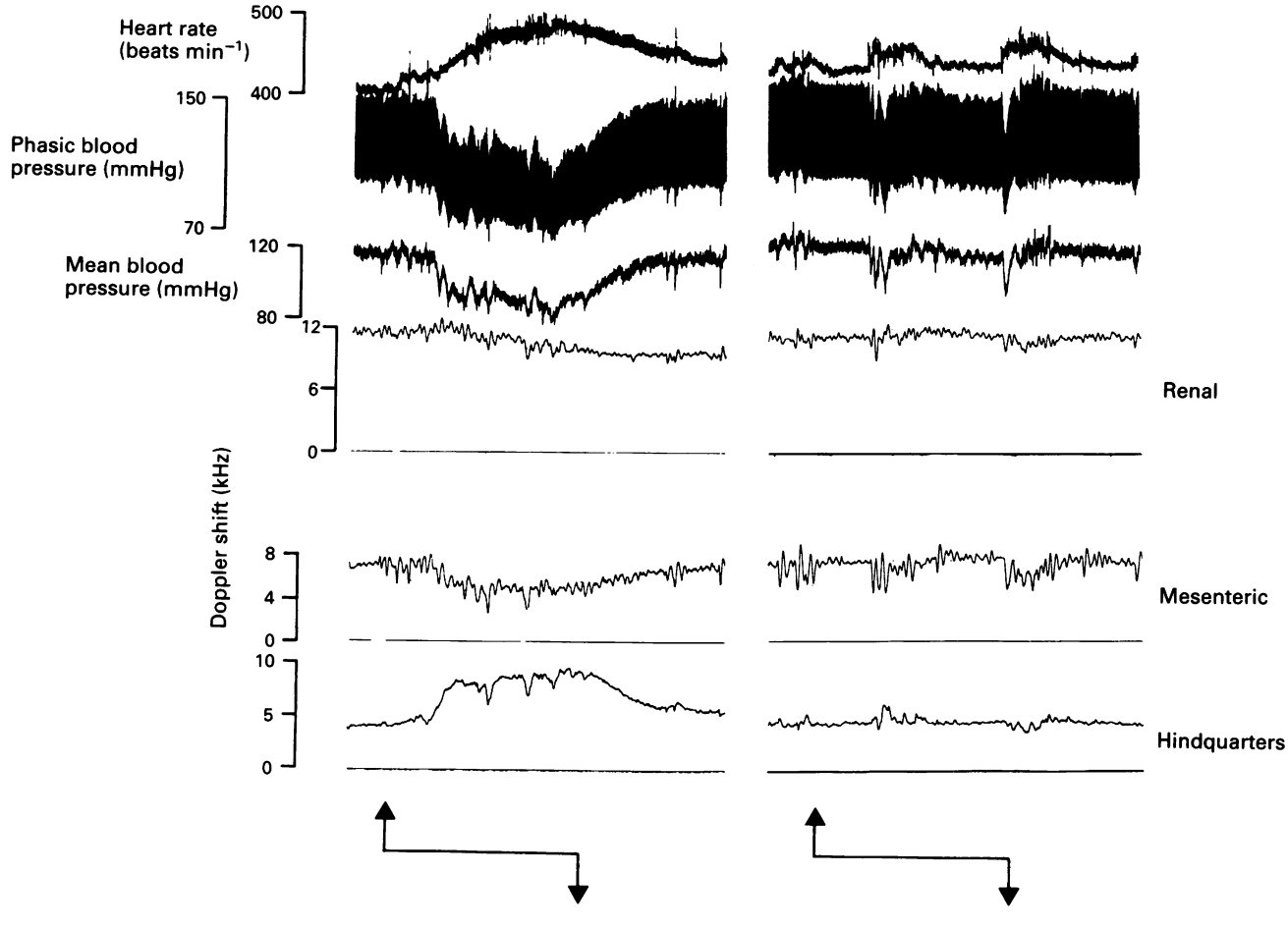


**Figure 3** Original tracings showing cardiovascular effects of 3 min infusions (between arrows) of salbutamol (2.1 nmol $kg^{-1}$ $min^{-1}$) before (left-hand panel) and 60 min after the start of ICI 118551 administration (670 nmol $kg^{-1}$ bolus, 335 nmol $kg^{-1}$ $h^{-1}$ infusion) (right-hand panel) in the same conscious, atropine-pretreated, Long Evans rat. The results are representative of 2 experiments.

adrenaline, isoprenaline and salbutamol the saline contained ascorbic acid (1 μmol $l^{-1}$).

All bolus injections were given in 0.1 ml and flushed in with 0.15 ml. Infusions were given at 0.3 ml $h^{-1}$. Administration of vehicle solutions alone in these volumes had no consistent cardiovascular effects.

### *Data analysis*

Within-group analysis of responses to bolus injections of bradykinin was by Friedman's test (Theodorsson-Norheim, 1987). Comparison of responses to bolus injections of bradykinin under different conditions was made by considering areas under or over curves (AUC, AOC, respectively) (Gardiner *et al.*, 1990b) and applying Wilcoxon's ranks sums test; a $P$ value $< 0.05$ was taken as significant.

In the case of the subsidiary experiments statistical analysis was not carried out because the group sizes were small.

## Results

### (1) *Effectiveness and selectivity of the antagonistic actions of human α-CGRP [8-37] and ICI 118551*

During infusion of human α-CGRP [8-37] (1.5 μmol $kg^{-1}$ $min^{-1}$) the haemodynamic effects of human α-CGRP (0.125 nmol $kg^{-1}$) were abolished, although a slight tachycardia persisted (Figure 1). Following cessation of the infusion of human α-CGRP [8-37], bolus injection of human α-CGRP once again elicited the usual effects (Figure 1). In the same animals human α-CGRP [8-37] had no effect on haemodynamic responses to bolus injections of adrenaline (Figure 2).

In other animals pretreated with atropine, infusion of salbutamol caused hypotension, tachycardia and falls in renal and mesenteric blood flows, together with a marked increase in hindquarters blood flow (Figure 3). All these effects were blocked by ICI 118551 (Figure 3).

Infusion of isoprenaline caused effects similar to those seen with salbutamol (Figure 4). ICI 118551 blocked all these effects except for the tachycardia, which was reduced (Figure 4).

All the cardiovascular changes seen during infusion of sodium nitroprusside were unaffected by ICI 118551 (data not shown).

### (2) *Haemodynamic effects of bolus injections of bradykinin*

The bolus dose of bradykinin used caused a small, but significant pressor effect (AUC, 18 ± 4 units), accompanied by a marked, significant tachycardia (AUC, 179 ± 10 units) (Figures 5 and 6). There was a slight, but significant rise in renal blood flow (AUC, 21 ± 4 units), whereas mesenteric and hindquarters flows showed marked, significant increases (AUC, 79 ± 15 and 91 ± 10 units, respectively) (Figures 5 and 6). The peak in mesenteric blood flow occurred about 30 s after administration of bradykinin, while the hindquarters hyperaemia did not peak until about 90 s after bradykinin injection (Figures 5 and 6). Similar patterns of change were seen in the vascular conductances, with a slight renal vasodilatation (AUC, 20 ± 6 units), an early, substan-

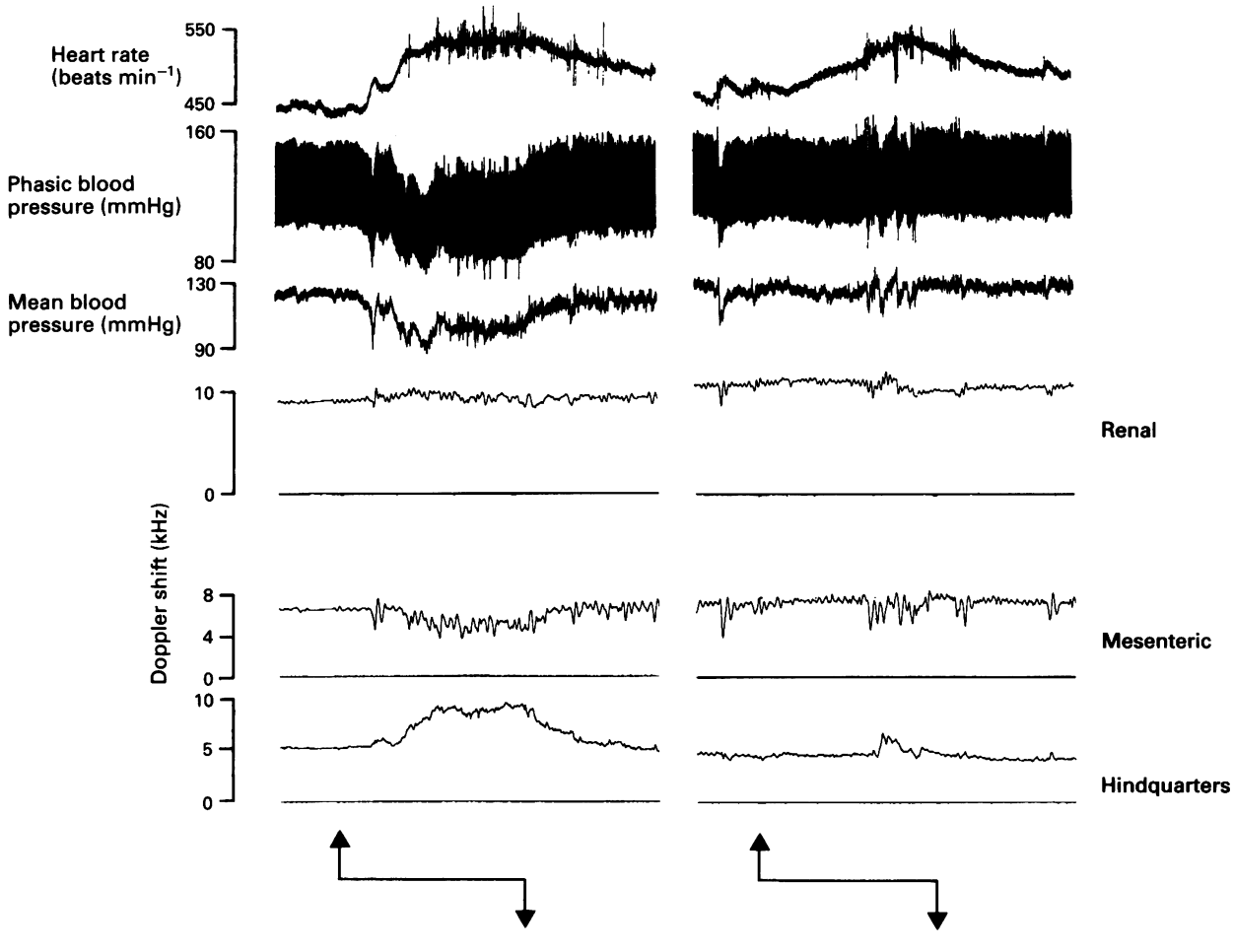


**Figure 4** Original tracings showing cardiovascular effects of 3 min infusions (between arrows) of isoprenaline (0.24 nmol $kg^{-1}$ $min^{-1}$) before (left-hand panel) and 60 min after the start of ICI 118551 administration (670 nmol $kg^{-1}$ bolus, 335 nmol $kg^{-1}$ $h^{-1}$ infusion) (right-hand panel) in the same conscious, atropine-pretreated, Long Evans rat. The results are representative of 2 experiments.

tial, mesenteric vasodilatation (67 ± 6 units) and a delayed, but even greater, hindquarters vasodilatation (AUC, 102 ± 14 units) (Figure 6). A second dose of bradykinin elicited similar responses to the first (AUC, heart rate = 213 ± 35 units; mean blood pressure = 21 ± 4 units; renal flow = 26 ± 6 units; mesenteric flow = 82 ± 10 units; hindquarters flow = 101 ± 17 units; renal conductance = 19 ± 6 units; mesenteric conductance = 63 ± 11 units; hindquarters conductance = 120 ± 21 units) (Figure 6).

During infusion of human α-CGRP [8-37], injections of bradykinin elicited similar patterns of response to those seen in the absence of human α-CGRP [8-37] (Figure 6). Although following infusion of the latter for 20 min there was a tendency for the injection of bradykinin to elicit an enhanced mesenteric vasodilatation (AUC, 86 ± 13 units) and a diminished hindquarters vasodilatation (AUC, 78 ± 11 units), these differences did not reach significance. However, following discontinuation of the infusion of human α-CGRP [8-37] these trends reversed and the responses to bradykinin were then numerically closer to those under control conditions (AUC, heart rate = 233 ± 17 units; mean blood pressure = 24 ± 5 units; renal flow = 20 ± 5 units; mesenteric flow = 98 ± 11 units; hindquarters flow = 101 ± 17 units; renal conductance = 10 ± 5 units; mesenteric conductance = 72 ± 11 units; hindquarters conductance = 106 ± 20 units) (Figure 6).

In the presence of ICI 118551, injection of bradykinin elicited a significantly enhanced pressor effect (AUC, 43 ± 6 units) accompanied by a reduced tachycardia (AUC, 146 ± 13 units) (Figures 5 and 6). The increase in renal flow was unchanged (AUC, 20 ± 4 units) but the mesenteric hyperaemia was significantly increased (AUC, 127 ± 11 units) (Figure 6). However, the increase in hindquarters flow was abolished (AUC, 15 ± 5 units) (Figures 5 and 6). Thus, there was a renal vasoconstriction (AOC, 22 ± 6 units) and a hindquarters vasoconstriction (AOC, 33 ± 8 units), rather than the vasodilatations seen in the absence of ICI 118551, whereas the mesenteric vasodilator response to bradykinin (AUC, 81 ± 15 units), was unchanged (Figure 6).

In the 4 animals that received the sixth injection of bradykinin in the absence of ICI 118551 the responses did not differ from those seen to the fifth injection of bradykinin (data not shown).

### (3) *Haemodynamic effects of infusions of bradykinin*

Infusion of bradykinin caused hypotension, tachycardia and increases in renal, mesenteric, and, particularly, hindquarters blood flow (Figure 7). In the presence of ICI 118551 the hypotensive effect of bradykinin infusion was inhibited, as was the tachycardia. Renal blood flow rose as before, whereas the mesenteric hyperaemia was increased. However, the increase in hindquarters blood flow was almost abolished (Figure 7).

### (4) *Haemodynamic effects of bolus injection and infusions of bradykinin following ganglion blockade*

In the presence of ganglion blockade there were substantial changes in haemodynamic status (Figures 8 and 9).

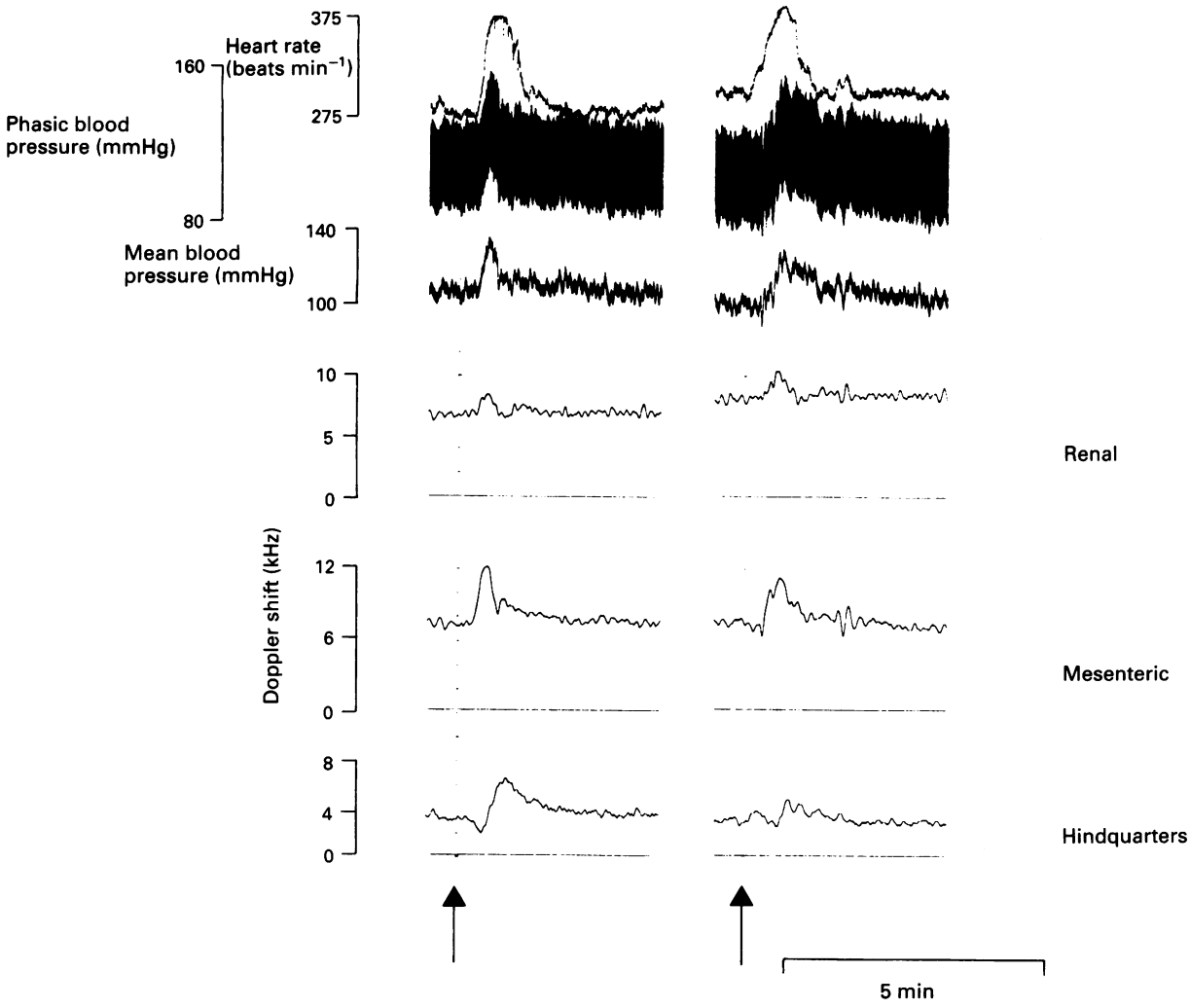


**Figure 5** Original tracings showing cardiovascular effects of bolus injections (at arrows) of bradykinin (3 nmol $kg^{-1}$) before (left-hand panel) and 60 min after the start of ICI 118551 administration (670 nmol $kg^{-1}$ bolus, 335 nmol $kg^{-1}$ $h^{-1}$ infusion) (right-hand panel) in the same conscious, Long Evans rat.

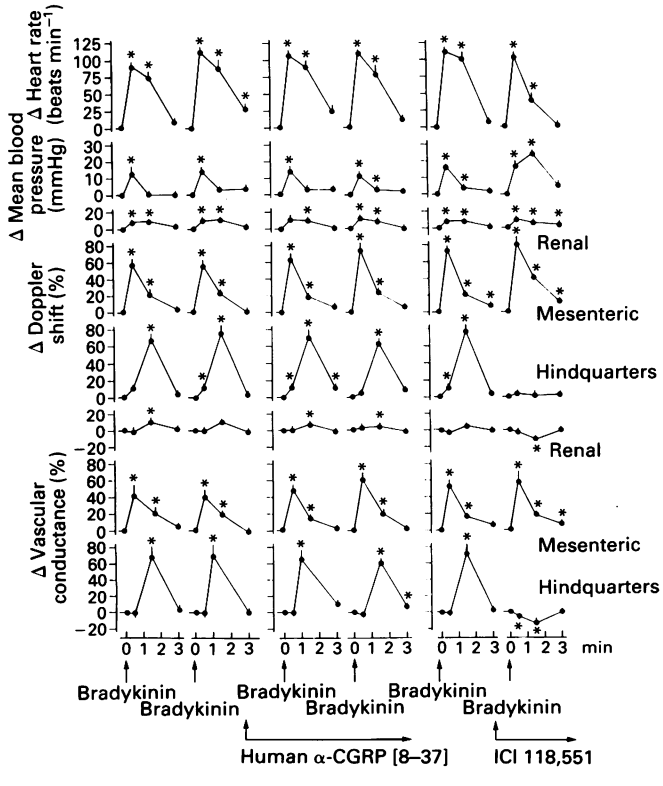


**Figure 6** Cardiovascular responses to i.v. bolus injections of bradykinin (3 nmol $kg^{-1}$). The left-hand panels show responses to control injections; the middle panels show responses to bradykinin injection 10 to 20 min after the start of infusion of human α-calcitonin gene-related peptide [8-37] (α-CGRP [8-37]) (1.5 μmol $kg^{-1}$ $h^{-1}$); the right-hand panels show responses to bradykinin injection before and 60 min after start of a primed infusion of ICI 118551 (670 nmol $kg^{-1}$ bolus, 335 nmol $kg^{-1}$ $h^{-1}$ infusion). All responses were measured in the same conscious, Long Evans rats ($n = 8$) and the sequence of the experiment was as shown (from left to right). Values are mean and vertical bars show s.e.mean; $^*P < 0.05$ versus baseline (Friedman's test).

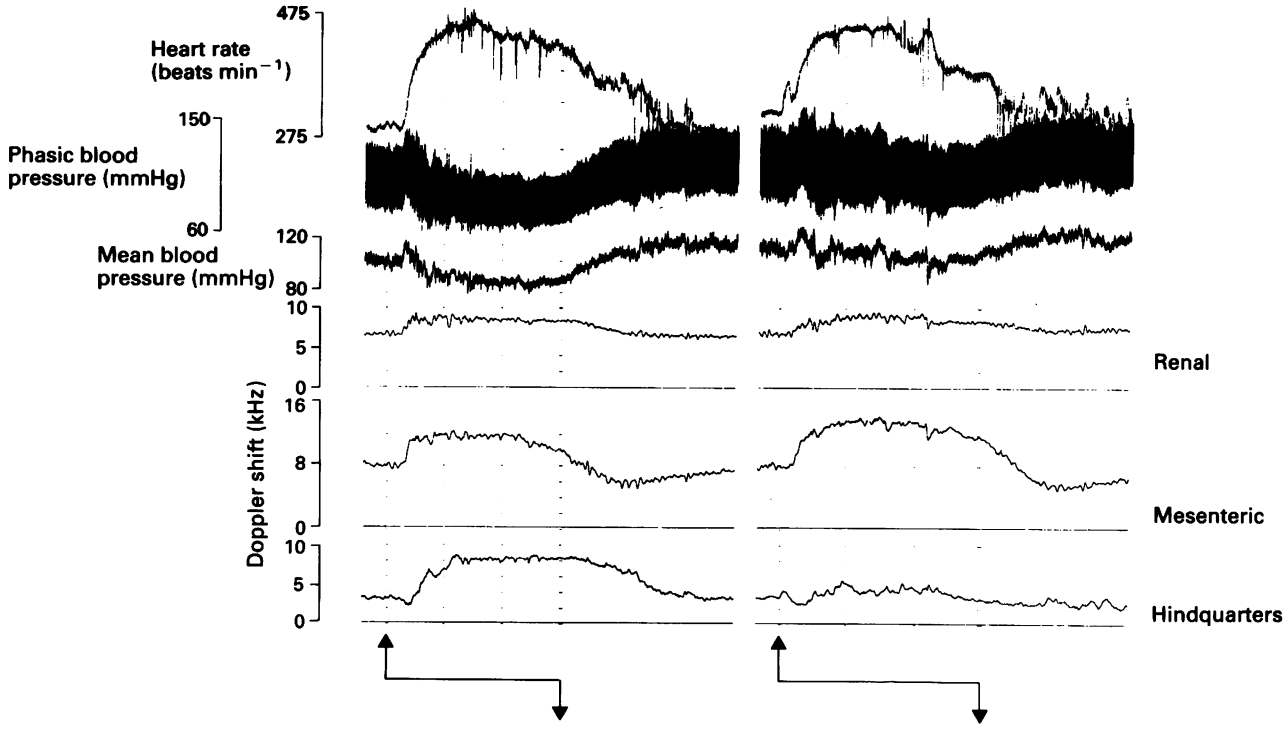


**Figure 7** Original tracings showing cardiovascular effects of infusions (between arrows) of bradykinin (36 nmol $kg^{-1}$ $min^{-1}$) before (left-hand panel) and 60 min after the start of ICI 118551 administration (670 nmol $kg^{-1}$ bolus, 335 nmol $kg^{-1}$ $h^{-1}$ infusion) (right-hand panel) in the same conscious, Long Evans rat. The results are representative of 2 experiments.

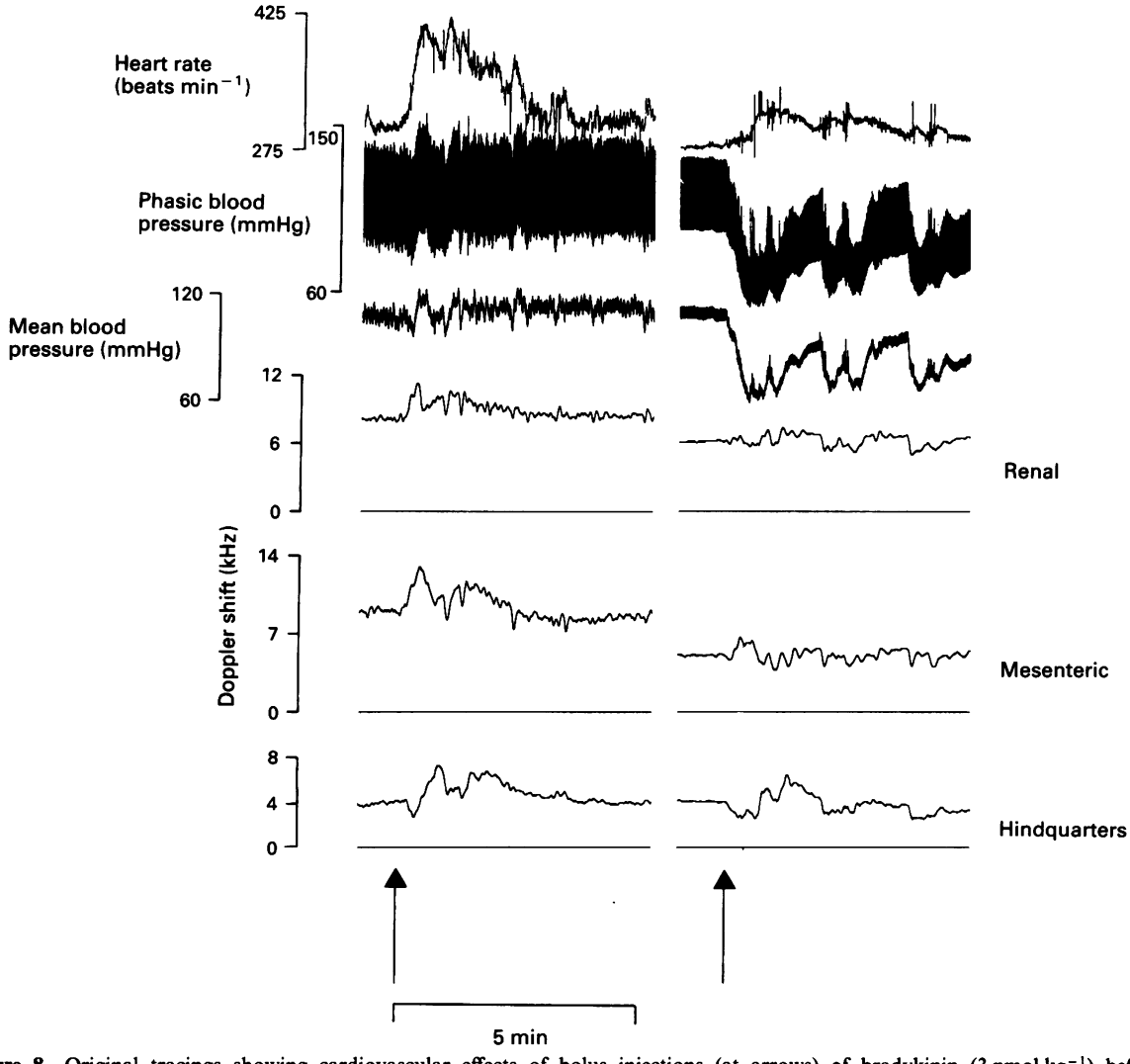


**Figure 8** Original tracings showing cardiovascular effects of bolus injections (at arrows) of bradykinin (3 nmol $kg^{-1}$) before (left-hand panel) and 20 min after the start of mecamylamine administration (50 μmol $kg^{-1}$ $h^{-1}$ infusion) (right-hand panel) in the same conscious, Long Evans rat. The results are representative of 2 experiments.

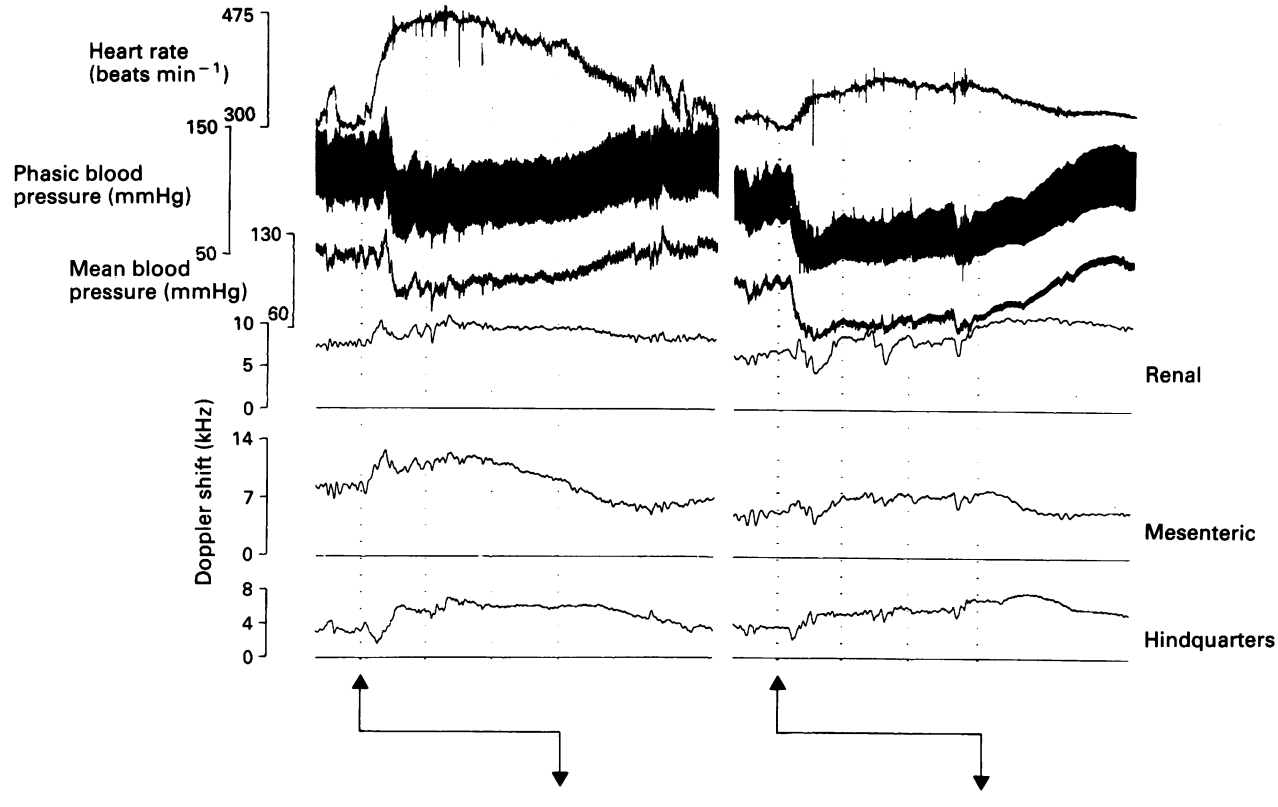


**Figure 9** Original tracings showing cardiovascular effects of infusions (between arrows) of bradykinin (36 nmol $kg^{-1}$ $min^{-1}$) before (left-hand panel) and 20 min after the start of mecamylamine administration (50 μmol $kg^{-1}$ $h^{-1}$ infusion) (right-hand panel) in the same conscious, Long Evans rat. The results are representative of 2 experiments.

Moreover, under these conditions the hypotensive effect of bradykinin was enhanced while its chronotropic effect was reduced (Figures 8 and 9). Because of the marked difference in the hypotensive response to bradykinin in the intact and ganglion-blocked state there were substantial differences in the patterns of change in regional blood flows (Figures 8 and 9). Nonetheless, there were still increases in renal, mesenteric and hindquarters blood flows following administration of bradykinin in the ganglion-blocked state, particularly when the peptide was infused (Figure 9). Thus, the hindquarters hyperaemic effect of bradykinin that was inhibited by ICI 118551 was not dependent upon ganglionic transmission.

### (5) *Haemodynamic effects of bolus injections and infusions of bradykinin in adrenal demedullated rats*

Bolus injection of bradykinin caused transient hypotension and slight tachycardia (Figure 10). There was little change in renal blood flow, but a clear increase in mesenteric blood flow; however, the normal hindquarters hyperaemic response was absent (Figure 10).

A similar pattern of haemodynamic changes was seen during infusion of bradykinin in the adrenal demedullated rats (Figure 10).

## Discussion

In the present work we assessed the possibility that release of CGRP (Gepetti *et al.*, 1990) and/or adrenal medullary catecholamines (Feldberg & Lewis, 1964) by bradykinin contributed to the regional haemodynamic effects of this peptide.

Human α-CGRP [8-37] has been shown to antagonize the cardiovasular effects of endogenous CGRP in the mesenteric vascular bed isolated from the rat (Han *et al.*, 1990) and the left atrium of the guinea-pig (Maggi *et al.*, 1991). Furthermore, it has marked inhibitory effects on the haemodynamic responses to infusions of human α-CGRP administered i.v. in conscious rats (Gardiner *et al.*, 1990d). In the present work we demonstrated that human α-CGRP [8-37] also blocked the haemodynamic effects of bolus injections of human α-CGRP, without affecting those to adrenaline. However, pretreatment with human α-CGRP [8-37] failed to affect significantly the responses to bolus doses of bradykinin in the present study, although there was a trend towards a diminution in the hindquarters, and an increase in the mesenteric, vasodilator responses to bradykinin in the presence of human α-CGRP [8-37]. Moreover, when infusion of human α-CGRP [8-37] was stopped these trends reversed. These findings make it difficult to dismiss the possibility that release of endogenous CGRP contributed to the haemodynamic responses to bradykinin in some animals (5/8 showed the changes described above). Although use of a higher dose of human α-CGRP [8-37] might, in theory, have produced a more clear-cut answer, this peptide at doses higher than used here has substantial effects itself (Gardiner *et al.*, 1990d).

ICI 118551 blocked the haemodynamic and tachycardic effects of salbutamol in atropine-pretreated rats, and blocked the haemodynamic, while only attenuating the chronotropic, effects of isoprenaline in the same animals. These results are consistent with specific inhibition of $\beta_2$-adrenoceptor-mediated vasodilatation (particularly in the hindquarters) by the selected dose of ICI 118551. This action, by suppressing the hypotensive responses to salbutamol and isoprenaline, would remove the stimulus for reflex tachycardia, although we cannot exclude the possibility that ICI 118551 antagonized any putative cardiac $\beta_2$-adrenoceptor effects (Brodde, 1991) of salbutamol or isoprenaline. However, the finding that ICI 118551 blocked all the effects of salbutamol while leaving a residual tachycardic response to isoprenaline, and having no influence on the positive chronotropic response to sodium nitroprusside in atropine-pretreated rats,

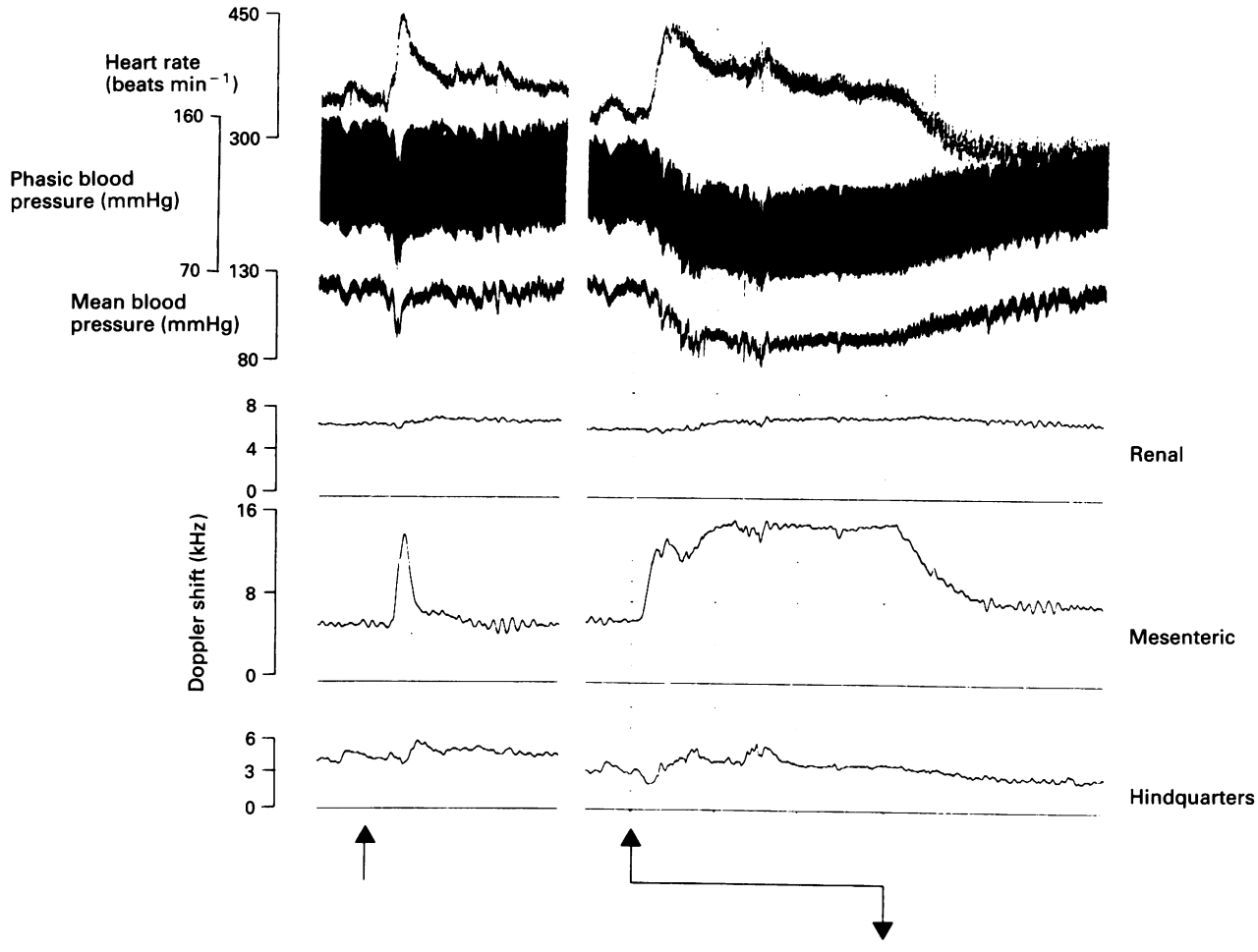


**Figure 10** Original tracings showing cardiovascular effects of bolus injection (3 nmol $kg^{-1}$) (at arrow, left-hand panel) or infusion (36 nmol $kg^{-1}$ $min^{-1}$) (between arrows, right-hand panel) of bradykinin in the same conscious, adrenal demedullated Long Evans rat. The results are representative of 4 experiments.

indicates that the dose of ICI 118551 used caused effective antagonism of $\beta_2$-adrenoceptor-mediated effects, and left cardiac $\beta_1$-adrenoceptor-mediated responses intact.

In the presence of ICI 118551, there was complete loss of the substantial hindquarters and the small renal vasodilator effects of bolus injections of bradykinin. Indeed, under these conditions, hindquarters and renal vasoconstrictions occurred in response to bradykinin. These results are consistent with activation of $\beta_2$-adrenoceptors causing the renal and hindquarters vasodilator responses to bolus injections of bradykinin under normal conditions. The difference in magnitude of the vasodilator responses in the two vascular beds is as would be predicted on the basis of their relative vasodilator responses to the $\beta_2$-adrenoceptor agonist, salbutamol (Gardiner *et al.*, 1991a), or to other interventions that activate $\beta_2$-adrenoceptors (Gardiner & Bennett, 1988c).

The blockade of the hindquarters hyperaemic effect of bradykinin by ICI 118551 was probably the major factor contributing to the enhanced pressor response to bolus doses of the peptide, and, under these circumstances, the attentuation of the tachycardic response to bradykinin was most likely baroreflex-mediated.

Infusions of bradykinin produced clear increases in blood flows in all three vascular beds studied. However, even under those circumstances, ICI 118551 caused marked attentuation of the hindquarters hyperaemic effects of bradykinin, although renal and mesenteric responses were not diminished. Thus, both the results with bolus injections and those with infusions of bradykinin indicate that the major component of the hindquarters hyperaemic vasodilator response, was $\beta_2$-adrenoceptor-mediated. The most likely explanation of this effect was that bradykinin released adrenaline from the adrenal medullae, and the adrenaline caused the hindquarters vasodilatation (Gardiner *et al.*, 1991b). This proposal is consistent with the finding that the hindquarters hyperaemic vasodilator effect of bradykinin was absent in adrenal demedullated rats, whereas the mesenteric vasocilator response was intact. The inability of mecamylamine to prevent the hindquarters hyperaemic response to bradykinin indicates the latter was probably acting directly on the chromaffin cells to release adrenaline (Warashina *et al.*, 1990).

In recent studies we observed that L-NAME reduced the hindquarters vasodilator action of bolus doses of bradykinin (Gardiner *et al.*, 1990c) and, furthermore, the hindquarters vasodilator effect of bradykinin was attenuated selectively in rats with streptozotocin-induced diabetes mellitus (Kiff *et al.*, 1991a). In addition, in the latter animals, the hindquarters vasoconstrictor response to L-NAME was diminished relative to that in control rats (Kiff *et al.*, 1991b). We interpreted these findings, together, as showing a particular involvement of nitric oxide in the hindquarters vasodilator effect of bradykinin, and a selective impairment of this process in streptozotocin-treated rats. At first sight, that interpretation seems to be at variance with the present findings. However, we now know that the hindquarters vasodilator effects of the $\beta_2$-adrenoceptor agonist, salbutamol (Gardiner *et al.*, 1991a)

and of adrenaline (Gardiner *et al.*, 1991b) are inhibited by L-NAME. So, these various findings can be reconciled by the proposition that the hindquarters vasodilator response to bradykinin that is mediated by adrenaline release involves nitric oxide at some point, and it is this process that is abnormal in streptozotocin-treated rats.

The mechanisms responsible for the mesenteric hyperaemic effects of bradykinin *in vivo* remain to be determined since other experiments (Gardiner *et al.*, 1990c) have shown this effect still occurs in the presence of L-NAME.

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# The effects of acute and repeated nicotine treatment on nucleus accumbens dopamine and locomotor activity

Maureen E.M. Benwell & David J.K. Balfour

Department of Pharmacology and Clinical Pharmacology, University of Dundee Medical School, Dundee, DD1 9SY

**1** The effects of acute and subchronic nicotine and (+)-amphetamine on the extracellular levels of dopamine and its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in nucleus accumbens (NAc) have been studied in conscious, freely-moving rats by use of *in vivo* microdialysis.

**2** In rats which had been habituated to the test apparatus for approximately 80 min, the acute subcutaneous (s.c.) administration of nicotine (0.1 or 0.4 mg $kg^{-1}$) caused a dose-dependent increase ($P<0.01$) in spontaneous activity and evoked significant increases ($P<0.05$) in the extracellular levels of DOPAC and HVA.

**3** Measurements made 24 h after the last injection of nicotine showed that pretreatment with the higher doses tested (0.4 mg $kg^{-1}$) resulted in increased basal levels of dopamine ($P<0.01$) and decreased basal levels of DOPAC ($P<0.05$) in the NAc dialysates.

**4** Pretreatment with nicotine (0.1 or 0.4 mg $kg^{-1}$ daily for 5 days) enhanced the effects of the drug on spontaneous locomotor activity and enhanced the effects of the drug on extracellular levels of dopamine to the extent that the response became significant ($P<0.05$).

**5** If a dopamine uptake inhibitor, nomifensine, was added to the Ringer solution used to dialyse the probe, the s.c. administration of both acute and subchronic nicotine (0.4 mg $kg^{-1}$) resulted in significant increases ($P<0.05$) in the dopamine concentration in the dialysate. Under these conditions, pretreatment with nicotine prior to the test day prolonged ($P<0.05$) the dopamine response to a challenge dose of nicotine.

**6** Subcutaneous injections of (+)-amphetamine (0.2 or 0.5 mg $kg^{-1}$) evoked dose-dependent increases in both spontaneous activity and the concentration of dopamine in NAc dialysates. These responses were unaffected by 5 days pretreatment with the drug.

**7** The results of this study support the conclusion that the enhanced locomotor response to nicotine observed in animals pretreated with the drug prior to the test day is associated with potentiation of its effects on dopamine secretion in the NAc.

**Keywords:** Nicotine; amphetamine; mesolimbic dopamine; sensitization; locomotor activity

## Introduction

There is substantial evidence to suggest that nicotine plays a pivotal role in maintaining the tobacco smoking habit and that many habitual smokers become dependent upon the drug (Balfour, 1990). Nicotine self-administration has also been demonstrated in infra-human species, (Goldberg *et al.*, 1981; Cox *et al.*, 1984; Hutchinson & Emley, 1985; Corrigal & Coen, 1991) although it appears to be a relatively weak substrate when compared with drugs such as (+)-amphetamine and cocaine (Pickens *et al.*, 1978; Collins *et al.*, 1984). Wise & Bozarth (1987) have hypothesized that the neural substrate which underlies drug self-administration is the mesolimbic dopamine system which arises in the ventral tegmental area and terminates on target structures within the nucleus accumbens and olfactory tubercle (Fallon & Moore, 1978). There is convincing evidence for the involvement of this system as a mediator of the self-administration and locomotor stimulant (Creese & Iversen, 1975; Kelly *et al.*, 1975; Lyness *et al.*, 1979) properties of (+)-amphetamine. The lesion studies of Singer *et al.* (1982) indicated that this system also appears to mediate nicotine self-administration. This conclusion is supported by more recent studies which suggest that exposure to both nicotine (Andersson *et al.*, 1981a,b; Imperato *et al.*, 1986; Di Chiara & Imperato, 1988; Damsma *et al.*, 1989) and tobacco smoke (Fuxe *et al.*, 1989) can evoke a preferential increase in dopamine secretion in the mesolimbic structures. In addition, there is increasing evidence to suggest that the locomotor stimulant properties of nicotine may also be mediated by this system (Clarke *et al.*, 1988; Vale & Balfour, 1988; Reavill & Stolerman, 1990). Other studies have shown that if rats are pretreated with nicotine for some days before the test day, the locomotor response to the drug is significantly enhanced (Stolerman *et al.*, 1973; Clarke & Kumar, 1983; Vale & Balfour, 1989). The purpose of the present study, therefore, was to employ the technique of *in vivo* microdialysis, which allows the simultaneous monitoring of extracellular neurotransmitter levels and behaviour, to test the hypothesis that the enhanced locomotor responses observed in rats given repeated injections of nicotine are associated with an increased mesolimbic dopamine response to the drug.

## Methods

### *Animals and drug pretreatments*

Male Sprague Dawley rats, bred in the Animal Services Unit, Ninewells Hospital and Medical School, from stock purchased from Interforna Ltd and weighing 250–350 g at the start of the experiments, were used throughout. The animals, which had free access to food and water, were housed in pairs prior to surgery and then individually following surgery. In the experiments involving the systemic administration of saline or drug solution on the test day, the rats were pretreated with 5 consecutive daily subcutaneous injections of sterile isotonic saline, (+)-amphetamine sulphate or nicotine hydrogen tartrate. The drugs were dissolved in saline and, when necessary, the pH was adjusted to 7.4 by the

addition of a small quantity of NaOH. Drug doses were expressed in terms of the free base.

### *Implantation of the microdialysis probe*

The animals were anaesthetised with Avertin (2,2,2, tribromoethanol: isoamyl alcohol: saline: ethanol in a ratio of 5:5:250:20) injected in a volume of 1 ml 100 $g^{-1}$, i.p. This procedure was carried out at least 3 h after the fifth daily injection. Dialysis loops (Figure 1) were implanted in the nucleus accumbens using the coordinates of 1.7 mm rostral and 1.5 mm lateral to bregma and 7.5 mm vertically from the surface of the brain according to Paxinos & Watson (1986). At the end of the experiment the position of the probes was routinely determined histologically from sections prepared at *postmortem* from tissue fixed in formalin.

### *Microdialysis and measurement of locomotor activity*

Eighteen to twenty four hours following implantation of the dialysis probe, the animals were placed in an activity box (40 cm square × 25 cm high) in which locomotor activity was assessed by photocells mounted 3 cm high on adjacent sides of the box, each infrared beam crossing being recorded as an activity count (Vale & Balfour, 1989). At this time, the dialysis loop was also connected via a liquid swivel to a syringe pump containing a Ringer solution (NaCl 147 mM, KCl 4 mM and $CaCl_2$ 1.25 mM) which was perfused at a constant rate of 1.7 μl $min^{-1}$. Samples were collected in small plastic tubes mounted above the outlet tubing. Samples were removed at 20 min intervals for 80 min in order to establish stable baseline levels of dopamine and its metabolites. At this time, treatments were administered either subcutaneously or directly into the nucleus accumbens via the dialysis loop. In the experiments designed to investigate the effects of KCl on dopamine release into the probe, the Ringer solution was changed to one containing KCl (100 mM), the osmolarity of the solution being maintained by an appropriate adjustment in the concentration of NaCl. For some of the experiments, the Ringer solution contained the dopamine uptake inhibitor, nomifensine (10 μM).

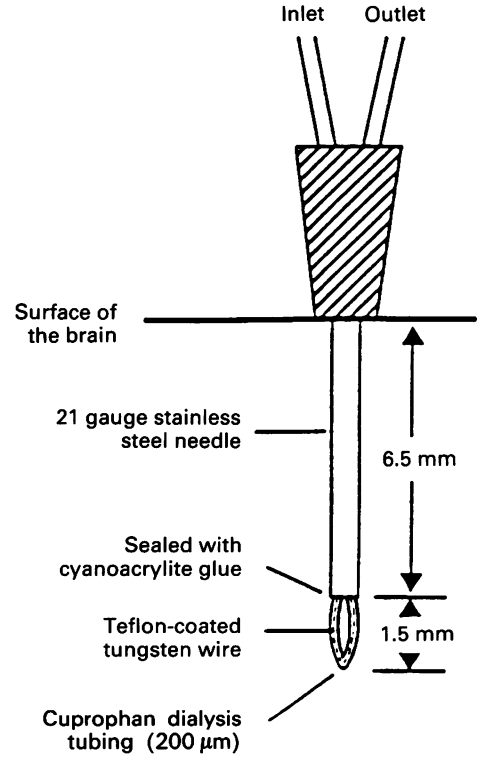


**Figure 1** The dialysis loop was constructed, in house, by feeding plastic-coated tungsten wire (0.075 mm o.d., Clarke Electromedical) through the lumen of cuprophan dialysis tubing (0.200 mm o.d., Medicell international). A 6 cm length of this was cut, folded loosely and fed through a 21 gauge stainless steel needle which had been cut to the appropriate length. The loop was sealed into the needle housing with cyanoacrylite glue (Loctite). One cm lengths of 21 gauge steel tubing were pushed down over the projecting ends of the dialysis tubing and into the needle cap. Loctite glue was introduced into the top of the needle cap to seal the dialysis fibre within this tubing by capillary action and fix the inlet and outlet tubes to the needle cap, the purpose of which was to facilitate the attachment of the perfusion tubing and the sample tubing.

Tissue levels of dopamine, dihyroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured in ultrafiltered supernatants prepared from homogenates of NAc in 0.1 N perchloric acid (100 mg of tissue per ml) by high performance liquid chromatography (h.p.l.c.) with electrochemical detection. The concentrations of dopamine, DOPAC and HVA in brain dialysates were assayed by injecting the dialysate samples directly into the h.p.l.c. The chromatography was performed on an ODS2 column with a solvent solution system composed of disodium orthophophosphate (0.1 M), EDTA (0.1 mM), sodium octane sulphonic acid (0.06 mM) and methanol (12% v/v). The pH was adjusted to 3.9 with citric acid. Dopamine and its metabolites were detected with a Coulochem Electrochemical (ESA) detector. DOPAC and HVA were measured by oxidation using the first detector set at 1 = + 0.35V. Dopamine was measured by reduction of the product of dopamine oxidation at detector 1 using detector 2 set at − 0.2V. The limit of detection of dopamine was approximately 0.02 pmol. Samples of modified Ringer solutions (containing 0.1 mM EDTA or 10 μM nomifensine) and standard solutions containing nicotine or amphetamine at a concentration of 10 μM, concentrations well in excess of those which could be expected to occur in the nucleus accumbens following systemic injections of these compounds at the highest doses employed in this study, were tested on the h.p.l.c. system and found to cause no extra or interfering peaks.

### *Data analysis*

In each experiment the mean concentrations of dopamine and its metabolites for the three samples collected before the test treatment were calculated and defined as 100%. The concentrations of dopamine and the metabolites in each fraction collected were then expressed as percentages of these mean values. The statistical analysis of the behavioural and biochemical data was performed by analysis of variance for repeated measures with treatment and time as the independent factors analysed. *Post hoc* analyses were performed with Duncan's test.

### *Drug sources*

Nomifensine hydrogen maleate was a generous gift from Hoescht Pharmaceuticals. Nicotine hydrogen tartrate, (+)-amphetamine sulphate and tetrodotoxin (TTX) were purchased from The Sigma Chemical Company. EDTA and sodium octane sulphonic acid were purchased from Fisons PLC; h.p.l.c. grade methanol was supplied by Rathburn Chemical Ltd. All other laboratory and h.p.l.c. grade reagents used were obtained from British Drug Houses.

## Results

### *Characterization of the release of dopamine and monamine metabolites into the dialysis probe*

The recoveries of dopamine, DOPAC and HVA, through the probe when determined *in vitro* before implantation, were 10 ± 3, 14 ± 3 and 13 ± 2% respectively. The basal levels of

dopamine and the monoamine metabolites in the dialysate samples, collected from rats which had received no injections, were unaffected by 5 daily injections of saline. The addition of tetrodotoxin (1 μM) to the dialysis fluid caused a rapid and marked ($F(1,7) = 40.4$; $P < 0.001$) reduction in the concentration of dopamine in the dialysate (Figure 2a) The dopamine concentration in the dialysate was also reduced substantially ($F(1,5) = 49.4$; $P < 0.001$) if the Ringer solution was changed to one containing 12 mM $MgCl_2$ (Figure 2b). If the Ringer solution was changed to one containing a depolarizing concentration of KCl (100 mM), the concentration of dopamine in the dialysate was increased significantly ($F(1,9) = 12.4$; $P < 0.01$) (Figure 2c). The response was rapidly reversed by changing the Ringer solution back to one containing 4 mM KCl. The increase in dialysate dopamine levels evoked by $K^+$ was attenuated significantly ($F$ treatment × time (7,42) = 3.0; $P < 0.05$) if the experiment was performed with a $CaCl_2$-free Ringer solution containing EDTA (0.1 mM).

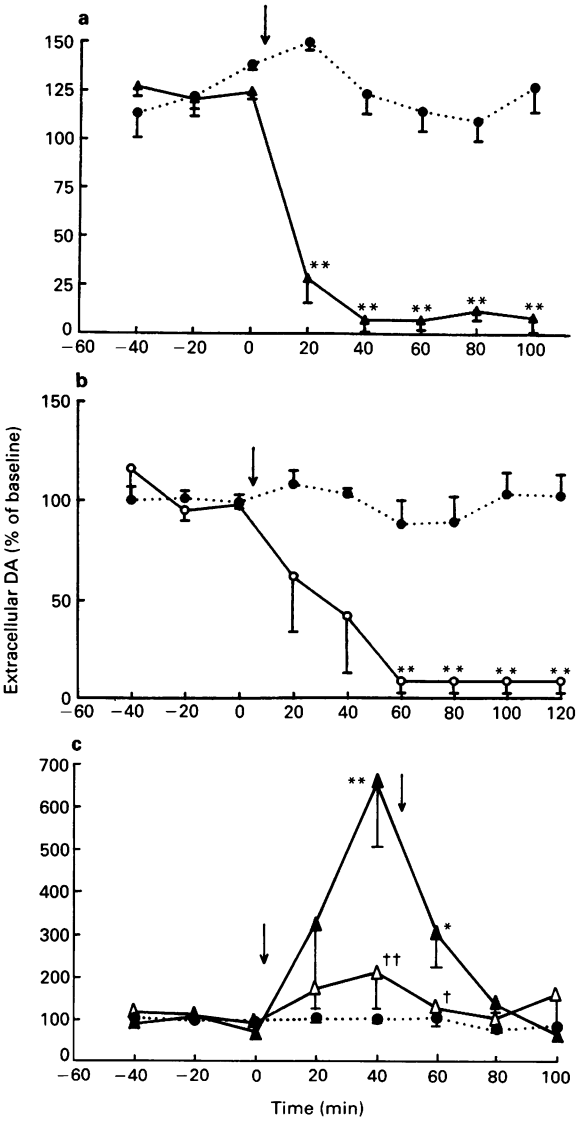


**Figure 2** The effects on extracellular dopamine (DA) of changing from a perfusion syringe containing normal Ringer to a second syringe containing normal Ringer (●) or (a) 1 μM tetrodotoxin (▲): (b) Ringer + 12 mM $MgCl_2$ (○) or (c) Ringer containing 100 mM KCl (▲) or $Ca^{2+}$-free Ringer containing 100 mM KCl and 100 μM EDTA (△) at the time = 0 min. In the experiments represented in (c), the solutions were changed back to normal Ringer after 40 min, at the time indicated by the second arrow. The results are means ($n = 5$ for figure (a); $n = 3$ for figure (b); $n = 4$ or 5 for (c)) expressed as % of the mean pretreatment levels measured in the three samples collected prior to time 0; vertical bars show s.e.mean. Significantly different from control: $*P < 0.05$, $**P < 0.01$; significantly different from KCl group: $\dagger P < 0.05$, $\dagger\dagger P < 0.01$.

### *The effect of acute and subchronic nicotine*

Under the conditions used, the acute administration of nicotine caused a dose-dependent increase ($F(2,19) = 7.02$; $P < 0.01$) in activity (Figure 3a). Acute nicotine (0.4 mg $kg^{-1}$) also appeared to increase the extracellular levels of dopamine in the dialysate (Figure 3b) although this effect was not statistically significant. The increases in extracellular DOPAC and HVA levels evoked by acute nicotine (Figure 3c and 3d) were significant ($F(2,19) = 3.52$; $P < 0.05$ and $F(2,19) = 5.45$; $P < 0.01$ for DOPAC and HVA respectively).

Pretreatment with nicotine (0.4 mg $kg^{-1}$) for 5 days before the test day resulted in a significant increase ($P < 0.01$) in the basal levels of dopamine in the dialysate measured 27 h after the last injection of nicotine (Table 1). Pretreatment with nicotine (0.1 and 0.4 mg $kg^{-1}$) also caused significant decreases in the basal concentrations ($P < 0.05$) of DOPAC in the NAc dialysates. The concentrations of HVA were not altered significantly by pretreatment with nicotine. The systemic administration of nicotine to rats pretreated with the compound resulted in significant increases ($F(2,22) = 8.22$; $P < 0.01$) in activity (Figure 4a) which were significantly greater ($F$ treatment by time (12,270) = 2.47; $P < 0.05$) than those observed in response to acute nicotine. Subchronic nicotine also evoked statistically significant increases ($F$ treatment by time (12,108) = 2.03; $P < 0.05$) in the concentrations of dopamine in the NAc dialysates (Figure 4b). Interestingly, under the conditions of the present study, neither the locomotor nor the mesolimbic dopamine responses to subchronic nicotine appeared to be dose-dependent. The concentrations of DOPAC and HVA were also increased significantly ($F(2,22) = 3.52$; $P < 0.05$ and $F(2,22) = 6.14$; $P < 0.05$ for DOPAC and HVA respectively) (Figure 4c and d).

### *Tissue levels of dopamine, dihydroxyphenylacetic acid and homovanillic acid*

The concentration of dopamine, DOPAC and HVA in the nucleus accumbens, 24 h after the last of 5 consecutive daily injections of nicotine remained unchanged when compared with those measured in saline-treated controls (Table 2).

### *Effect of nomifensine on responses to nicotine*

The inclusion of nomifensine (10 μM) in the perfusate caused a significant ($F$ treatment (1,8) = 46, $P < 0.01$) and sustained elevation in extracellular dopamine concentrations in the NAc (Figure 5). The intra-probe administration of nomifensine, however, had no significant effects on the concentrations of DOPAC or HVA in the dialysates. When nomifensine was present in the Ringer solution perfusing the dialysis probe both the acute and chronic administration of nicotine (0.4 mg $kg^{-1}$) resulted in a significant increase in extracellular dopamine ($F$ acute nicotine (1,9) = 8.9; $P < 0.05$; $F$ chronic nicotine (1,15) = 5.1; $P < 0.05$) (Figure 6). In the presence of nomifensine the peak dopamine response to nicotine did not appear to be enhanced by pretreatment with the drug. The duration of the response to nicotine, however, was prolonged ($F$ treatment × time (8,80) = 2.8; $P < 0.01$) when compared with that to acute nicotine if the animals were pretreated with the alkaloid. In addition, the increase in basal dopamine levels evoked by 5 days pretreatment with nicotine was still observed when the experiment was performed with nomifensine (Table 3). However, the increase in activity evoked by either acute or chronic nicotine was not altered significantly by the addition of nomifensine to the probe.

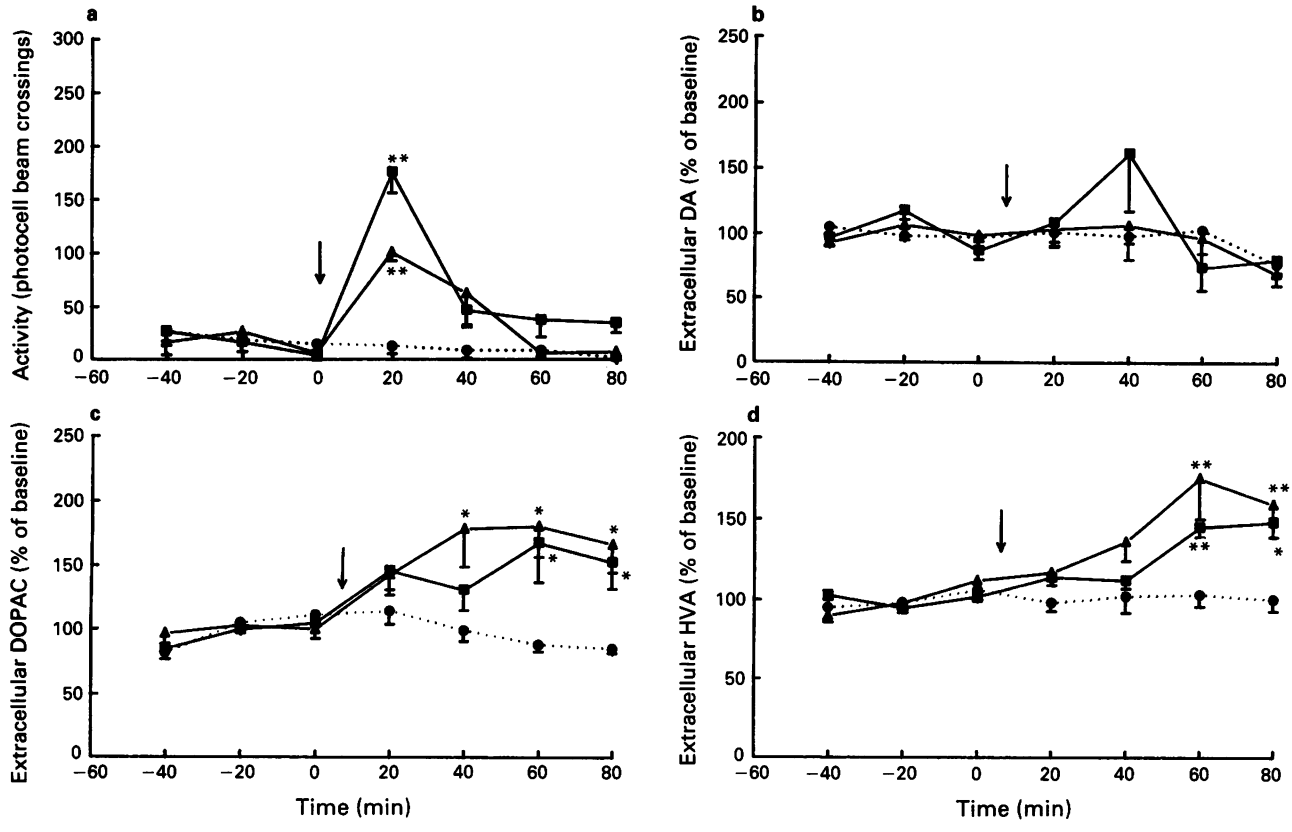


**Figure 3** The effects of acute subcutaneous injections of nicotine on spontaneous activity and the concentrations of dopamine (DA) and its metabolites in nucleus accumbens dialysates. Subcutaneous injections of saline (●, $n = 6$), 0.1 mg kg$^{-1}$ nicotine (▲, $n = 6$) or 0.4 mg kg$^{-1}$ nicotine (■, $n = 8$) were given at the point indicated by the arrow (time 0). The results are expressed as means of the numbers of observations shown above in parentheses; vertical bars show s.e.mean. The data for DA, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) are expressed as percentages of the mean pretreatment value. Significantly different from control: *$P<0.05$, **$P<0.01$.

**Table 1** The influence of pretreatment with nicotine or (+)-amphetamine on the concentrations of dopamine and its metabolites in nucleus accumbens dialysates

| | | *Dialysate DA and metabolite concentrations* | | |
|---|---|---|---|---|
| | | *DA* | *DOPAC* | *HVA* |
| *Pretreatment* | (n) | (fmol 20 µl$^{-1}$) | (pmol 20 µl$^{-1}$) | (pmol 20 µl$^{-1}$) |
| Saline | (14) | 86.7 ± 12.4 | 12.84 ± 2.05 | 8.46 ± 1.32 |
| Nic (0.1) | (7) | 98.6 ± 24.9 | 5.20 ± 1.40* | 8.78 ± 1.67 |
| Nic (0.4) | (10) | 365.9 ± 85.1** | 6.16 ± 1.68* | 5.46 ± 0.56 |
| Amphet (0.5) | (4) | 70.1 ± 14.1 | 13.69 ± 1.68 | 7.68 ± 1.11 |

Pretreatment protocols consisted of 5 consecutive daily injections of saline, 0.1 or 0.4 mg kg$^{-1}$ nicotine (Nic 0.1; Nic 0.4) or 0.5 mg kg$^{-1}$ (+)-amphetamine (Amphet 0.5). The basal levels were measured in dialysate samples collected approximately 24 h after the last injection of the pretreatment protocol. Basal levels were calculated from the 3 samples collected before the administration of the challenge drug on the final experimental day and represent the mean ± s.e.mean of the numbers of observations in parentheses. Significantly different from rats receiving daily injections of saline: *$P<0.05$; **$P<0.01$.

### *Effects of acute and subchronic (+)-amphetamine*

The subcutaneous administration of amphetamine induced a significant and dose-dependent ($F(2,13) = 6.5$; $P<0.01$) increase in the extracellular levels of dopamine in the NAc (Figure 7b) which peaked during the third 20 min period following amphetamine treatment. The time course for the increase in the dialysate dopamine content paralleled, very closely, the increase in locomotor activity observed in these animals ($F(2,13) = 12.05$; $P<0.01$) (Figure 7a). Statistical analysis of the data obtained from rats pretreated with (+)-amphetamine (0.5 mg kg$^{-1}$) daily for 5 days before the test day showed that the pretreatment regimen had no effects on the basal levels of dopamine and its metabolites in the NAc dialysates (Table 1) or on the locomotor or NAc dopamine responses to a challenge dose of (+)-amphetamine (0.5 mg kg$^{-1}$) (Figure 7a and b).

## Discussion

The preliminary results showed that the extracellular levels of dopamine in the dialysate were substantially reduced if tetrodotoxin or excess $Mg^{2+}$ ion were added to the perfusion fluid, data which suggest that under the conditions used, most of the basal dopamine sampled from the extracellular space by the dialysis probe had been secreted from dopaminergic nerve terminals via impulse-dependent mechanisms (Westerink & De Vries, 1988; Di Chiara, 1990). In addition it

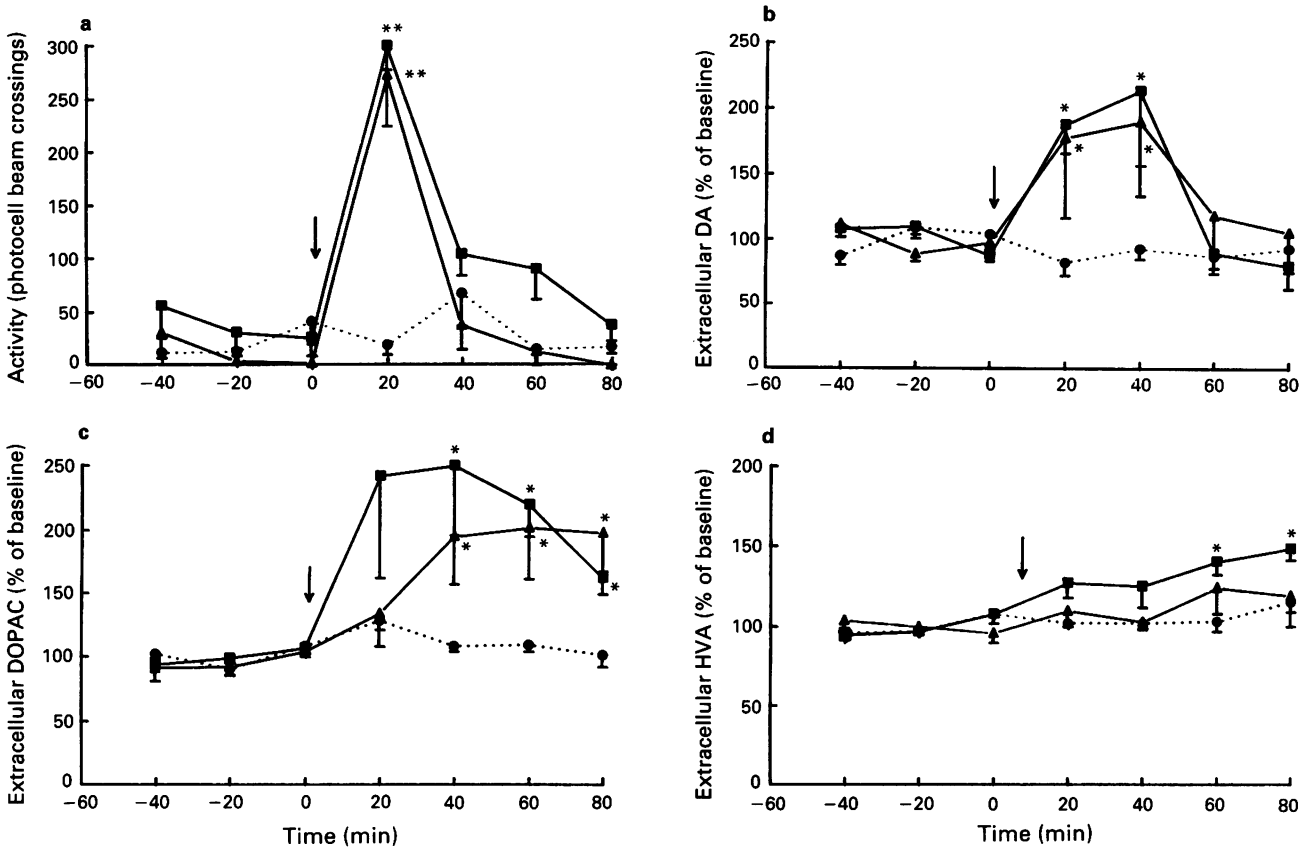


**Figure 4** The effects of subchronic nicotine on spontaneous activity and the concentrations of dopamine (DA) and its metabolites in nucleus accumbens dialysates. The rats were pretreated with daily subcutaneous injections of saline (●, $n = 6$), 0.1 mg kg$^{-1}$ nicotine (▲, $n = 6$) or 0.4 mg kg$^{-1}$ nicotine (■, $n = 10$) for 5 days before the test day. On the test day the animals were given injections of saline or nicotine (0.1 or 0.4 mg kg$^{-1}$) respectively and the time indicated by the arrow (time 0). The results are expressed as means of the numbers of observations shown above in parentheses; vertical bars show s.e.mean. The data for DA, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) are expressed as percentages of the mean pretreatment value. Significantly different from control: $*P<0.05$, $**P<0.01$. Peak levels at time = 40 min were 84.9 ± 4.2, 187.3 ± 55.2 and 789.0 ± 208.0 fmol 20 µl$^{-1}$ for rats challenged with saline, 0.1 or 0.4 mg kg$^{-1}$ nicotine respectively.

**Table 2** Tissue concentrations of dopamine (DA) and its metabolites in the nucleus accumbens of rats pretreated subchronically with nicotine

| | *Tissue DA and metabolite concentrations* (pmol mg$^{-1}$ wet tissue) | | |
|---|---|---|---|
| *Pretreatment* | *DA* | *DOPAC* | *HVA* |
| Saline | 31.6 ± 5.1 | 9.5 ± 1.4 | 4.4 ± 1.9 |
| Nic (0.1) | 37.5 ± 5.4 | 8.8 ± 1.7 | 5.0 ± 3.5 |
| Nic (0.4) | 32.9 ± 3.4 | 7.8 ± 0.8 | 3.5 ± 1.7 |

Results are the means ± s.e.mean of 6 observations. Rats received 5 consecutive daily injections of saline or nicotine (Nic 0.1 or 0.4 mg kg$^{-1}$) and were killed 24 h following their last treatment.

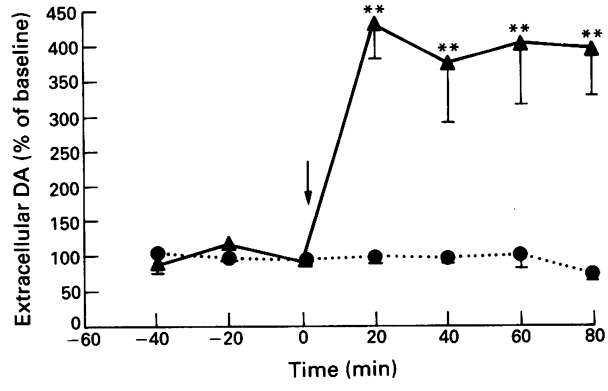


**Figure 5** The effect of nomifensine on dopamine (DA) levels in the nucleus accumbens dialysates. At the time indicated by the arrow (time 0), the Ringer solution used to perfuse the dialysis probe was changed to one containing nomifensine (10 µM) (▲) or the same Ringer solution (●). The results are the means of at lest 4 experiments and are expressed as a percentage of the mean pretreatment value; vertical bars show s.e.mean. Significantly different from control: $**P<0.01$.

was found that the increase in extracellular dopamine levels evoked by perfusing a depolarizing concentration of KCl through the probe was attenuated to a significant extent if a $Ca^{2+}$-free solution containing a small quantity of EDTA was used in place of the normal Ringer solution. Results very similar to these have been taken by others (Kalivas & Duffy, 1990) as evidence that the increase in dopamine secretion evoked by depolarization of the nerve terminals is calcium-dependent. In addition the studies which showed that the inclusion of the dopamine uptake inhibitor, nomifensine, in the Ringer solution used to perfuse the probe greatly increased the concentration of dopamine in the dialysate, suggest that much of the dopamine released in the NAc is normally rapidly recaptured by the dopaminergic terminals in the structure.

In contrast to results reported by some other groups (Imperato *et al.*, 1986; Di Chiara & Imperato, 1988; Damsma *et al.*, 1989), the present study failed to demonstrate a

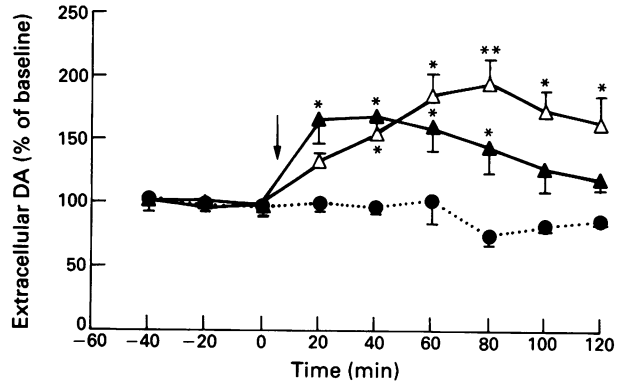


**Figure 6** The effects of nomifensine on the responses to acute and subchronic nicotine. The rats were given daily injections of saline or nicotine (0.4 mg kg$^{-1}$) for 5 days before the test day. On the test day the animals pretreated with saline were given saline (●) or an acute injection of nicotine (0.4 mg kg$^{-1}$, ▲) at the point indicated by the arrow (time 0). At this time the rats pretreated with nicotine were also given an injection of nicotine (△). The results are the means of at least 4 experiments and are expressed as a percentage of the mean pretreatment value; vertical bars show s.e.mean. Significantly different from control: $^{**}P<0.01$.

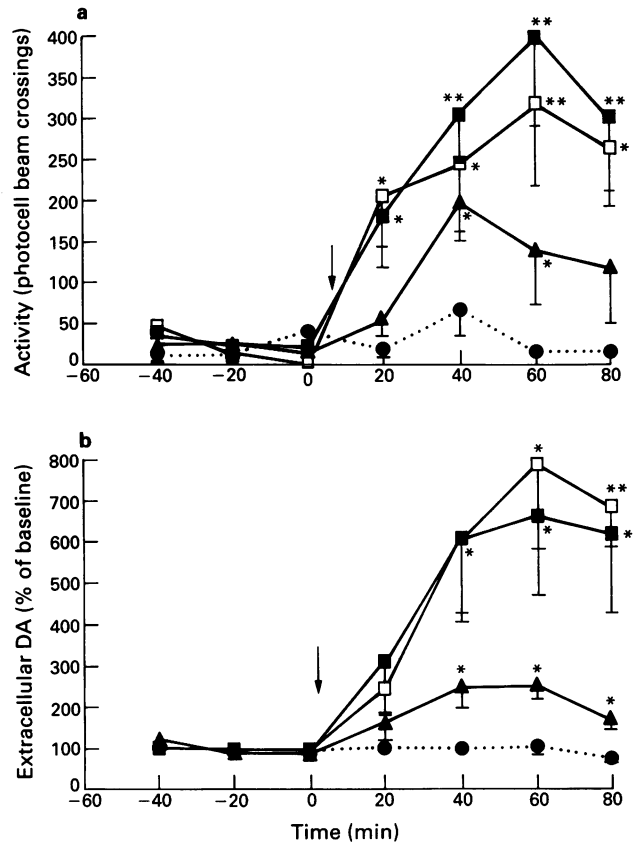


**Figure 7** The effects of (+)-amphetamine on spontaneous activity and the concentration of dopamine (DA) in nucleus accumbens dialysates. The rats were pretreated with daily subcutaneous injections of saline or (+)-amphetamine (0.5 mg kg$^{-1}$) for 5 days. On the test day the rats pretreated with saline were given saline (●, $n=6$) 0.2 mg kg$^{-1}$ (+)-amphetamine (▲, $n=6$) or 0.5 mg kg$^{-1}$ (+)-amphetamine (■, $n=5$) at the point indicated by the arrow (time 0). At this time the rats pretreated with 0.5 mg kg$^{-1}$ (+)-amphetamine were given 0.5 mg kg$^{-1}$ (+)-amphetamine (□, $n=6$). The results are the means of the numbers of observations shown in parentheses; vertical bars show s.e.mean. The data for dopamine are expressed as a percentage of the mean pretreatment value. Significantly different from control: $^{*}P<0.05$; $^{**}P<0.01$.

significant increase in extracellular dopamine levels in the NAc in response to the acute administration of nicotine when compared with the control response observed in rats given saline although the apparent increase in extracellular dopamine levels observed in rats given the higher dose of nicotine tested (0.4 mg kg$^{-1}$) was of a similar magnitude to that reported by Damsma and colleagues (1989). Recent studies have shown that the dopaminergic innervation of the NAc is heterogeneous to the extent that the fibres which innervate the caudal NAc also contain cholecystokinin whereas those which innervate the rostral NAc do not (Hokfelt *et al.*, 1980). It is possible, therefore, that acute nicotine acts preferentially on one of the pathways which supply the NAc and that, in this study, the probes which were located in the rostral rather than the caudal NAc, were not located in the terminal field of this pathway. Acute nicotine, however, did increase the concentrations of DOPAC and HVA in the dialysate, data which support the conclusion that acute nicotine did increase dopamine turnover in the area of the brain sampled by the dialysis probe. Although increased dopamine turnover does not necessarily reflect increased dopamine release, the results suggest this probably was the case in these experiments because, when neuronal dopamine uptake was antagonized with nomifensine, acute nicotine administration did result in increased extracellular levels of dopamine. Therefore, these data imply that in the area of NAc sampled by the dialysis probe in this study, dopamine uptake is probably fairly rapid and that in the absence of an uptake inhibitor, the effects of acute nicotine were not sufficiently great to result in substantial leakage from the synaptic cleft into the extracellular space, the compartment actually sampled by microdialysis (Benveniste, 1989; Di Chiara, 1990).

In contrast to the results obtained with rats treated acutely with nicotine, subchronic administration of the drug did result in a significant increase in extracellular dopamine. For the rats pretreated with 0.4 mg kg$^{-1}$ nicotine this represented a substantial enhancement of the response to the drug since pretreatment with this dose also caused increased basal

**Table 3** The concentrations of dopamine (DA) and its metabolites in dialysates of nucleus accumbens measured in the presence of nomifensine

| | | *Dialysate DA and metabolite concentrations* | | |
|---|---|---|---|---|
| *Pretreatment* | (n) | *DA* (fmol 20 µl$^{-1}$) | *DOPAC* (pmol 20 µl$^{-1}$) | *HVA* (pmol 20 µl$^{-1}$) |
| Saline | (10) | 263 ± 32 | 10.47 ± 2.34 | 5.25 ± 0.48 |
| Nicotine (0.4 mg kg$^{-1}$) | (10) | 386 ± 87* | 9.21 ± 0.96 | 4.58 ± 0.75 |

Rats received 5 daily injections of saline or nicotine before implantation of the dialysis probe. The dialysate samples were collected 27 h after the last injection of the pretreatment protocol using a Ringer solution containing nomifensine (10 µM). Basal levels were calculated from the 3 samples obtained on the final experimental day prior to the administration of the challenge drug and represent the means ± s.e.mean of the numbers of observations in parentheses. Significantly different from the saline-pretreated group: $^{*}P<0.05$.

dopamine levels prior to the injection of the challenge dose. These results are consistent with the potentiation of mesolimbic dopamine responses observed in tissue slices prepared from the NAc of nicotine-pretreated rats (Fung, 1989) but do not entirely agree with the results obtained by Damsma and colleagues (1989) who obtained no significant change in the mesolimbic dopamine response to s.c. nicotine, as measured by microdialysis, following a period of chronic treatment. The reason for the difference between the two studies remains to be established although they could reflect differences in the experimental procedures such as the use of transcerebral cannulae by Damsma's group as opposed to the loop probe employed in the present study or, as has been suggested above, the area of the NAc sampled by the probe. However, Damsma *et al.* (1989) did note a tendency for the basal dopamine levels to be raised in nicotine-treated rats. Elevated endogenous levels of NAc dopamine have previously been reported after 5 (Fung & Lau, 1988) and 14 (Fung, 1989) days of nicotine infusion. However, the enhanced basal extracellular dopamine, observed in the present study, was not accompanied by increased tissue levels of dopamine since the more moderate dosing regimen used here had no significant influence on the concentration of dopamime or its metabolites measured *post-mortem*. This finding confirms that *post mortem* tissue levels do not adequately reflect the activity of dopamine systems *in vivo*. Recent studies, however, suggest that nicotine at low doses can act as a non-competitive antagonist of the neuronal dopamine transporter (Izenwasser *et al.*, 1991) and it is possible, therefore, that the increased dopamine response observed in the animals pretreated with nicotine could be the result of a nicotine-induced attenuation of neuronal dopamine reuptake which persists for at least 24 h after the last nicotine injection. For the higher dose of nicotine at least, this hypothesis is consistent with the fact that pretreatment results in increased basal levels of dopamine and decreased levels of DOPAC in the NAc dialysates prior to the administration of the challenge dose of nicotine and with the observation that sub-chronic nicotine elevates NAc dopamine to a lesser extent in nomifensine-treated rats than it does in untreated animals. Furthermore, the studies using the Ringer solution containing nomifensine suggested that pretreatment with nicotine increases the duration of the response to a challenge dose of the alkaloid on the mesolimbic dopaminergic system although, clearly, further experiments are necessary to confirm this conclusion.

Studies in other laboratories suggest that the locomotor stimulant properties of both nicotine and amphetamine are mediated by their effects on dopamine secretion in the mesolimbic dopamine system (Kelly *et al.*, 1975; Imperato *et al.*, 1986; Clarke *et al.*, 1988). In the present study the effects of (+)-amphetamine on the extracellular levels of dopamine in the NAc paralleled both quantitatively and temporally the changes in locomotor activity evoked by the drug, data which are clearly consistent with the results of the earlier studies. The finding that the enhanced effects of nicotine on NAc dopamine, seen in animals pretreated with the drug, were accompanied by enhanced locomotor responses to the drug suggests that the behavioural sensitization could be mediated by potentiation of its effects on mesolimbic dopamine secretion. Interestingly Lapin *et al.* (1987) have reported that nicotine-induced circling behaviour in rats with unilateral lesions of the nigrostriatal system is only observed in animals which have been pretreated with the drug for 5 days, results which suggest that pretreatment with nicotine over a period of time similar to that used in the present studies may also result in sensitization of its effects on the nigrostriatal dopamine system.

The mesolimbic dopaminergic system is also thought to mediate the reinforcing properties of nicotine (Singer *et al.*, 1982; Wise & Bozarth, 1987) and, if this is the case, the present data suggest that pre-exposure to nicotine may enhance the reinforcing properties of a subsequent injection. This interesting possibility is supported by the results of recent studies which suggest that pretreatment with nicotine, for 7 days, enhances its ability to evoke a conditioned place preference response (M. Shoaib & I.P. Stolerman, personal communication). In the present study, the short period of pretreatment with a relatively low dose of (+)-amphetamine did not influence its effects on either extracellular dopamine levels in the NAc or locomotor activity. Studies in other laboratories, however, have shown that pretreatment with other psychostimulant drugs can enhance their effects on the secretion of dopamine in the mesolimbic system (Robinson *et al.*, 1988; Kalivas & Duffy, 1980; Pettit & Justice, 1991) although, in the case of amphetamine, this appears to occur in animals which are tested some weeks after cessation of treatment with relatively high doses of the drug (Robinson *et al.*, 1988). Thus, sensitization of the mesolimbic dopamine response following chronic treatment may be a property which is common to most, if not all, psychostimulant drugs of dependence although it seems likely that different mechanisms mediate sensitization to the different groups of drugs since pretreatment with nicotine does not appear to result in sensitization of the responses to other psychostimulant drugs (Schenk *et al.*, 1991).

In conclusion, these results suggest that the mesolimbic dopaminergic system does not develop tolerance to nicotine and indeed may become sensitized following repeated exposure to the alkaloid. In addition, the evidence presented suggests that this neural pathway may subserve, to some extent at least, the enhanced behavioural effects seen in these animals.

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# Evidence for an atypical, or $\beta_3$-adrenoceptor in ferret tracheal epithelium

S.E. Webber & M.J. Stock

Department of Physiology, St George's Hospital Medical School, Tooting, London SW17 ORE

**1** A preparation of the ferret trachea *in vitro* was used to examine the effects of three selective β-adrenoceptor agonists on lysozyme secretion from submucosal gland serous cells and epithelial albumin transport into tracheal mucus following sustained, submaximal stimulation of mucus production with methacholine (20 μM)

**2** Prenalterol, salbutamol and BRL 37344 all enhanced methacholine-induced albumin output. BRL 37344 was 10,000 times more potent than salbutamol, and salbutamol was slightly more potent than prenalterol. The concentrations required to increase albumin output by 100% ($EC_{100\%}$) were 1.4 nM, 0.7 mM and approximately 1.0 mM for BRL 37344, salbutamol and prenalterol, respectively. All three agonists inhibited methacholine-induced lysozyme output, with salbutamol being 60 times more potent than BRL 37344, and BRL 37344 being approximately 100 times more potent than prenalterol.

**3** The selective $\beta_2$-adrenoceptor antagonist, ICI 118551, inhibited the increase in albumin output produced by BRL 37344, but was much more potent at inhibiting the response to salbutamol; the $pA_2$ for ICI 118551 was 5.55 and 7.18 ($P<0.001$) when the agonist was BRL 37344 and salbutamol, respectively. ICI 118551 also attentuated the inhibition of lysozyme output produced by the two agonists, but was 10–30 times more potent at inhibiting this response than the albumin response to BRL 37344 and salbutamol.

**4** The greater potency (4–5 orders of magnitude) of BRL 37344, compared to the $\beta_1$- (prenalterol) and $\beta_2$ - (salbutamol) adrenoceptor selective agonists, in stimulating methacholine-induced albumin transport suggests that tracheal epithelium possess an atypical, or $\beta_3$-adrenoceptor similar to that previously reported for adipocytes and gastrointestinal smooth muscle. The weak antagonism of the response to BRL 37344 by ICI 118551 would also be consistent with an atypical adrenoceptor mediating the albumin transport response. Inhibition of methacholine-induced serous cell lysozyme output would appear to be mediated predominantly by $\beta_2$-adrenoceptors.

**5** In view of the possible beneficial protective effects of albumin in airway surface liquid, selective $\beta_3$-agonists like BRL 37344 might have potential value in the prevention and/or treatment of inflammatory airway disease.

**Keywords:** Trachea; β-adrenoceptors; salbutamol; BRL 37344; albumin; lysozyme; epithelium; submucosal glands

## Introduction

Ahlquist (1948) first proposed that there was more than one adrenoceptor. His hypothesis was based on a study of the relative abilities of several adrenoceptor agonists to cause either contraction or relaxation of smooth muscle. Receptors on the smooth muscle were designated either α or β depending on whether catecholamines produced either excitatory or inhibitory responses, respectively. This initial classification was corroborated by the finding that certain antagonists can selectively block the effects of sympathomimetic agents at α- or β- adrenoceptors. β-Adrenoceptors were later subdivided into $\beta_1$ and $\beta_2$ on the basis of the differences in potency (10 to 50 fold) between adrenaline and noradrenaline in different tissues (Lands *et al.*, 1967), and many antagonists that discriminate between $\beta_1$ and $\beta_2$ -receptors have since been developed.

Although most functional studies can be reconciled with the involvement of $\beta_1$ or $\beta_2$-adrenoceptors, or a mixture of the two, there have been an increasing number of reports incompatible with such a simple division (see Zaagsma & Nahorski, 1990). Some of the strongest evidence for a third, or atypical β-adrenoceptor came from studies with a series of novel agonists (e.g. BRL 26830, BRL 35135) that had potent and selective thermogenic anti-obesity activities in animal models. The active metabolites of these compounds (e.g. BRL 37344) were found to be 20 to 400 times more potent on brown adipose tissue (BAT) thermogenesis than on classical $\beta_1$- (atrial rate) and $\beta_2$- (tracheal relaxation) functions (Arch *et al.*, 1984). These potency ratios, and the low $pA_2$ values of conventional selective and non-selective β-adrenoceptor antagonists (Arch *et al.*, 1984; Stock & Sudera, 1989; Zaagsma & Hollenga, 1991), suggest that BAT possesses an atypical adrenoceptor. A human gene has now been identified that encodes for a third (i.e. $\beta_3$) adrenoceptor (Emorine *et al.*, 1989), and cells transfected with this receptor are 10 times more sensitive to noradrenaline than adrenaline, resistant to blockade by conventional β-antagonists and highly sensitive to the novel, thermogenic β-agonists. Thus, the atypical adrenoceptor on BAT is now thought to be the same, or similar to the human $\beta_3$-adrenoceptor, and thermogenic BAT-selective agonists, such as BRL 37344, are commonly refered to as $\beta_3$-agonists. Apart from brown (and white) adipocytes, there is evidence also for atypical, or $\beta_3$-adrenoceptors in the gastrointestinal tract that mediate relaxation of guinea-pig gastric fundus (Coleman *et al.*, 1987), inhibition of longitudinal muscle tension in guinea-pig ileum (Bond & Clarke, 1988) and relaxation of rat distal colon (McLaughlin & McDonald, 1989).

In the airways, activation of $\beta_2$-adrenoceptors leads to relaxation of tracheal and bronchial smooth muscle and β-agonists are still the standard treatment used for obstructive airways diseases such as asthma. However, asthma is now characterized as a disease of chronic inflammation, involving

mucosal tissues such as submucosal glands and epithelium, as well as airway smooth muscle. β-Adrenoceptors have been shown to be present on the epithelium and glands (Barnes *et al.*, 1982), and $\beta_2$-agonists such as salbutamol are weak stimulants of gland secretion (Webber & Widdicombe, 1989) and ion transport across the epithelium (Feldman *et al.*, 1990). However, relatively little is known of the effects of β-agonists on airway mucosal tissues and the receptors, or receptor subtypes mediating these effects.

In the present study, the ferret whole trachea *in vitro* preparation (Webber & Widdicombe, 1987) was used to examine the effects of agonists selective for each of the β-receptor subtypes on submucosal gland secretion (lysozyme secretion from submucosal gland serous cells) and the active transport of albumin across the tracheal epithelium (Webber & Widdicombe, 1989). Since these agents are only weak secretagogues in their own right, baseline mucus secretion was promoted with the muscarinic agonist, methacholine. The effects of a selective $\beta_2$-antagonist on the responses to the agonists was also studied.

## Methods

### *The ferret trachea* in vitro

Ferrets of either sex, weighing 0.5–1.5 kg, were anaesthetized by an intraperitoneal injection of sodium pentobarbitone (50 mg $kg^{-1}$, Sagatal, May & Baker). The trachea was exposed and cannulated about 5 mm below the larynx with a perspex cannula containing a conical collecting well (Webber & Widdicombe, 1987). The ferret was then killed with an overdose of sodium pentobarbitone injected into the heart. The chest was opened along the midline and the trachea exposed to the carina, cleared of adjacent tissue, removed and cannulated just above the carina. The trachea was mounted, laryngeal end down, in a jacketed organ bath with Krebs-Henseleit buffer restricted to the submucosal side. The composition of the Krebs-Henseleit solution was (mM): NaCl 120.8, KCl 4.7, $KH_2PO_4$ 1.2, $MgSO_4$ $7H_2O$ 1.2, $NaHCO_3$ 24.9, $CaCl_2$ 2.4, glucose 5.6. The buffer was maintained at 37°C and gassed with 95% $O_2$/5% $CO_2$. The lumen of the trachea remained air-filled. Before the start of an experiment each trachea was allowed to equilibrate for 20 min, and during this time changes of bathing medium were made every 5 min. Secretions were carried by gravity and mucociliary transport to the lower cannula, where they pooled and could be withdrawn periodically into a polyethylene catheter which was inserted into the lower cannula to form an airtight seal. The catheters containing the secretions were sealed at both ends with bone wax, numbered and stored frozen until required.

After defrosting, the secretions were washed out of the catheters into labelled plastic vials with 0.5 ml distilled $H_2O$. The vials were frozen and stored for use in the albumin and lysozyme assays. Preliminary experiments had shown that frozen storage for up to 6 months does not affect the enzymatic activity of lysozyme or the albumin content of the samples. Secretion volumes were estimated by the differences in weights of the catheters with secretions and dried without secretions, and the secretion rates were expressed as μl $min^{-1}$ (assuming 1 g of secretion was equivalent to 1 ml).

### *Assay for lysozyme*

The lysozyme concentrations of the mucus samples were measured by a turbidimetric assay which relies on the ability of lysozyme to break down the cell wall of the bacterium *Micrococcus lysodeikticus*. Addition of lysozyme to a solution of the bacteria reduces the turbidity of the solution, thereby leading to a fall in optical density (OD) measured at 450 nm.

A stock suspension of *M. lysodeikticus* of 3 mg $ml^{-1}$ was prepared. When diluted 10 fold (the dilution in the assay) this suspension gives an OD of approximately 0.6 at 450 nm. To produce a standard curve, various concentrations of hen egg white lysozyme (0.5 to 100 ng $ml^{-1}$) were incubated in duplicate in 1.5 ml potassium phosphate buffer (50 mM, pH 7.4) containing *M. lysodeikticus* (0.3 mg $ml^{-1}$), sodium azide (1 mg $ml^{-1}$) and bovine serum albumin (BSA, 1 mg $ml^{-1}$). The BSA was included in the assay for its protein stabilizing effects and the sodium azide was added to prevent the growth of bacteria in the incubating solutions. The reaction mixtures were incubated for 18 h at 37°C. After incubation, the OD of each solution was measured at a wavelength of 450 nm with potassium phosphate buffer (pH 7.4) containing BSA (1 mg $ml^{-1}$) as a blank. The standard curve was constructed by plotting the fall in OD (reduction in turbidity) against the concentration of lysozyme in the solution.

To estimate the concentration of lysozyme in a mucus sample, 20 μl of sample were incubated in 1.5 ml potassium phosphate buffer (50 mM, pH 7.4), exactly as described above for the known concentrations of lysozyme used in the preparation of the standard curve. The lysozyme concentrations (equivalent to hen egg white lysozyme) of the 20 μl samples and hence of the original mucus samples were estimated from the standard curve. The rate of output of lysozyme was then calculated by dividing the total amount of lysozyme in a mucus sample by the time over which the sample accumulated.

### *Albumin transport*

To examine the effect of β-receptor stimulation on the transport of albumin across the ferret trachea, BSA was added to the buffer bathing the submucosal surface of the trachea in a concentration of 4 mg $ml^{-1}$. Fluorescent BSA (0.02–0.03 mg $ml^{-1}$) was also added to the buffer as a marker and enabled an estimate to be made of the total amount of albumin which appeared in the mucus samples.

The fluorescence of the mucus samples was measured with a fluorimeter, using an excitation wavelength at 550 nm and an emission wavelength of 490 nm. The fluorescent albumin concentration of the mucus samples was estimated from a standard curve relating fluorescence (arbitrary units) to the concentration of fluorescent BSA (range 25 ng $ml^{-1}$ to 3 μg $ml^{-1}$). The total concentration of albumin in the mucus samples was obtained by multiplying the fluorescent albumin concentration (estimated from the standard curve) by the ratio of non-fluorescent to fluorescent albumin used in the experiment. The rate of output of albumin was determined by dividing the total amount of albumin in a mucus sample by the time over which that sample accumulated.

### *Experimental protocol*

*Effect of β-agonists on responses to methacholine* Previous studies have shown that methacholine produces concentration-dependent increases in lysozyme and albumin outputs from the ferret trachea (Webber & Widdicombe, 1987; 1989). After a 30 min control period, methacholine (20 μM) was added to the buffer bathing the trachea. This concentration of methacholine produces 70–80% of the respective maximum responses for lysozyme and albumin outputs. Mucus samples were taken every 30 min until a steady 'maintained' mucus volume output was obtained (typically 2.5–3 h). After each 30 min period the buffer surrounding the trachea was replaced with fresh buffer containing methacholine.

In the first series of experiments, and after a maintained mucus volume output to methacholine had been established, five concentrations of a β-agonist were added in ascending order to the methacholine-containing buffer surrounding the trachea. The concentrations of agonist used in these experiments covered a wide concentration range (at least four log units). Each concentration of β-agonist was left in contact with the trachea for 30 min. After 30 min, the secretion produced was withdrawn and processed. The buffer surrounding the trachea was then replaced with fresh buffer

containing methacholine and the next concentration of β-agonist.

In order to compare the relative potencies of salbutamol and BRL 37344 more accurately, a second series of experiments was carried out. In these experiments, the same procedure was used as described above, but with a much narrower concentration range of the β-agonists (one to two log units). The range used for each agonist was chosen to cover the linear segment of the concentration-response that would produce a 100% increase in albumin transport ($EC_{100\%}$), or a 50% inhibition of lysozyme secretion ($IC_{50\%}$); the $EC_{100\%}$ and $IC_{50\%}$ values obtained were used in the next series of experiments.

*Effect of ICI 118551 on responses to salbutamol and BRL 37344* As in the previous experiments, a steady mucus volume output was obtained with methacholine, and then the concentration of salbutamol or BRL 37344 producing a 100% increase in methacholine-induced albumin output ($ED_{100\%}$, determined in the previous experiment) was added with methacholine in the buffer. This concentration of salbutamol or BRL 37344 was present in the buffer with the methacholine for the remainder of the experiment. Four concentrations of the $\beta_2$-antagonist, ICI 118551 (0.1–100 μM), were then added in ascending order to the buffer surrounding the trachea. Each concentration of ICI 118551 was left in contact with the trachea for 30 min, after which time the secretion was withdrawn and processed. In another series of experiments, exactly the same procedure was followed using concentrations of salbutamol and BRL 37344 that produced a 50% inhibition of methacholine-induced lysozyme output ($IC_{50\%}$). Finally, an attempt was made to estimate an approximate $pA_2$ value for ICI 118551 by determining the concentration-response for albumin secretion using BRL 37344 and salbutamol, with and without ICI 118551 (10 μM) in the incubation buffer.

### *Analysis of results*

The change in methacholine-induced lysozyme or albumin output produced by a β-agonist was calculated as the difference in output obtained between the period immediately before the β-agonist was added and the period when the agonist was in the organ bath, expressed as a percentage. The $EC_{100\%}$ and $IC_{50\%}$ values for salbutamol and BRL 37344, and the $IC_{50\%}$ values for ICI 118551 (concentration producing a 50% inhibition of response to salbutamol or BRL 37344) were obtained by linear regression analysis of the data. MacKay's method (1978) was used to calculate the $pA_2$ for ICI 118551 from the $ED_{100\%}$ concentration-ratio of both salbutamol and BRL 37344.

### *Drugs*

The following drugs were used sources are given in parenthesis: Salbutamol (Glaxo); prenalterol methacholine (Hässle); ICI 118551 (ICI); BRL 37344 (SKB).

## Results

### *Baseline values*

The mean baseline outputs of lysozyme and albumin in all experiments ($n = 48$) before the addition of any drugs were $29 \pm 15$ ng min$^{-1}$ and $0.15 \pm 0.09$ μg min$^{-1}$ respectively.

### *Effects of methacholine*

In the 30 min period immediately after the addition of methacholine, the lysozyme and albumin outputs increased from baseline levels to $358 \pm 33$ ng ml$^{-1}$ ($n = 48$) and $2.12 \pm 0.16$ μg min$^{-1}$ ($n = 48$), respectively. On continued application of methacholine the lysozyme and albumin outputs fell, but reached a steady maintained level after 2.5–3 h. The mean level of the maintained methacholine-induced lysozyme and albumin output immediately before the addition of any further drugs was $168 \pm 17$ ng min$^{-1}$ and $0.64 \pm 0.07$ μg min$^{-1}$.

### *Concentration-response effects of β-adrenoceptor agonists*

Prenalterol (10 μM–1 mM), salbutamol (0.1 μM–1 mM) and BRL 37344 (0.1 nM–10 μM) produced concentration-dependent increases in methacholine-induced albumin output (Figure 1a). Judging from the concentration required to produce a 100% increase in albumin output, BRL 37344 was approximately five orders of magnitude more potent than salbutamol, which was only slightly more potent than prenalterol. All three agonists also produced concentration-dependent reductions in methacholine-induced lysozyme output. However, the potency order was changed, with salbutamol being approximately 60 fold more potent than BRL 37344, which was 100 fold more potent than prenalterol.

Based on these initial results, more precise $EC_{100\%}$ values for salbutamol and BRL 37344-induced enhancement of methacholine-stimulated albumin output were determined with a narrower range of concentrations (salbutamol 0.1–1 mM; BRL 37344 0.3–10 nM). These produced linear responses (Figure 2), and estimated $EC_{100\%}$ values of 1.4 nM and 0.74 mM for BRL 37344 and salbutamol, respectively. These $EC_{100\%}$ concentrations of BRL 37344 and salbutamol increased the concentration of albumin in the mucus from ($n = 6$) $1.8 \pm 0.4$ to $9.2 \pm 1.3$ μg μl$^{-1}$, and from $2.1 \pm 0.3$ to $8.4 \pm 1.1$ μg μl$^{-1}$, respectively (cf. 4 μg μl$^{-1}$ in submucosal buffer).

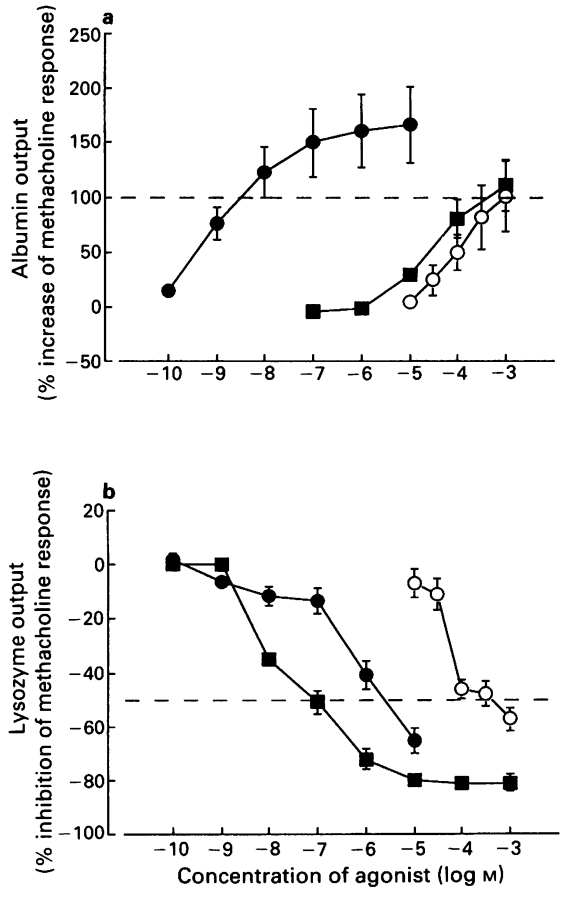


**Figure 1** Concentration-response curves showing the effect of BRL 37344 (●), salbutamol (■) and prenalterol (○) on maintained methacholine-induced albumin (a) and lysozyme (b) outputs. Points are the means of 6–8 determinations, vertical bars = s.e.mean.

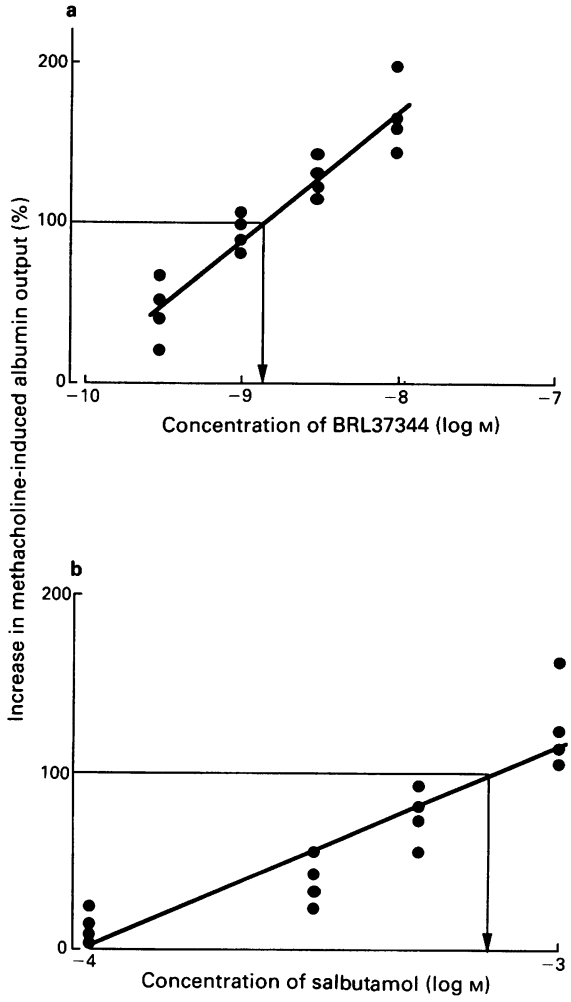


**Figure 2** Linear regression analysis of the increase in methacholine-induced albumin output by (a) BRL 37344 and (b) salbutamol. Individual data points are shown. The arrows indicate the concentrations of BRL 37344 and salbutamol which increase albumin output by 100% ($EC_{100\%}$).

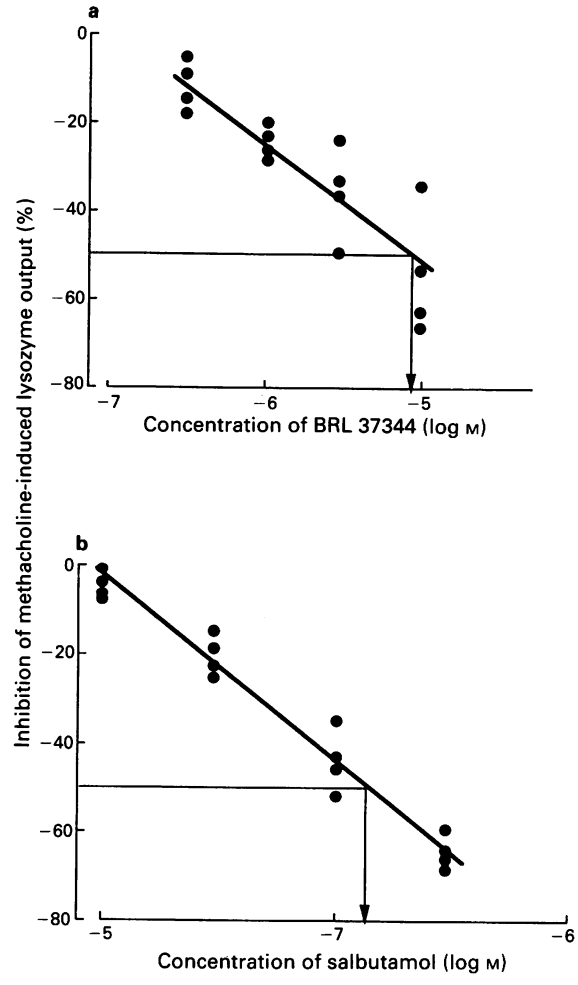


**Figure 3** Linear regression analysis of the inhibition of methacholine-induced lysozyme output produced by (a) BRL 37344 and (b) salbutamol. Individual data points are shown. The arrows indicate the concentrations of BRL 37344 and salbutamol which inhibit lysozyme output by 50% ($IC_{50\%}$).

A different range of concentrations (salbutamol 10 nM–1 μM; BRL 37344 0.3–10 μM) was used to produce the results for inhibition of lysozyme secretion shown in Figure 3. The estimated $IC_{50\%}$ values were 0.14 μM and 8.14 μM for salbutamol and BRL 37344, respectively.

### *Effects of ICI 118551 on responses to salbutamol and BRL 37344*

ICI 118551 produced a concentration-dependent inhibition of albumin output in response to $EC_{100\%}$ concentrations of BRL 37344 and salbutamol (Figure 4). However, the concentration of ICI 118551 required to inhibit the response to BRL 37344 by 50% ($IC_{50\%}$) was 10 times greater (4.3 μM) than that (0.43 μM) required to inhibit the salbutamol response. ICI 118551 also produced a concentration-dependent attenuation of the inhibitory effects of $IC_{50\%}$ concentrations of BRL 37344 and salbutamol on lysozyme secretion (Figure 5). In this case, ICI 118551 was 30 times more potent at inhibiting the lysozyme response to salbutamol than to BRL 37344; $IC_{50\%}$ for the antagonist was 25 nM and 0.8 μM for salbutamol and BRL 37344, respectively.

Having obtained these results with different concentrations of ICI 118551, a single concentration (10 μM) of the antagonist was selected and the $EC_{100\%}$ concentration-ratio for stimulation of albumin secretion by salbutamol and BRL 37344 determined. These data were used to calculate a $pA_2$ for ICI 118551 and produced a mean value of 5.55 ± 0.07 ($n = 4$) when BRL 37344 was the agonist, but a significantly ($P < 0.001$) higher value (7.18 ± 0.06; $n = 4$) when salbutamol was the agonist.

## Discussion

In the present study, agonists presumed from previous work to be relatively selective for each of the three β-adrenoceptor subtypes all produced a concentration-dependent inhibition of methacholine-induced lysozyme output. Lysozyme is a specific marker for submucosal gland serous cell secretion (Bowes & Corrin, 1977), and these results therefore suggest that β-receptor activation (of whatever subtype) results in an inhibition of serous cell secretion produced by muscarinic receptor stimulation. These results are in direct contact to the effects of β-agonists on baseline secretion. Thus, the $\beta_2$-agonist, salbutamol, is a weak stimulant of baseline mucus volume output and lysozyme output from the ferret trachea (Webber & Widdicombe, 1989), dobutamine ($\beta_1$-agonist) and salbutamol both increase the output of radiolabelled sulphated glycoprotein from cat trachea (Peatfield & Richardson, 1982), and the $\beta_2$-agonist, terbutaline, increases the output of radiolabelled macromolecules from ferret trachea (Borson *et al.*, 1984). It is not clear how activation of β-adrenoceptors can increase baseline submucosal gland secre-

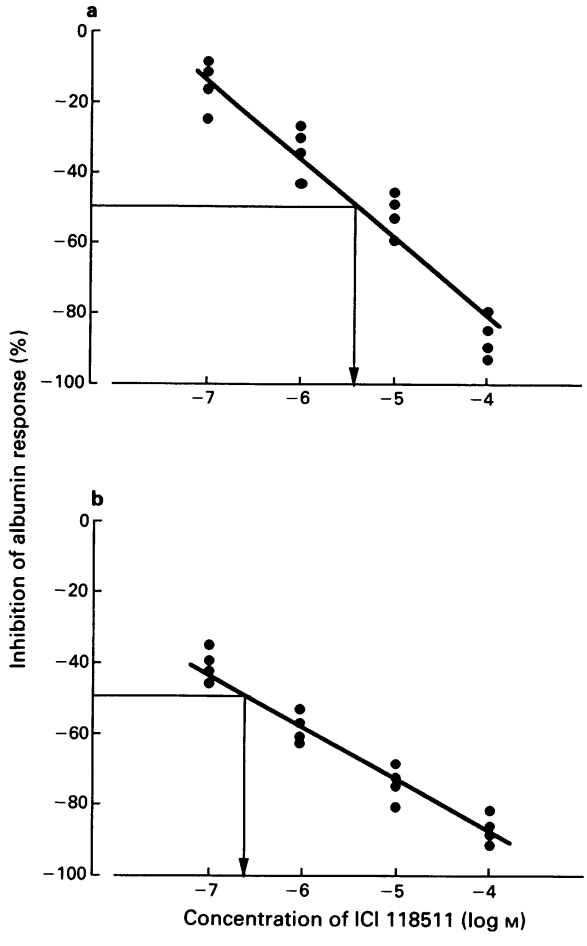


**Figure 4** The effect of ICI 118511 on the enhancement of methacholine-induced albumin output produced by $EC_{100\%}$ concentrations of (a) BRL 37344 (1.4 nM) and (b) salbutamol (0.7 mM). Individual data points are shown. The arrows indicate the concentration ($IC_{50\%}$) of ICI 118551 inhibiting the response to BRL 37344 or salbutamol by 50%.

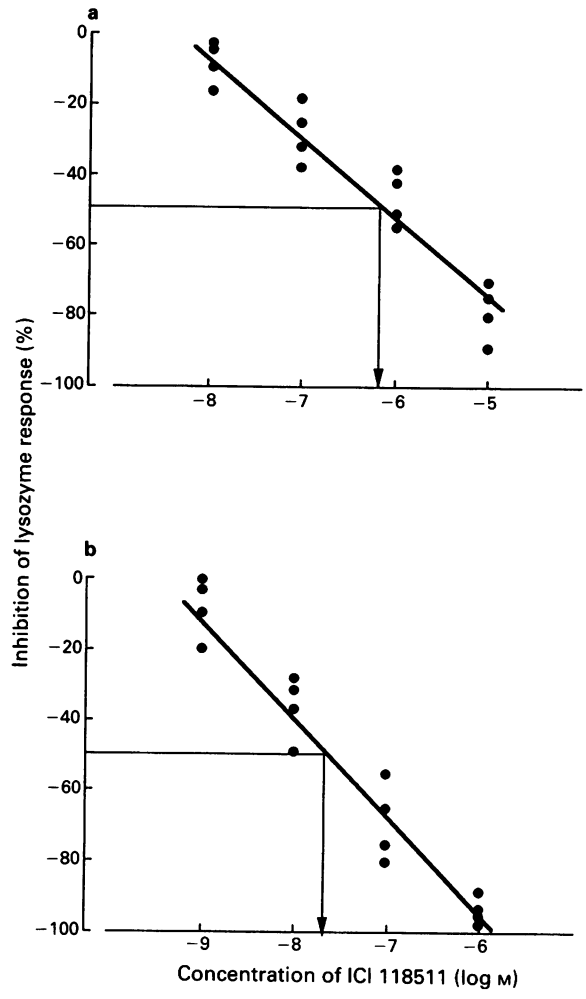


**Figure 5** The effect of ICI 118551 on the inhibition of methacholine-induced lysozyme output produced by (a) BRL 37344 and (b) salbutamol. Individual data points are shown. The arrows indicate the concentration ($IC_{50\%}$) of ICI 118551 inhibiting the response to BRL 37344 or salbutamol by 50%.

tion, while also being capable of inhibiting the secretion resulting from muscarinic stimulation. The most likely explanation is that an intracellular messenger of the β-adrenoceptor response (possibly adenosine 3':5'-cyclic monophosphate, cyclic AMP) antagonizes the intracellular regulation of muscarinic-induced secretion (possibly involving $IP_3$ and release of $Ca^{2+}$ from intracellular stores). In other tissues, such as airway smooth muscle, it is known that β-adrenoceptor-induced release of cyclic AMP can inhibit phosphoinositide hydrolysis, and thus the release of intracellular $Ca^{2+}$ by $IP_3$ (Offer *et al.*, 1990).

Salbutamol was 50 times more potent than BRL 37344 and 10,000 times more potent than prenalterol at inhibiting methacholine-induced lysozyme output. The high potency of salbutamol in the present study strongly suggests that this agonist is inhibiting submucosal gland serous cell secretion by activation of $\beta_2$-receptors. The selective $\beta_2$-adrenoceptor antagonist, ICI 118551, produced concentration-dependent inhibitions of the lysozyme response to BRL 37344 and salbutamol. However, ICI 118551 was 30 times more potent at inhibiting the lysozyme response to salbutamol. A 50% inhibition of the salbutamol response was achieved at a concentration of 25 nM providing further evidence that salbutamol inhibits muscarinic-induced lysozyme output by activation of $\beta_2$-receptors. The low potency of BRL 37344 and the extremely low potency of prenalterol on lysozyme secretion was probably due to residual non-selective activity on $\beta_2$-receptors, although effects on $\beta_3$- and $\beta_1$-adrenoceptors, respectively, cannot be completely ruled out.

All three selective β-agonists produced concentration-dependent increases in albumin output above that sustained by methacholine, suggesting enhancement of muscarinic-receptor-stimulated active transport of albumin across the tracheal epithelium into the lumen. Salbutamol alone (i.e. without methacholine) is also known to stimulate the baseline transport of albumin across the ferret and rabbit tracheal epithelium (Webber & Widdicombe, 1989; Price *et al.*, 1990), but this may be because it also increases mucus production, whereas BRL 37344 and prenalterol alone have no effect on mucus volume. Thus, it is not possible to determine whether the latter two agonists stimulate albumin production by themselves, since it is not possible to collect sufficient mucus for albumin analysis. However, BRL 37344 was approximately 10,000 times more potent than salbutamol, and 100,000 times more potent than prenalterol at enhancing methacholine-induced albumin output.

It is now generally accepted that activation of BAT by BRL 37344 and other thermogenic β-agonists occurs via the atypical, or $\beta_3$-adrenoceptor (Zaagsma & Hollenga, 1991), where BRL 37344 is 3 orders of magnitude more potent than salbutamol (Arch *et al.*, 1984). The fact that BRL 37344 was 4 orders of magnitude more potent than salbutamol in activating albumin transport strongly suggests the existence of the same, or simiar $\beta_3$-adrenoceptors in tracheal epithelium. Further support for the presence of an atypical adrenoceptor comes from the observation that higher concentrations of ICI

118551 were required to inhibit the effects of BRL 37344 on albumin transport than those required to inhibit the salbutamol responses.

It was not feasible (practically or ethically) to determine $pA_2$ values for ICI 118551 in this preparation by Schild analysis, and the $pA_2$ values determined by MacKay's method have to be considered approximate. However, the difference obtained when using the two agonists (over 1.5 log units) lends further support to the notion that BRL 37344 stimulates albumin secretion via a different adrenoceptor from that stimulated by salbutamol. The combination of BRL 37344 plus ICI 118551 gave a $pA_2$ (5.55) that was almost identical (5.4) to that determined for brown adipocyte thermogenesis (Stock & Sudera, 1989). These values have to be compared with a $pA_2$ of 8.7 obtained for tracheal relaxation with the same combination of agonist and antagonist (Arch *et al.*, 1984), which suggests that the adrenoceptor subtype involved in albumin secretion is more akin to the adipocyte $\beta_3$-adrenoceptor, than the tracheal smooth muscle $\beta_2$-adrenoceptor. The observation that 17 fold higher concentrations of ICI 118551 were required to produce 50% inhibition of the albumin response than the lysozyme reponse to salbutamol might suggest that activation of albumin transport by salbutamol may also involve an atypical receptor. The somewhat low $pA_2$ value (7.18) for ICI 118551 inhibition of the effects of salbutamol on albumin would be consistent with this.

Previous work (Webber & Widdicombe, 1989; Price *et al.*, 1990) has established that the appearance of albumin in the tracheal lumen is not due to passive transudation, but involves active transport across the epithelial cell layer. This is supported in the present study by the fact that the $EC_{100\%}$ concentrations of BRL 37344 and salbutamol both increased the concentration of albumin in the secreted mucus to a value up to twice that in the submucosal buffer. These results suggest strongly the presence of an active transport process. Mucus albumin probably has several protective functions (antioxidant, binding inflammatory mediators), apart from contributing to the general gel-forming and rheological properties of mucus (Webber & Widdicombe, 1989). Likewise, the serous cell secretion of lysozyme contributes to its bactericidal properties. It would appear that cholinergic stimulation of these two protective functions are independently modulated by adrenergic influences, with $\beta_2$-adrenoceptor activation causing an inhibition of lysozyme secretion and $\beta_3$-adrenoceptor activation causing a potentiation of albumin output. The therapeutic use of $\beta_2$-agonists could therefore, be considered potentially detrimental in terms of lysozyme secretion, whereas stimulation of albumin transport by a selective $\beta_3$-agonist, having little or no $\beta_2$-activity, might be of value in the prevention and/or treatment of inflammatory airway disease.

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# Neuroendocrine response to clonidine and 8-OH-DPAT in rats following chronic administration of desipramine or sertraline

[1]J.M. O'Donnell & M. Grealy

Department of Pharmacology, University College, Galway, Ireland

**1** Rats were administered either desipramine (DMI) or sertraline daily at doses 7.5 mg kg$^{-1}$ or 10 mg kg$^{-1}$, i.p., respectively and the effects on the functional state of hypothalamic neuroendocrine control mechanisms assessed by measurements of plasma hormones following acute drug challenge. The effects of treatment on gross behaviour and brain adrenoceptor density were also determined.

**2** Both DMI and sertraline caused significant reduction in activity measured as ambulation and rearing at 14 days of treatment.

**3** All animals were chronically cannulated after 14 days of treatment and tested for neuroendocrine response to acute i.v. clonidine (50 μg kg$^{-1}$) or 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, 250 μg kg$^{-1}$) after 21 or more days of treatment.

**4** Rats treated with DMI but not sertraline showed a virtually complete suppression of the growth hormone (GH) secretion elicited by clonidine in controls, while the secretion of corticosterone was augmented.

**5** Treatment with DMI but not sertraline led to a significantly greater 8-OH-DPAT-induced secretion of prolactin than in the control rats, while the plasma concentrations of corticosterone following 8-OH-DPAT were not influenced by either DMI or sertraline treatment.

**6** The density (but not the affinity) of cerebral cortical binding of [$^3$H]-dihydroalprenolol was significantly reduced by DMI treatment.

**7** These results show that DMI treatment blunted the sensitivity of post-synaptic $\alpha_2$-adrenoceptors, accompanied by complex interactions manifested as increased responsiveness of $\alpha_1$-adrenoceptors and 5-HT$_{1A}$ receptors. Sertraline had no significant neurendocrine effects at a dose which significantly reduced gross activity.

**Keywords:** Clonidine; growth hormone; 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT); prolactin; desipramine; sertraline

## Introduction

Administration of the α-adrenoceptor agonist, clonidine, to rats elicits a characteristic pulse of secretion of growth hormone (GH), a pituitary response which has been firmly identified as a primary neural effect through an activation of hypothalamic $\alpha_2$-adrenoceptors (Krulich *et al.*, 1982). A similar response to clonidine has been found in man and employed as a sensitive, non-invasive probe of central adrenoceptor sensitivity in endogenous depression (Checkley *et al.*, 1984). Chronic administration of antidepressant drugs has been reported to blunt the sensitivity of the hypothalamic-pituitary response to clonidine in both animals (Eriksson *et al.*, 1982) and man (Checkley *et al.*, 1986). As a variety of antidepressant drugs of differing pharmacological type have been found to have blunting effects on GH responses to clonidine it was desirable to examine the effects of two drugs, of known different primary action, on the GH response to clonidine and related neuroendocrine phenomena.

In addition to a clonidine challenge, rats may be tested by means of 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT); this agonist elicits secretion of prolactin through a presumed activation of 5-hydroxytrytpamine (5-HT) receptors (Aulakh *et al.*, 1988a). The concentration of corticosterone in plasma may be used as a further test in combination with either clonidine or 8-OH-DPAT, in order to provide a measure of generalised stress or arousal in parallel with the activation of more specific mechanisms.

The antidepressant drugs selected for investigation were desipramine (DMI) which primarily acts to inhibit neuronal reuptake of noradrenaline (Tang & Seeman, 1980) and sertraline, which is relatively selective in acting as an inhibitor of neuronal 5-HT reuptake (Koe *et al.*, 1983). The drugs were administered for 21 or more days before neuroendocrine challenges were performed. The animals were cannulated after 14 days of drug treatment, which permitted the subsequent challenges to be made without anaesthetic and with the minimum disturbance to the animals.

In order to establish the effectiveness of the drug regimens, simple behavioural tests were applied after the first drug administration and again after 14 days dosing; the second assessment had to be made before cannulation, since cannulation involved a change of housing conditions. At the conclusion of the neuroendocrine tests the animals were killed and the brains removed for assay of the binding properties of cerebral cortical membranes of [$^3$H]-dihydroalprenolol ([$^3$H]-DHA) as an additional monitor of antidepressant drug action.

## Methods

### *Chronic drug treatment*

Experiments were performed on male Wistar rats of weight range 250–280 g at the start of drug treatment. Animals were randomly assigned to vehicle-control or drug groups and housed 4 per cage with food and water *ad libitum*. The control group were administered 0.9% NaCl and the drug groups either DMI 7.5 mg kg$^{-1}$ or sertraline 10 mg kg$^{-1}$ by i.p. injection once daily.

[1] Author for correspondence.

*Cannulation*

After 14 days of drug administration the animals were implanted with permanent cannulae in the right external jugular, which allowed for subsequent drug challenges to be given intravenously, and for serial blood sampling in conscious and unrestrained animals. The cannulation procedure was as previously described (Grealy & O'Donnell, 1991). Once cannulated the animals were housed one per cage in a controlled light-dark environment with lights on at 08 h 00 min and off at 20 h 00 min.

*Drug challenges*

On day 21 of treatment the rats were challenged with clonidine or 8-OH-DPAT, and 3 days later with the alternate drug. At 09 h 30 min the cannula was fitted with a length of flexible tubing sufficient to extend out of the cage. Two pre-challenge blood samples were drawn via the tubing at 10 h 35 min and 10 h 50 min and at 11 h 00 min the challenging drug was injected. The dose of clonidine was 50 $\mu g\,kg^{-1}$ and of 8-OH-DPAT 250 $\mu g\,kg^{-1}$, as these doses had been found in preliminary investigations to be capable of eliciting reliable neuroendocrine secretory responses. Blood samples were taken at intervals 5, 20, 35, 50 and 65 min post-challenging; sample volume was 0.4 ml, drawn into syringes previously rinsed with heparin (1000 u $ml^{-1}$), then transferred to polypropylene centrifuge tubes and plasma separated. Cells were resuspended in sterile saline and returned to the donor animal following the next sampling. All plasma samples were stored frozen (−20°C) prior to assay.

*Hormone assays*

Plasma concentrations of GH and PRL were measured by radioimmunoassay (RIA) using assay materials supplied by Dr A.F. Parlow and the NIDDK, Bethesda, Maryland, U.S.A. Purified rat GH and PRL were iodinated with $^{125}I$ (Amersham International), and RIA performed by Protein A (Sigma Chemical Co.) to separate free from antibody-bound hormones. Plasma corticosterone was assayed by a fluorometric method adapted from the micro-method of Glick *et al.* (1964) as described previously (Grealy & O'Donnell, 1991).

*Ligand-binding*

The determination of the binding characteristics of crude membrane preparations for [$^3$H]-DHA (Amersham International) was carried out as described by Bylund & Synder (1976), with minor modifications.

*Behavioural studies*

The effects of drug treatment on locomotor activity were assessed by use of the open-field apparatus, as described by Gray & Lalljee (1974). A white circular base was divided into a grid of 10 × 10 cm squares and surrounded by a 75 cm wall. The diameter was 90 cm. The field was illuminated by a 60 W bulb suspended 45 cm above centre and measurements were performed in a darkened quiet room between 08 h 00 min and 12 h 00 min. Individual animals were observed over a 4 min period. Ambulation was scored as the number of squares crossed, and rearing by the simultaneous raising of both forepaws.

*Drugs*

Desipramine hydrochloride and clonidine hydrochloride were purchased from Sigma Chemical Co.; 8-OH-DPAT was purchased from SEMAT Technical Ltd., St. Albans, Herts., and sertraline was a gift from Pfizer Corp., U.S.A.

*Analysis of data*

Concentrations of GH and PRL in plasma were calculated as ng $ml^{-1}$; these values were found not to follow a statistically normal distribution and therefore group values were treated as medians with interquartile range, and non-parametric tests were applied in deciding the statistical significance of any observed differences (Clarke & Cooke, 1983). In order to decide whether a significant secretory response had occurred following a drug challenge, post-challenge hormone concentrations at each time interval were compared with the pre-challenge values (−10 min) using Wilcoxon's matched pairs signed-rank test, with each animal acting as its own control. For comparison of the magnitude of secretory response between groups of animals which had received chronic antidepressant drug treatment, the pre-challenge hormone concentration was subtracted from each post-challenge value in turn, and the peak change (ΔGH or ΔPRL) obtained for each animal. Group median values were then calculated and the significance of any between-group differences was determined by the Mann-Whitney U-test; group data from behavioural tests were also expressed as medians and the same test of statistical significance applied.

Plasma corticosterone concentrations showed normal distribution and these were expressed as mean $\mu g\,dl^{-1}$. Any significance of a change in plasma corticosterone following drug challenge was assessed by comparison of post with pre-challenge values using Student's paired $t$ test (two-tailed). Group means for corticosterone and ligand-binding data comparison between drug treatments were assessed by Student's unpaired $t$ test.

## Results

*Behavioural effects of antidepressants*

The results of behavioural assessments at the first drug administration and then after 14 days of treatment are illustrated in Figure 1. Following the first drug dose, there was a significant reduction in rearing by both DMI and sertraline-treated rats. After 14 days both drugs were still causing significant reductions in rearing compared to controls; these effects were seen in the drug-treated groups even though the rearing score in the controls was itself significantly lower at 14 days than at the start of the experiment. Activity scored as ambulation (Figure 1b) was significantly reduced following the first administration of DMI, with the median value in the sertraline group not being significantly different to control. At 14 days of treatment, both the drugs were showing significant reductions compared to the saline controls. As in the case of rearing activity, ambulation in all three groups was significantly less on day 14 than at the start of drug treatment.

*Neuroendocrine response to clonidine*

Figure 2 shows the time-course of the GH secretory response to a clonidine challenge. In the control rats there was a characteristic pattern of GH secretory response, manifested as a significant rise in plasma hormone by 20 min, which was sustained until 65 min. In the DMI-treated group there was no significant change in GH at any sampling point. The sertraline-treated group showed increases which were less marked than in controls, but nonetheless still significant.

An alternative criterion of secretory response is furnished by the peak change following challenge. In the DMI-tested rats, peak ΔGH was also less than in the controls, with median value 15 (interquartile range −9 to 49) versus 83 (range 46 to 93); the reduction was significant at $P<0.05$ (Mann-Whitney U test). In the rats treated with sertraline the reduction was less marked (median 29, range 15 to 50), and did not attain statistical significance, in agreement with the

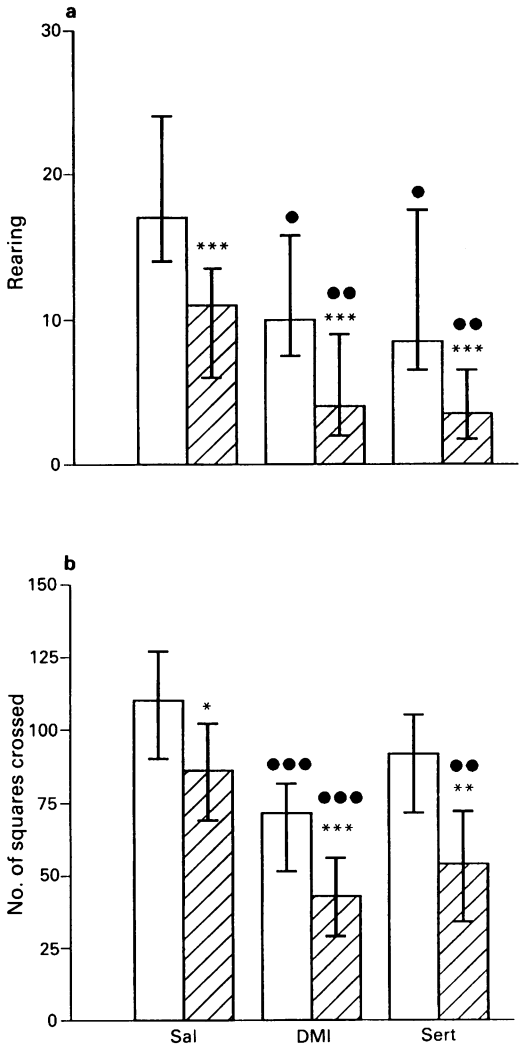


**Figure 1** Effects of desipramine (DMI, 7.5 mg kg$^{-1}$, i.p.) or sertraline (Sert, 10 mg kg$^{-1}$, i.p.) on rearing (a) and ambulation (b) in rats on day 1 (open columns) and day 14 (hatched column) of treatment. Both activities were measured in the open field. Scores are medians with interquartile range. *$P<0.05$; **$P<0.01$; ***$P<0.001$, day 14 vs. day 1. ●$P<0.05$; ●●$P<0.01$; ●●●$P<0.001$, DMI ($n = 14$) or sertraline ($n = 14$) vs. saline ($n = 13$); (Mann-Whitney U test).

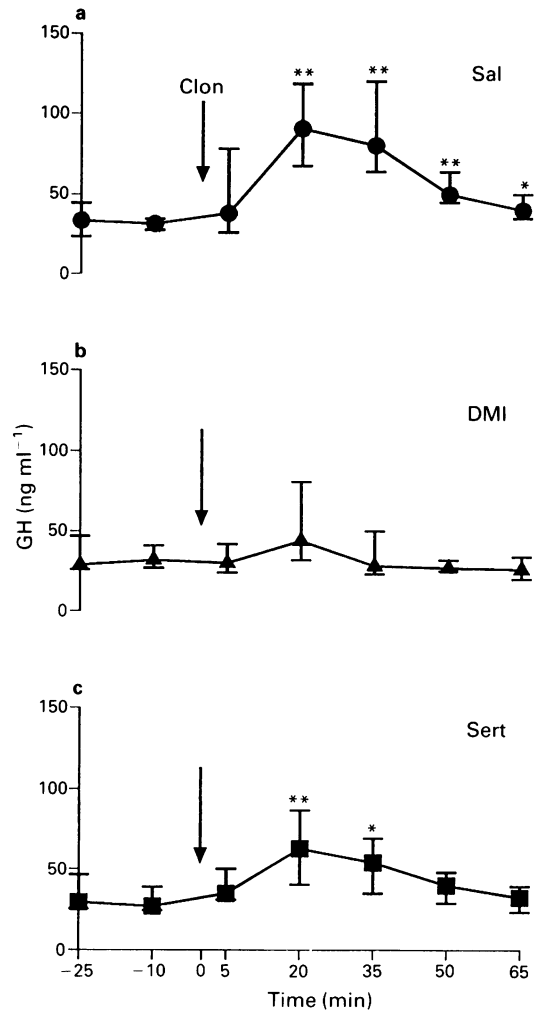


**Figure 2** Plasma growth hormone (GH) concentrations (medians with interquartile range) following clonidine (Clon) challenge (50 μg kg$^{-1}$) in cannulated rats. Animals had been administered (a) saline (Sal, $n = 7$), (b) desipramine (DMI, 7.5 mg kg$^{-1}$ i.p., $n = 7$) or (c) sertraline (Sert, 10 mg kg$^{-1}$ i.p., $n = 8$) for 21 days prior to being challenged. Significant increases over pre-challenge values are shown thus *$P<0.05$; **$P<0.01$ (Wilcoxon's signed-rank test).

trend shown by the overall time-course of secretion.

Plasma prolactin was not significantly altered by clonidine challenge, except that in the sertraline group, and only in that group, prolactin levels fell by a small yet significant amount at 50 and 65 min. The response of plasma corticosterone to clonidine is shown in Figure 3. The control rats had a slightly elevated concentration at 20 min, while an essentially similar pattern without any significant change, was shown by the sertraline-treated group. In contrast, the animals which had received DMI showed a rise at 20 min followed by further significantly increased concentrations which were sustained until 65 min after challenge.

### *Responses to 8-OH-DPAT*

A significant increase in plasma prolactin was elicited by 8-OH-DPAT in control animals (Figure 4); while of lesser magnitude than the GH reponse to clonidine, the prolactin effect was significant as early as 5 min post-injection, and was shorter-lived than the GH response. The sertraline group showed a pattern of prolactin secretion very similar to the controls. The DMI-treated rats, in contrast, showed prolactin markedly elevated at 5 and 20 min, although the effect was again short-lived. The peak changes in secretion of prolactin showed median 7.5 (5 to 9) in controls. Comparison of the drug-treated animals with controls shows that the DMI group had a significantly increased response to 8-OH-DPAT, with the median value 19 (12 to 34), $P<0.05$. Sertraline-group values were 14 (4 to 21).

Plasma GH measured after 8-OH-DPAT showed very small fluctuations from pre-challenge values with slight decreases at 20 min, significant in the case of controls and the DMI group. Corticosterone levels in plasma were significantly increased by 8-OH-DPAT in all three groups (Figure 5). In the saline controls the response was evident at 5 min and concentrations remained significantly elevated for the whole of the sampling period. Substantially the same pattern of response was found in the drug-treated groups, although the sertraline group showed only non-significant increases at 5 min and corticosterone was no longer significantly raised in either group at 65 min.

### *Cerebral cortical membrane [$^3$H]-dihydroalprenolol binding*

The results of determination of the ligand-binding characteristics of crude membranes prepared from cerebral cortex

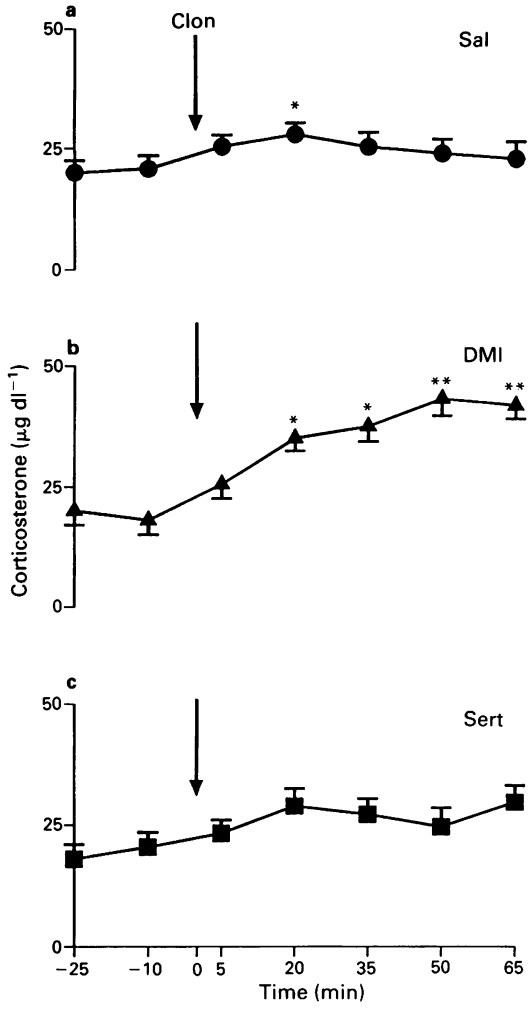


**Figure 3** Plasma corticosterone concentrations (means with s.e.mean shown by vertical bars) in rats challenged with clonidine (Clon, 50 μg kg$^{-1}$), following treatment with desipramine (DMI) or sertraline (Sert) as in Figure 2. Significant increases over pre-challenge values are shown thus: *$P<0.05$; **$P<0.01$ (Student's paired $t$ test, two-tailed).

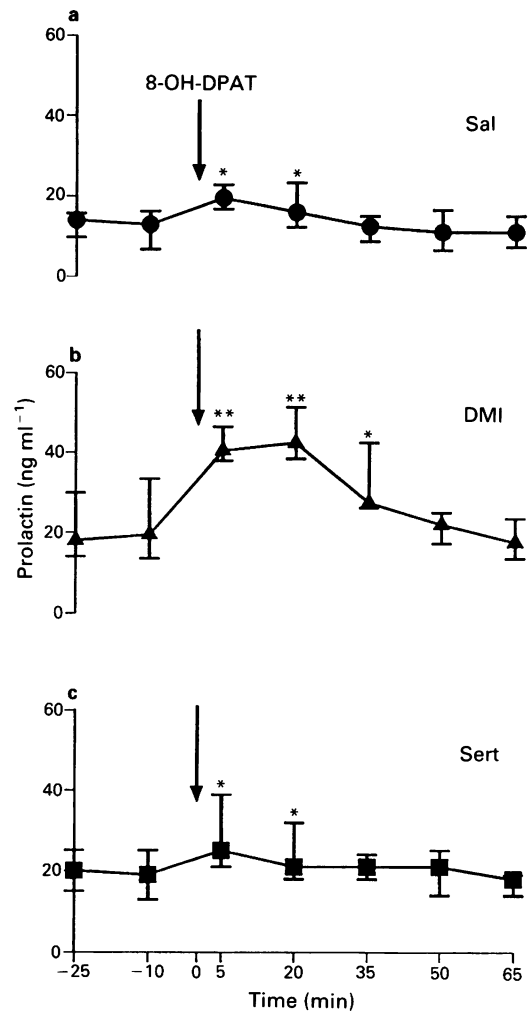


**Figure 4** Plasma prolactin concentrations (medians with interquartile ranges) following 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) challenge (250 μg kg$^{-1}$), in rats treated as in Figure 2. Significant increases over pre-challenge values are shown thus: *$P<0.05$; **$P<0.01$ (Wilcoxon's signed-rank test).

of these animals are given in Table 1. The density of binding sites for [$^3$H]-DHA was significantly less in membranes prepared from the DMI group than in control preparations; the mean value for the sertraline group was not significantly different from controls. Neither treatment significantly altered the affinity for the ligand.

## Discussion

The behavioural assessments in this study, necessarily performed at 14 days of treatment since single-housing could alter responses (Einon *et al.*, 1975; Dalrymple-Alford & Benton, 1981; Gentsch *et al.*, 1982), provided independent confirmation of the efficacy of drug treatments. Sedative and anti-anxiety effects are commonly associated with the administration of tricyclics to patients or animals; such actions need not correlate with antidepressant activity (Tucker & File, 1986; File & Tucker, 1986). Clinically effective doses of sertraline do not show sedative effects (Doogan & Caillard, 1988).

Control rats showed the GH response to clonidine that has been well characterized (Durand *et al.*, 1977; Eden & Modigh, 1977; Krulich *et al.*, 1982; Erikkson *et al.*, 1982; Grealy & O'Donnell, 1991), and DMI treatment significantly reduced this response. Literature reports of effects of tricyclic drugs on this response are lacking for rat. Corn *et al.* (1984) found that DMI increased GH response in man at one week, with a return to basal sensitivity at three weeks. In baboons a reduced sensitivity after two days was reversed at three weeks (McWilliam *et al.*, 1983). The sedation due to clonidine was found to be attenuated by chronic desipramine in the rat (Heal, 1990) while imipramine treatment was found to counteract clonidine-induced hypothermia (Pilc & Vetulani, 1982). These effects of clonidine are mediated via central $\alpha_2$-adrenoceptors, and chronic tricyclic administration clearly desensitizes to clonidine, most probably by increasing noradrenaline concentrations at central receptors. The reduced sensitivity of GH response to clonidine following DMI is also most likely to be due to agonist-desensitization following on inhibition of reuptake of neurotransmitter. Sertraline, which has a selective action on reuptake of 5-HT, did not have a significant effect on GH secretion although the effects on behaviour were evident. Differences in the effects of selective noradrenaline and 5-HT reuptake inhibitors on postsynaptic $\alpha_2$-adrenoceptors have been recently described (Heal *et al.*, 1991). Treatment with sertraline led to a small yet consistent decrease in prolactin following clonidine injection. This effect may involve interaction between 5-HT and $\alpha$-adrenoceptors.

The enhanced corticosterone response to clonidine after

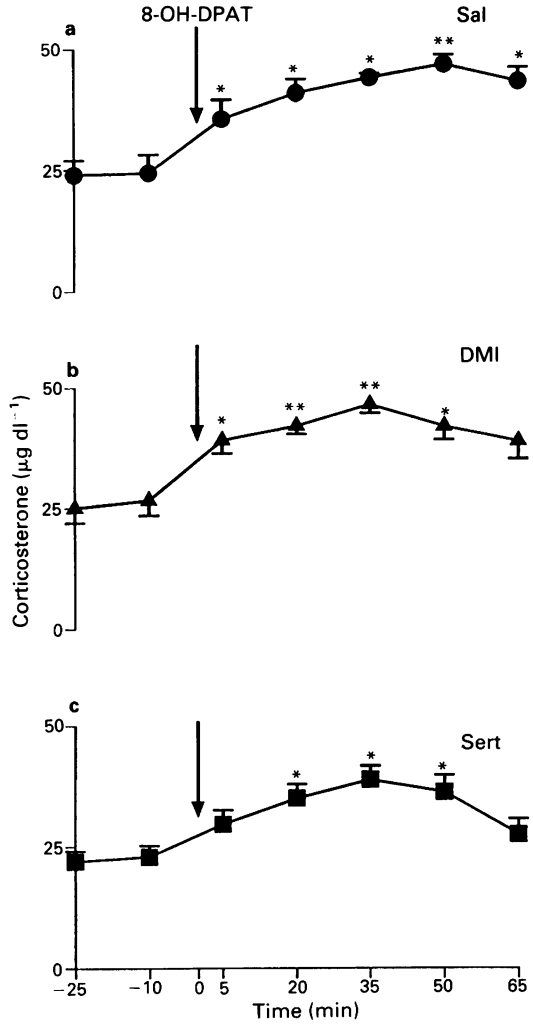


**Figure 5** Plasma corticosterone concentrations (means with s.e.mean shown by vertical bars) in rats challenged with 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, 250 μg kg$^{-1}$) following treatment with (b) desipramine (DMI) or (c) sertraline (Sert) as in Figure 2. Significant increases over pre-challenge values are shown thus: *$P<0.05$; **$P<0.01$ (Student's paired $t$ test, two-tailed).

DMI treatment is difficult to reconcile with the established effects of this antidepressant on α-adrenoceptors. The involvement of adrenoceptors in corticosterone secretion in rats is complex (Saphier & Feldman, 1989). If an α-adrenoceptor is implicated in the enhancement effect it could be through an $\alpha_1$-agonist action. Such an effect could possibly occur following intravenous clonidine at the dose employed, if $\alpha_1$-adrenoceptor sensitivity were increased above normal. While the effects of DMI on $\alpha_1$-receptors in rat brain are controversial (Green, 1990) there are several reports of increased $\alpha_1$-adrenoceptor ligand binding after chronic administration of DMI and other antidepressant drugs (Rehavi *et al.*, 1980; Cohen *et al.*, 1982; Menkes *et al.*, 1983; Vetulani *et al.*, 1984; Maj *et al.*, 1985; Nowak & Przegalinski, 1988). In addition, there is some behavioural evidence for a functional supersensitivity in treated animals (Davis *et al.*, 1981; Plaznik & Kostowski, 1985; Mogilnicka *et al.*, 1987).

**Table 1** Effects of chronic desipramine (DMI) or sertraline (Sert) on binding of [$^3$H]-dihydroalprenolol to crude membranes prepared from cerebral cortex

| | n | $B_{max}$ (fmol mg$^{-1}$) | $K_d$ (nM) |
|---|---|---|---|
| Saline controls | 11 | 164 ± 11 | 7.6 ± 0.6 |
| DMI | 10 | *113 ± 7 | 7.6 ± 0.9 |
| Sert | 7 | 127 ± 13 | 5.7 ± 0.8 |

*Significantly less than control, $P<0.005$ (Student's $t$ test, two-tailed).

Rats treated with DMI also showed an enhanced pulse of prolactin when challenged with 8-OH-DPAT. A similar effect following clomipramine has been reported (Aulakh *et al.*, 1988b). An increased sensitivity of postsynaptic 5-$HT_{1A}$ receptors is a possible explanation for this enhancement, since these receptors are thought to mediate prolactin secretion (Simonovic *et al.*, 1984). The mechanism by which 8-OH-DPAT elicits corticosterone secretion is less clear. Matsuda *et al.* (1990) postulated that higher dose (2 mg kg$^{-1}$) 8-OH-DPAT caused corticosterone secretion via presynaptic 5-$HT_{1A}$ receptors, with lower doses activating a postsynaptic effect. If this is so then a presynaptic mechanism cannot be invoked to explain the different effects of DMI on prolactin and corticosterone responses to 8-OH-DPAT, since a low dose (250 μg kg$^{-1}$) was employed in the present study. As in the case of the principal response to clonidine, sertraline did not alter the response to 8-OH-DPAT, except for the absence of a small fall in GH that was seen in the controls; when secretory function was assessed by peak response no significant effect of sertraline was found.

In conclusion, treatment of rats with DMI led to an apparent desensitization of central adrenoceptors, and to significantly increased secretory response of both prolactin and corticosterone which could imply changes in mechanisms involving adrenoceptors and 5-HT. These neuroendocrine effects of DMI were accompanied by both behavioural effects and a reduction in cerebral cortical binding associated with the β-adrenoceptor antagonist, DHA. The results are consistent with a primary action of increasing the availability of neurotransmitter. Sertraline caused similar effects on behaviour but did not alter neuroendocrine sensitivity to either adrenoceptor or 5-hydroxytryptamine receptor agonists.

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# Effect of α-human atrial natriuretic peptide on the synthesis of dopamine in the rat kidney

[1]P. Soares-da-Silva & [2]M. Helena Fernandes

Department of Pharmacology and Therapeutics, Faculty of Medicine, 4200 Porto, Portugal

**1** The present study has examined the influence of α-human atrial natriuretic peptide (α-hANP) on the synthesis of dopamine and its deamination into 3,4-dihydroxyphenylacetic acid (DOPAC) in rat kidney slices loaded with exogenous L-dihydroxyphenylalanine (L-DOPA).

**2** α-hANP (3.3 and 330 nM) was found to produce a marked reduction (63– 78% reduction) in the time-dependent accumulation of newly-formed dopamine and of its deaminated metabolite DOPAC in kidney slices loaded with 10 μM L-DOPA. α-hANP (330 nM) was also found to decrease the accumulation of newly-formed dopamine (45– 66% reduction) and DOPAC (38– 61% reduction) in experiments in which increasing concentrations (1– 100 μM) of L-DOPA were used. This inhibitory effect was found to be potentiated by zaprinast (M&B 22,948; 10 μM), a guanosine cyclic 3',5'-monophosphate (cyclic GMP) phosphodiesterase inhibitor. Alone, zaprinast also decreased the accumulation of both dopamine (54–71% reduction) and DOPAC (73–92% reduction).

**3** In kidney homogenates, α-hANP (330 nM) was found to affect neither the formation of dopamine nor its deamination to DOPAC.

**4** Both α-hANP (330 nM) and zaprinast (10 μM) were found not to affect the formation of dopamine and DOPAC in kidney slices obtained from rats on a high salt diet during the previous 6 weeks. A similar situation was also found to occur when kidney slices obtained from 24-months old rats were used.

**5** The results obtained suggest that the inhibitory effect of α-hANP on the renal synthesis of dopamine is dependent on the activation of a membrane-operated mechanism, coupled to the enzyme guanylate cyclase, controlling the entry of L-DOPA into the cells.

**Keywords:** α-Human atrial natriuretic peptide; kidney; dopamine; zaprinast; high salt diet; aging

## Introduction

Atrial natriuretic peptides (ANPs) exert profound effects on renal function and play an important role in the regulation of sodium balance and central blood volume. Although a full expression of the natriuretic effects of ANPs is thought to depend predominantly on their renal haemodynamic actions, they may also directly alter tubular sodium reabsorption through the activation of specific receptors (Salazar *et al.*, 1986; Sonneberg *et al.*, 1986). However, several studies performed in the rat have shown that the natriuretic response to ANPs was completely blocked by pretreatment with dopamine receptor antagonists; the most effective compounds were those blocking $D_1$-receptors (for review see Murphy & Bass, 1990). Since some of the renal effects of ANPs, namely those concerning vasodilatation and inhibition of tubular sodium reabsorption are also shared by the catecholamine dopamine, it has been suggested that the renal effects of ANPs might be mediated through enhanced renal dopaminergic activity. Conversely, other authors have demonstrated, in dogs and man, that the natriuretic effect of α-human ANP (α-hANP) did not depend on a permissive role of dopamine (Murphy *et al.*, 1988; Freestone *et al.*, 1989; Lewis *et al.*, 1989).

In the kidney, ANPs activate specific receptors coupled to the particulate form of guanylate cyclase, the activation of which results in an increase in the generation of guanosine 3'-5'-cyclic monophosphate (cyclic GMP) (Kim *et al.*, 1989). By contrast, type $D_1$-receptors in renal vascular smooth muscle cells and tubular epithelial cells are coupled to the enzyme adenylate cyclase the activation of which is accompanied by an increase in the formation of adenosine 3',5'-cyclic monophosphate (cyclic AMP) (for review see Felder *et al.*, 1989). Since natriuresis induced by the infusion of α-hANP closely parallels the increase in the urinary excretion of cyclic GMP (Lewis *et al.*, 1988), one possible explanation for the permissive role of dopamine on the natriuretic effects of ANPs, as has been found to occur in the rat, might be related to the possibility that peptide-induced natriuresis, may be dependent on the mobilization of dopamine of renal origin. In fact, renal tissues are endowed with an enormous capacity to synthesize dopamine; tubular epithelial cells, namely those of proximal convoluted tubules, are endowed with a high aromatic L-amino acid decarboxylase (AAAD) activity and filtered dihydroxyphenylalanine (DOPA) is converted to dopamine after being taken up into this cellular compartment (Baines & Chan, 1980). Considering that endogenous intrarenal dopamine may act physiologically in the control of the renal excretion of sodium, as a result of activation of $D_1$-receptors located in tubular epithelial cells (Siragy *et al.*, 1989), the present study was undertaken to examine the effects of α-hANP on the synthesis of dopamine in rat renal tissues loaded with exogenous L-DOPA. A preliminary account of these findings was reported initially at the 3rd International Conference on Peripheral Dopamine (Soares-da-Silva & Fernandes, 1990a).

## Methods

Male Wistar rats (Biotério do Instituto Gulbenkian de Ciência, Oeiras, Portugal) aged 45–60 days and weighing 200–280 g were used in the experiments. Animals were kept two per cage under controlled environmental conditions (12 h light/dark cycle and room temperature, 24°C). Food and tap

[1] Author for correspondence.
[2] Permanent address: Faculty of Medical Dentistry, 4200 Porto, Portugal.

water were allowed *ad libitum*. The experiments were all carried out during day time.

The rats were killed by decapitation under ether anaesthesia and both kidneys were removed and rinsed free from blood with saline (0.9% NaCl). The kidneys were placed on an ice cold glass plate, the kidney poles removed and renal slices approximately 1.5 mm thick, containing both the cortex and the medulla, and weighing about 90 mg wet weight were prepared with a scalpel. Thereafter, renal slices were pre-incubated during 60 min in warm (37°C) and gassed (95% $O_2$ and 5% $CO_2$) 10 ml Krebs solution. The Krebs solution had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 25, $KH_2PO_4$ 1.2, EDTA 0.4, ascorbic acid 0.57 and glucose 11; (−)-α-methyl-*p*-tyrosine (50 μM) and copper sulphate (10 μM) were also added to the Krebs solution to inhibit the enzyme tyrosine hydroxylase and the endogenous inhibitors of dopamine β-hydroxylase, respectively. After preincubation, renal slices were incubated for 5, 10, 20 or 30 min in gassed and warm Krebs solution with added L-DOPA (10 μM). In another set of experiments, kidney slices were incubated for 15 min with increasing concentrations of L-DOPA (1–100 μM) added to the medium. In experiments in which the effects of α-hANP (3.3 and 330 nM) and zaprinast (M&B 22,948; 10 μM), a relatively specific guanosine cyclic GMP phosphodiesterase inhibitor, were tested, the compounds were present during the preincubation and incubation periods. The preincubation and incubation were carried out in a shaking water bath at 37°C, in an atmosphere of 95% $O_2$ and 5% $CO_2$; renal slices were incubated individually in glass vials containing 10 ml Krebs solution. After the incubation, renal slices were collected, washed for 30 min in ice cold Krebs solution, blotted with filter paper, minced with fine scissors and placed in 2 ml of 0.2 M perchloric acid at 4°C for the next 24 h, before quantification of tissue catecholamines.

Some experiments with kidney slices were performed with tissues taken from animals on a high salt diet (1% sodium chloride in the drinking fluid) during the previous 6 weeks. The daily sodium intake of rats on the high salt and normal salt diet averaged, respectively, 0.5 and 5 mmol 100 $g^{-1}$ body weight. In another series of experiments, kidney slices were obtained from 24-months old rats (considered to be old).

In another set of experiments, kidney homogenates, instead of tissue slices, were used. Whole kidneys were homogenized in a modified Krebs solution with Duall-Kontes homogenizers and kept continuously on ice. The modified Krebs solution consisted of a medium similar to that described above with the exception that NaCl was reduced to 50 mM; the osmolarity of the medium was kept constant by the addition of 68 mM choline chloride. Aliquots of 1.0 ml of kidney homogenates plus 1.5 ml Krebs solution were placed in glass test tubes incubated for 60 min; thereafter, L-DOPA (0.1–10.0 μM) was added to the medium for a further 15 min. In experiments in which the effect of α-hANP (330 nM) was tested, the peptide was present during the preincubation and incubation periods. During preincubation and incubation, kidney homogenates were continuously shaken and gassed (95% $O_2$ and 5% $CO_2$) at a constant temperature of 37°C. The reaction was stopped by adding 250 μl of 2 M perchloric acid and the preparations placed at 4°C for 60 min; thereafter, kidney homogenates were centrifuged (2000 r.p.m., 2 min, 4°C) and aliquots of 1.5 ml of the supernatant used for the assay of L-DOPA, dopamine and DOPAC.

The assay of L-DOPA, dopamine, noradrenaline and DOPAC in renal tissues and kidney homogenates was performed by means of high performance liquid chromatography with electrochemical detection, as previously described (Soares-da-Silva & Fernandes, 1991a). In brief, aliquots of 1.5 ml perchloric acid in which the tissues had been kept or 1.5 ml of supernatant of kidney homogenates were placed in 5 ml conical-based glass vials with 50 mg alumina and the pH of the samples immediately adjusted to pH 8.6 by the addition of Tris buffer. The adsorbed catecholamines were then eluted from the alumina with 200 μl of 0.2 M perchloric acid on Millipore microfilters (MF1); 50 μl of the eluate was injected into a high performance liquid chromatograph (Gilson Medical Electronics, Villiers le Bel, France). The lower limits for detection of L-DOPA, dopamine, noradrenaline and DOPAC were 1.0, 1.4, 0.9 and 2.5 pmol $g^{-1}$, respectively.

The protein content of the homogenates (mg of protein per g of tissue) was determined by the method of Lowry *et al.* (1951), with human serum albumin as a standard.

Mean values ± s.e.mean of $n$ experiments are given. Significance of differences between two means was estimated by Student's $t$ test for unpaired data. Significance of difference between one control and several experimental groups was evaluated by the Tuckey-Kramer method (Sokal & Rohlf, 1981). A $P$ value less than 0.05 was assumed to denote a significant difference.

3,4-Dihydroxyphenylacetic acid (DOPAC), L-dihydroxyphenylalanine (L-DOPA), dopamine hydrochloride, α-human atrial natriuretic peptide, (−)-α-methyl-*p*-tyrosine and noradrenaline bitartrate were purchased from Sigma Chemical Comany (St. Louis, Mo, U.S.A.) and zaprinast (M&B 22,948; 2-*o*-propoxyphenyl-8-azapurin-6-one) was kindly donated by the manufacturer (May & Backer, Ltd, Dagenham, Essex).

## Results

Incubation of renal tissues with 10 μM L-DOPA for 5, 10, 20 and 30 min or with increasing concentrations of L-DOPA for 15 min resulted, respectively, in a time- (Figure 1) and concentration-dependent (Figure 2) accumulation of newly-formed dopamine; the amount of the amine accumulated in the tissue reached values as high as 11 nmol $g^{-1}$ when 100 μM L-DOPA was added to the medium (Figure 2). The accumulation of DOPAC, the deaminated metabolite of dopamine, in kidney slices was also dependent on the incubation time (Figure 1) and on the concentration of L-DOPA used (Figure 2) reaching values as high as 32 nmol $g^{-1}$ when 100 μM L-DOPA was used (Figure 2). In kidney slices incubated in the absence of exogenous L-DOPA, the dopamine and noradrenaline tissue concentrations were 0.018 ± 0.004 and 0.709 ± 0.041 nmol $g^{-1}$ ($n$ = 8), respectively; DOPAC was not detectable under these experimental conditions. The tissue levels of noradrenaline in kidney slices did not significantly change in the course of these experiments even when 100 μM L-DOPA was used (data not shown, but see Fernandes & Soares-da-Silva, 1990). Incubation of kidney homogenates with increasing concentrations of L-DOPA (0.1–10.0 μM) for 15 min resulted in a concentration-dependent accumulation of newly-formed dopamine (Figure 3); the formation of DOPAC was dependent on the concentration of L-DOPA added to the medium (Figure 3).

The addition of α-hANP (330 nM) to the incubation medium resulted in a marked decrease (63–78% reduction) in the time-dependent accumulation of dopamine and DOPAC in kidney slices incubated with 10 μM L-DOPA (Figure 1). The inhibitory effect of the peptide on the accumulation of newly-formed dopamine and DOPAC was also observed when 3.3 nM α-hANP was used; this only achieved statistical significance at 20 and 30 min of incubation (Figure 1). As shown in Figure 2, α-hANP (330 nM) was also found to decrease the accumulation of newly-formed dopamine in experiments in which increasing concentrations of L-DOPA were added to the medium during the 15 min incubation period. Under these experimental conditions, the inhibitory effect of α-hANP (45–66% reduction) could be observed at all concentrations of L-DOPA, even at 100 μM L-DOPA (Figure 2). The inhibitory effect of α-hANP on the accumulation of newly-formed dopamine was also accompanied by a reduction in the formation of DOPAC; this effect was similar (38–61% reduction) to that observed for

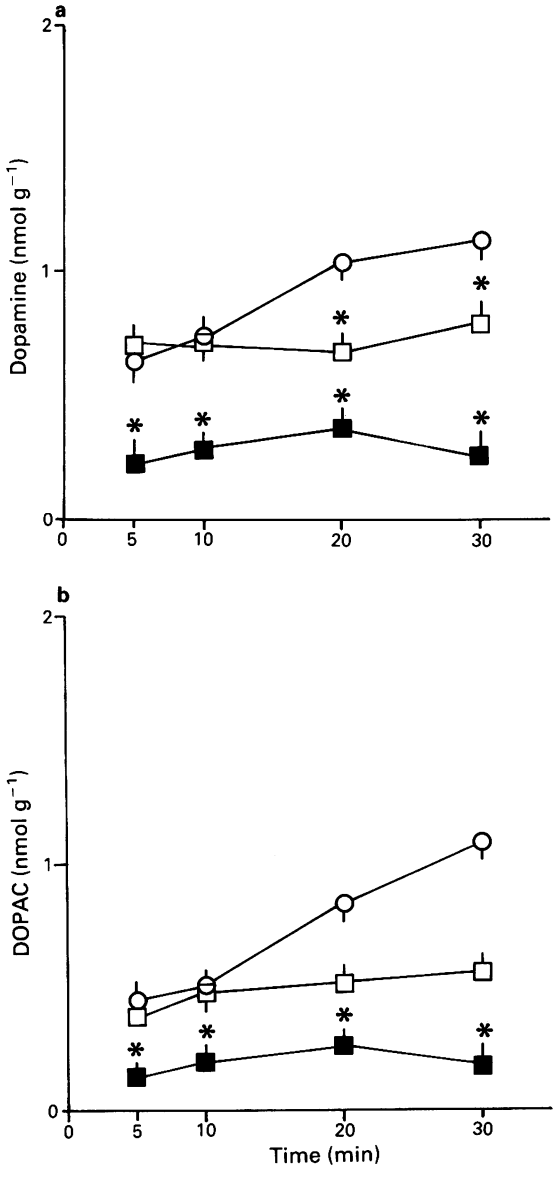


**Figure 1** Effect of α-human atrial natriuretic peptide (α-hANP) on the time-dependent accumulation of (**a**) dopamine and (**b**) 3,4-dihydroxyphenylacetic acid (DOPAC) (in nmol $g^{-1}$) in rat kidney slices incubated with exogenous L-DOPA (10 μM) for 5, 10, 20 and 30 min. Each point represents the mean of five experiments per group; vertical lines show s.e.mean. Significantly different from corresponding values of control using the Tuckey Kramer method (*$P < 0.01$). Control, (○); 3.3 nM α-hANP, (□); 330 nM α-hANP, (■).

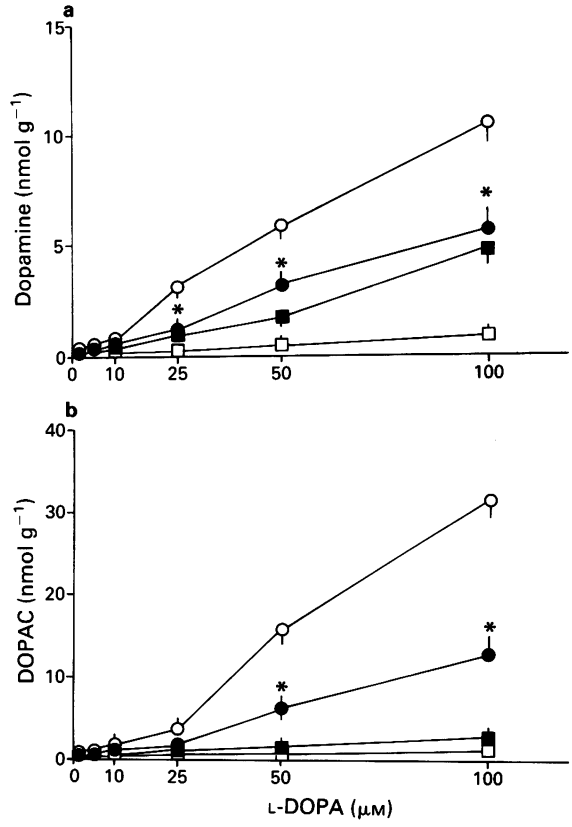


**Figure 2** Effect of α-human atrial natriuretic peptide (α-hANP; 330 nM), zaprinast (10 μM) and α-hANP plus zaprinast on the accumulation of (**a**) dopamine and (**b**) 3,4-dihydroxyphenylacetic acid (DOPAC) (in nmol $g^{-1}$) in rat kidney slices incubated with increasing concentrations of exogenous L-DOPA (1–100 μM) for 15 min. Each point represents the mean of five to six experiments per group; vertical lines show s.e.mean. Significantly different from corresponding values of control using the Tuckey Kramer method (*$P < 0.01$). Control, (○); α-hANP, (●); zaprinast, (■); α-hANP plus zaprinast (□).

dopamine (Figure 2). In the kidney, ANPs stimulate specific receptors coupled to the enzyme guanylate cyclase, the activation of which results in an increased accumulation of cyclic GMP (Kim *et al.*, 1989). The addition of zaprinast (10 μM), a cyclic GMP phosphodiesterase inhibitor, to the incubation medium was found to potentiate the inhibitory effects of α-hANP on the accumulation of dopamine and DOPAC in kidney slices incubated with L-DOPA (1–100 μM) (Figure 2). When added alone to the incubation medium, zaprinast, which has also been reported to inhibit at least one isoform of $Ca^{2+}$/calmodulin-dependent phosphodiesterase (Torphy & Cieslinski, 1991), also decreased the accumulation of dopamine (54–71% reduction) and to a greater extent (73–92% reduction) its deamination into DOPAC; in fact, zaprinast alone nearly abolished the formation of DOPAC (Figure 2). By contrast to results obtained in experiments performed in kidney slices, α-hANP (330 nM) was devoid of any inhibitory effect on the formation of dopamine in rat kidney homogenates (Figure 3a). The deamination into DOPAC of the newly-formed dopamine in kidney homogenates was also found not to be reduced by α-hANP (Figure 3).

Figure 4 shows the results of experiments in which the effects of α-hANP (330 nM) and zaprinast (10 μM) were examined on the accumulation of dopamine and DOPAC in kidney slices obtained from rats given a high salt diet during the previous 6 weeks. α-hANP (330 nM) was found to affect neither the formation of dopamine nor its deamination into DOPAC in kidney slices loaded with increasing concentrations of L-DOPA (10–100 μM). Similarly, zaprinast (10 μM) was also found not to reduce significantly the accumulation of newly-formed dopamine and of its deaminated metabolite, DOPAC (Figure 4). A similar situation was also found to occur with kidney slices obtained from 24-month old rats. In this set of experiments, as shown in Figure 5, α-hANP and zaprinast were found to affect neither the decarboxylation of L-DOPA into dopamine nor the accumulation of DOPAC in kidney slices loaded with L-DOPA (Figure 5); the addition of α-hANP plus zaprinast was found to reduce slightly the accumulation of newly-formed dopamine and of DOPAC. The total amount of dopamine formed, as indicated by the algebraic sum of dopamine and DOPAC tissue levels, at 25, 50 and 100 μM L-DOPA was significantly less in kidney slices from 24-months old rats and in rats on high salt diet, than in kidney slices obtained from 2-month old rats or animals on normal salt diet (see Table 1).

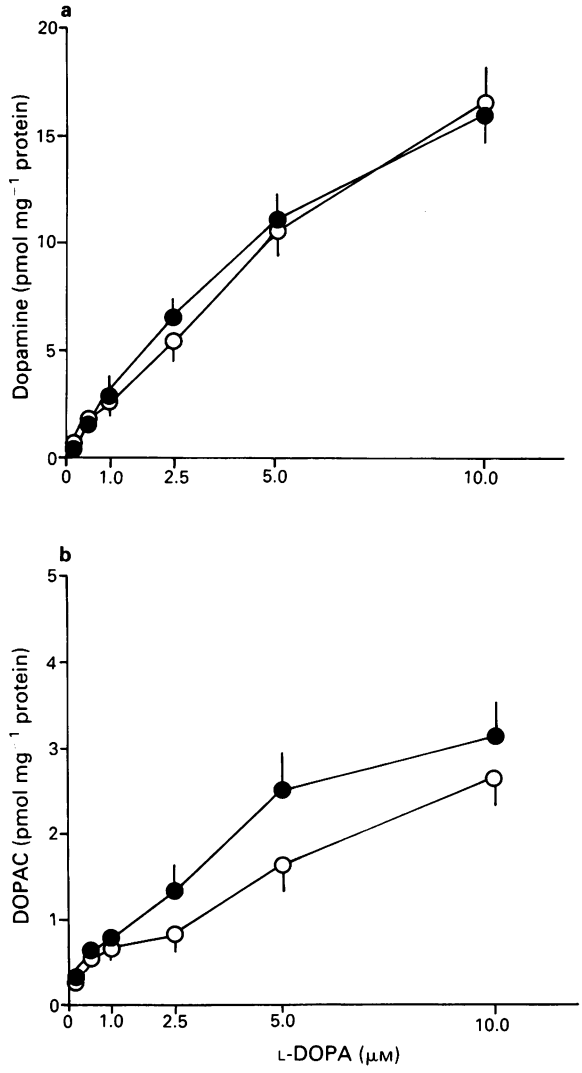


**Figure 3** Levels (in pmol $mg^{-1}$ protein) of (**a**) dopamine and (**b**) 3,4-dihydroxyphenylacetic acid (DOPAC) in whole kidney homogenates incubated with increasing concentrations of L-DOPA (0.1–10.0 μM) for 15 min in control conditions and in the presence of α-human atrial natriuretic peptide (α-hANP; 330 nM). Each point represents the mean of five to six experiments per group; vertical lines show s.e.mean. Control, (○); α-hANP, (●).

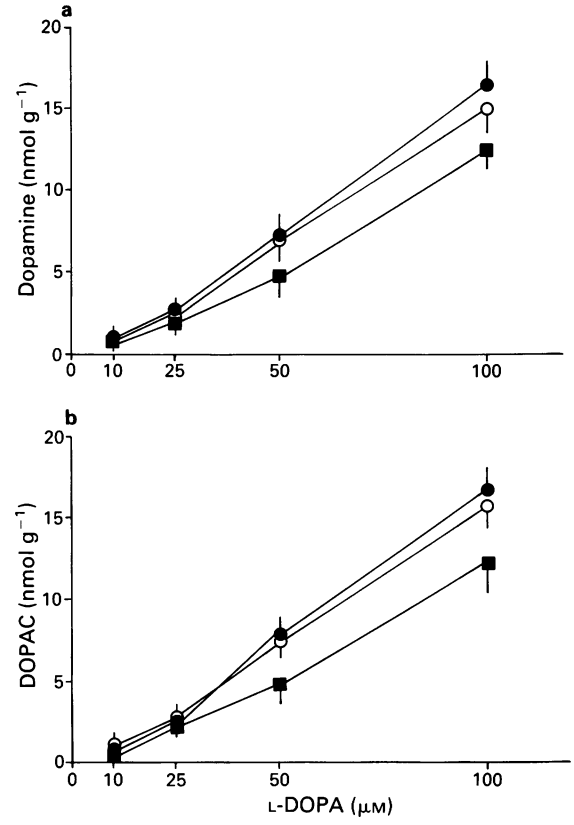


**Figure 4** Effect of α-human atrial natriuretic peptide (α-hANP; 330 nM) and zaprinast (10 μM) on the accumulation of (**a**) dopamine and (**b**) 3,4-dihydroxyphenylacetic acid (DOPAC) (in nmol $g^{-1}$) in kidney slices of rats on a high salt diet during the previous six weeks and incubated with increasing concentrations of exogenous L-DOPA (1–100 μM) for 15 min. Each point represents the mean of five experiments per group; vertical lines show s.e.mean. Control (○); α-hANP (●); zaprinast (■).

## Discussion

The results of the present study show that pharmacological concentrations of α-hANP (3.3 and 330 nM) decrease in a concentration- and time-dependent manner the accumulation of newly-formed dopamine in rat kidney slices loaded with L-DOPA. This effect was found not to depend on the concentration of L-DOPA added to the incubation medium, i.e. a similar inhibitory effect of α-hANP could be observed at all concentrations (1–100 μM) of L-DOPA used. Two different sets of results suggest that the inhibitory effect of α-hANP on renal synthesis of dopamine does not depend on a direct effect on the enzyme AAAD, but most probably on a reduced uptake of L-DOPA into the tubular cell. Firstly, the experiments performed in kidney homogenates clearly show that α-hANP requires tubular cell integrity to exert its inhibitory effect on the formation of dopamine. Secondly, the potentiation by zaprinast, a cyclic GMP phosphodiesterase inhibitor, of the inhibitory effect of α-hANP on the renal formation of dopamine in kidney slices indicates that the peptide activates a membrane operated mechanism controlling the entry of L-DOPA into the cells. In fact, receptors for ANPs have been shown to be coupled to the particulate form of guanylate cyclase, the activation of which increases the accumulation of cyclic GMP in renal tissues (Kim *et al.*, 1989). Since phosphodiesterases represent the sole pathway for the degradation of cyclic GMP it is expected that the effects obtained during the inhibition of this metabolic process might be related to an increased accumulation of the cyclic nucleotide (Waldman & Murad, 1987). Previous studies on the renal formation of dopamine have shown that neither 8-bromo cyclic GMP, a stable analogue of cyclic GMP, nor zaprinast exert a direct inhibitory effect upon the enzyme AAAD; the inhibitory effect of these two compounds was observed only when tissue slices were used (Soares-da-Silva & Fernandes, 1991a).

Although there is no doubt about the presence of specific receptors for ANPs in the kidney, there has been some dispute over whether the natriuretic action of ANP is the result of a tubular effect of the peptide. Initially, it was reported that the high-affinity binding sites for ANPs in renal tissues were mainly found in the glomerulus (Healy & Fanestyl, 1986) and that the direct application of ANP in microperfused tubules was found not to inhibit sodium reabsorption (Baum & Toto, 1986). More recently, however, it has been demonstrated that ANPs, at concentrations which do not affect glomerular filtration rate, are endowed with marked tubular effects, at both proximal and distal parts of the nephron (Sonnenberg *et al.*, 1986; Nonoguchi *et al.*, 1988; Winaver *et al.*, 1990; Ortola *et al.*, 1990; Salazar *et al.*, 1986; Hammond *et al.*, 1985; Harris *et al.*, 1987; Garvin *et al.*, 1989); some of the proximal tubular effects of ANP were

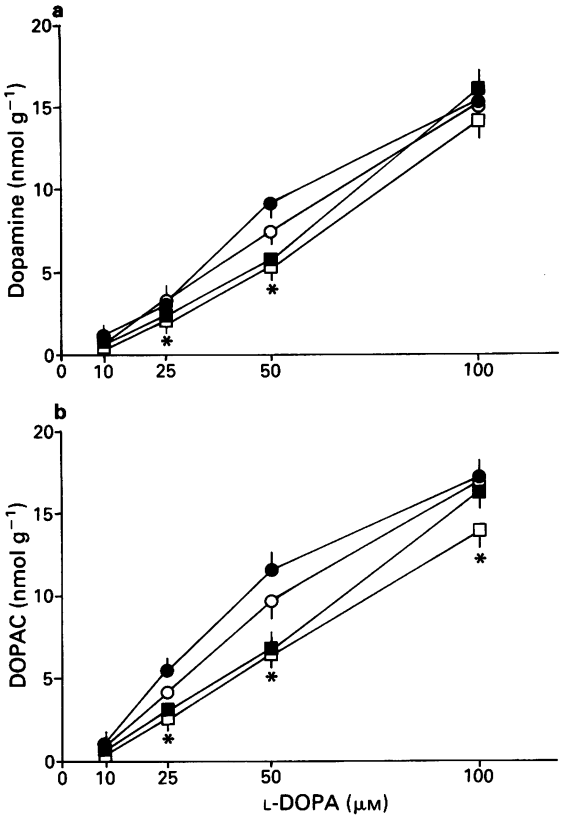


**Figure 5** Effect of α-human atrial natriuretic peptide (α-hANP; 330 nM), zaprinast (10 μM) and α-hANP plus zaprinast on the accumulation of (**a**) dopamine and (**b**) 3,4-dihydroxyphenylacetic acid (DOPAC) (in nmol $g^{-1}$) in kidney slices obtained from 24-month old rats and incubated with increasing concentrations of exogenous L-DOPA (1–100 μM) for 15 min. Each point represents the mean of five experiments per group; vertical lines show s.e.mean. Significantly different from corresponding values of control using the Tuckey Kramer method (*$P < 0.01$). Control, (○); α-hANP, (●); zaprinast, (■); α-hANP plus zaprinast, (□).

even mimicked by dibutyryl cyclic GMP (Garvin *et al.*, 1989). This appears, therefore, to suggest that ANPs mediate tubular effects at the proximal segments of the nephron and agrees with the results presented here, suggesting that the inhibitory effect of α-hANP on the renal formation of dopamine is probably related to the activation of a membrane operated mechanism involving the activation of a guanylate cyclase.

In kidney, most of the filtered L-DOPA or that present in the extracellular medium is decarboxylated after being taken up into tubular epithelial cells, namely those of the proximal convoluted tubules. The tubular transport of L-DOPA, an active process with great structural selectivity (Chan, 1974), appears to represent the rate-limiting step for the production of tubular dopamine, as the $K_m$ for AAAD is in the millimolar range (Hayashi *et al.*, 1990; Seri *et al.*, 1990; Soares-da-Silva & Fernandes, 1990a; Grenader & Healy, 1991). Sodium, however, appears to play a determining role in the renal formation of dopamine as has been clearly demonstrated *in vivo* (for reviews see Lee, 1982; Bass & Murphy *et al.*, 1990; Young, 1990) and *in vitro* (Hagege & Richet, 1985; Soares-da-Silva & Fernandes, 1990b; Soares-da-Silva *et al.*, 1990). It might, therefore, be hypothesized that changes in the renal production of dopamine during modifications in the renal delivery of sodium would be related to modifications in the membrane transport of DOPA, affecting, subsequently the intracellular availability of the amino acid. Support for this view comes from the fact that cyclic GMP, an effective inhibitor of the tubular transport of sodium (Lewis *et al.*, 1988), decreases the intracellular availability of L-DOPA in rat kidney slices and reduces the renal formation of dopamine (Soares-da-Silva & Fernandes, 1991a). Decreased tubular sodium reabsorption has been reported to occur in chronically sodium-loaded animals; this is a well known phenomenon and has also been found to be responsible for the lack of natriuretic effects of diuretic drugs in sodium-loaded animals (Kahn *et al.*, 1980). Inhibition of sodium transport during sodium load appears to result from an overriding mechanism, which has its origin in an increased tubular hydrostatic pressure, decreased peritubular colloidal osmotic pressure and passive sodium backleakage (Bonventre & Leaf, 1982). To test the hypothesis that the inhibitory effect of α-hANP on the renal synthesis of dopamine was related to the inhibition of tubular reabsorption of both sodium and L-DOPA, the effect of the peptide was tested in kidney slices obtained from rats fed a high salt diet (5 mM/100 g body weight) for 6 weeks. Under these experimental conditions, the formation of dopamine and of its deaminated metabolite DOPAC in kidney slices loaded with L-DOPA was not affected by α-hANP (330 nM) and was only slightly atenuated by zaprinast. These results suggest that inhibition of dopamine production by tubular epithelial cells induced by increased cyclic GMP tissue accumulation might only occur under conditions of normal sodium tubular transport. Renal tissues taken from aged rats (24 months old) have also been shown to produce less dopamine when incubated in the presence of L-DOPA and this appears not to depend on a reduced activity of the enzyme AAAD, but is most probably the result of a decreased cellular uptake of L-DOPA (Soares-da-Silva & Fernandes, 1991b). As in kidney slices taken from rats on a high salt diet, renal tissues obtained from aged rats were found to be insensitive to the inhibitory effects of both α-hANP (330 nM) and zaprinast (10 μM).

In conclusion, the results presented here demonstrate that α-hANP reduces, in a time- and concentration-dependent manner, the formation of dopamine in renal tissues loaded with L-DOPA. This effect appears to result from the activation of a membrane operated mechanism coupled to the enzyme guanylate cyclase and requires a normally operating

**Table 1** Decarboxylation of added L-DOPA in kidney slices obtained from 2- and 24-month old rats and from animals on normal salt diet (0.5 mM/100 g body weight) and high salt diet (5 mM/100 g body weight).

| *L-DOPA* | *2-months old* | *24-months old* | *Normal salt diet* | *High salt diet* |
|---|---|---|---|---|
| 10 μM | 2.1 ± 0.3 | 1.3 ± 0.1 | 2.2 ± 0.2 | 1.5 ± 0.2 |
| 25 μM | 6.3 ± 0.3 | 4.1 ± 0.3* | 7.5 ± 0.7 | 4.7 ± 0.6* |
| 50 μM | 20.9 ± 2.1 | 16.3 ± 1.7* | 19.9 ± 1.5 | 13.6 ± 1.4* |
| 100 μM | 41.1 ± 2.3 | 30.4 ± 1.6* | 40.6 ± 2.1 | 28.9 ± 2.5* |

The results are expressed as the algebraic sum of tissue contents of newly-formed dopamine and DOPAC (in nmol $g^{-1}$). Values are means ± s.e.mean of five experiments per group. Significantly different from corresponding control values by Student's *t* test (*$P < 0.02$).

transport system for the precursor, L-DOPA. It should be recalled, however, that the inhibitory effect of a α-hANP on the renal synthesis of dopamine does not necessarily conflict with the evidence that the natriuretic effects of ANPs might be mediated through the utilization of endogenous renal dopamine. In fact, both effects could be different epiphenomena of the action of ANPs at proximal renal tubules.

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# Characterization of the receptor mediating relaxation to substance P in canine middle cerebral artery: no evidence for involvement of substance P in neurogenically mediated relaxation

[1]C.M. Stubbs, G.J. Waldron, H.E. Connor & W. Feniuk

Department of Neuropharmacology, Glaxo Group Research, Park Road, Ware, Herts, SG12 0DP

**1** The aim of this study was to characterize the neurokinin receptor which mediates relaxation of dog isolated middle cerebral artery by the use of selective agonists and antagonists and to establish whether substance P is involved in the neurogenically mediated relaxant response in this vessel.

**2** Substance P caused concentration-related, endothelium-dependent relaxations of dog isolated middle cerebral artery, contracted with prostaglandin $F_{2\alpha}$. The selective $NK_1$ receptor agonists, GR73632 and substance P methyl ester (SPOMe), also caused relaxation with similar maximum effects to those of substance P. GR73632 and SPOMe were approximately 20 times and 6 times less potent respectively than substance P. The selective $NK_2$ and $NK_3$ receptor agonists, GR64349 and senktide, were only weakly active in causing relaxation being at least 425 times and 245 times less potent respectively than substance P.

**3** The selective $NK_1$ receptor antagonist, GR82334, was a potent, specific, competitive antagonist of the relaxant effects of substance P. In contrast, the selective $NK_2$ receptor antagonist, R396 (10 $\mu$M) had no effect on the response to substance P.

**4** Electrical field stimulation of dog isolated middle cerebral artery, contracted with prostaglandin $F_{2\alpha}$, caused neurogenically mediated, non-adrenergic non-cholinergic (NANC) relaxations. These NANC relaxations were unaffected by endothelium removal, GR82334 (10 $\mu$M) or by capsaicin (10 $\mu$M) treatment. However, the nitric oxide synthesis inhibitor, L-$N^G$-monomethyl arginine methyl ester (L-NMMA) (100 $\mu$M) markedly attenuated the response to electrical stimulation.

**5** These results suggest that substance P causes relaxation of dog isolated middle cerebral artery via activation of $NK_1$ receptors. However, substance P does not appear to be involved in NANC neurotransmission. In contrast, the marked inhibitory effect of L-NMMA on NANC relaxations implicates nitric oxide in NANC neurotransmission in this vessel.

**Keywords:** Substance P; $NK_1$ receptors; neurogenic relaxation; nitric oxide

## Introduction

It has been known for some time that large cerebral arteries are supplied with both sympathetic and parasympathetic nerve fibres (Neilson & Owman, 1967; Edvinsson *et al.*, 1972). More recently, immunohistochemical studies have shown that these vessels are also densely innervated with sensory nerve fibres containing a variety of peptides including substance P and calcitonin gene-related peptide (CGRP, for review see Uddman & Edvinsson, 1989). Sensory neurones have been implicated in neurogenic vasodilatation and extravasation and can be depleted of their neuropeptides by the neurotoxic agent, capsaicin (Lembeck, 1985). Electrical stimulation of isolated cerebral arteries causes neurogenic relaxations which are resistant to atropine and guanethidine (Lee *et al.*, 1978; Toda, 1982). The transmitter involved in this relaxant response has not been defined although both vasoactive intestinal polypeptide (VIP) and CGRP have been implicated in certain cerebral vessels (Lee *et al.*, 1984; Saito *et al.*, 1989).

Substance P is a member of the tachykinin group of peptides which share a common C-terminal sequence (Erspamer, 1980). In mammalian tissues the biological effects of endogenous tachykinins are mediated via activation of at least three receptor subtypes (Regoli *et al.*, 1989). These receptors have been chiefly characterized by use of the naturally occurring tachykinins with substance P, neurokinin A and neurokinin B acting preferentially at $NK_1$, $NK_2$ and $NK_3$ receptors respectively (Regoli *et al.*, 1988). However, since these natural peptides are not selective, definitive characterization of the receptor type mediating the numerous physiological effects of the naturally occurring tachykinins has awaited the development of selective agonists and antagonists.

Substance P is a potent, endothelium-dependent dilator of cerebral blood vessels both *in vitro* and *in situ* (Edvinsson, 1985; Alafaci *et al.*, 1989). However, the neurokinin receptor mediating this effect has not been fully characterized. In this study we have examined the effects of recently available, selective agonists and antagonists in order to characterize the receptor mediating the relaxation to substance P in the dog isolated middle cerebral artery. Additionally, we have investigated whether the release of neuropeptides from capsaicin-sensitive nerves is involved in the response to electrical stimulation in this vessel. A preliminary account of some of these findings has been presented to the British Pharmacological Society (Stubbs *et al.*, 1991).

## Methods

### *General*

Brains were removed from beagle dogs killed by sodium pentobarbitone (100 mg $kg^{-1}$, i.v.). Middle cerebral arteries were dissected and stored overnight in modified Krebs solution (Apperley *et al.*, 1976) at 4°C. Preliminary experiments showed that this storage procedure did not affect the sen-

[1] Author for correspondence.

sitivity of preparations compared to freshly obtained tissue. Each artery was cut into ring segments approximately 4 mm in length and suspended between two 200 μm parallel wires in 10 ml organ baths containing modified Krebs solution at 37°C and gassed with 5% $CO_2$ in $O_2$. Isometric tension changes were recorded. A resting tension of 0.3 g was initially applied and re-adjusted every 10 min for 1 h and then tension was increased to 1 g for the remainder of the experiment.

### *Determination of agonist potency*

In order to assess relaxant effects, tissues were contracted with prostaglandin $F_{2\alpha}$ (1–10 μM) to gain an active tone of approximately 1 g. Preliminary experiments showed that this concentration caused approximately 80–90% of the maximal contraction to prostaglandin $F_{2\alpha}$. Substance P was added to the bathing solution using a cumulative concentration schedule. Sufficient time was allowed for the effect of each concentration of substance P to plateau before adding higher concentrations, until a maximum response was obtained or contraction started to occur. Tissues were then washed and allowed at least 30 min before rechallenging with substance P and the procedure repeated. For the third concentration-effect curve one tissue was again challenged with substance P to assess any change in tissue sensitivity whilst concentration-effect curves to test agonists were constructed in the other three preparations. At the end of all experiments papaverine (200 μM) was added to tissues. Responses were expressed as a % of this papaverine-induced relaxation. Measurements of agonist relative potency were made at the level of 30% of the papaverine-induced relaxation and are expressed as an $EC_{30}$ value. Relative potency estimates were calculated by dividing the $EC_{30}$ value of the test agonist by the $EC_{30}$ value of substance P (previous curve in that preparation).

### *Determination of antagonist affinity*

Two cumulative concentration-effect curves to substance P were obtained in all tissues. Preparations were washed and at least 30 min later a single concentration of antagonist was added to 3 baths whilst one preparation was left as an untreated control. Antagonists were equilibrated for 30 min before rechallenging with substance P. Papaverine (200 μM) was added at the end of the experiment. Equi-effective concentration ratios (C-R) were determined by comparing $EC_{30}$ values for substance P in the presence and absence of antagonist.

### *Electrical field stimulation studies*

Atropine (1 μM) and guanethidine (1 μM) were added to the bathing solution 15 min before each curve to inhibit cholinergic and sympathetic responses respectively. Preparations were contracted with prostaglandin $F_{2\alpha}$ (1–10 μM) and stimulation parameters that gave a neurogenically mediated response were determined in pilot experiments by constructing voltage and pulse width response curves, at 8 Hz, to obtain responses which were abolished by tetrodotoxin (TTX, 1 μM). In all subsequent experiments, 10 V and a square wave pulse of 0.3 ms pulse width were used which corresponded to a current of approximately 500 mA. The validity of these parameters was tested at the start of every experiment and any vessel which still responded to electrical stimulation in the presence of TTX was discarded (< 1%). Frequency-response curves were constructed by stimulating tissues at 8 Hz and subsequently at 2, 4, and 8 Hz, each stimulation consisting of a train of 80 pulses. The influence of an antagonist on the response to electrical stimulation was studied by adding the antagonist after the initial 8 Hz stimulation had returned to the pre-response level. Antagonists were incubated for 10 min: 7–10 min were left between all stimulations (see Figure 4). In some experiments a combination of antagonists was added and their combined effect assessed in subsequent frequency-response curves. In each experiment one tissue was not treated with an antagonist and thus acted as a time matched control.

### *Other studies*

To determine the effect of endothelium removal on the response to electrical stimulation, arteries were perfused for 80 s with Triton X-100 solution (0.1% in distilled water) at a flow rate of 2 ml $min^{-1}$ to remove the endothelium (Connor & Feniuk, 1989). In each experiment the integrity of the endothelium was assessed by examining the response to substance P (10 nM). Tissues that responded to substance P with greater than 15% of the papaverine (200 μM)-induced relaxation were discarded.

The effect of capsaicin was determined by comparing the response to electrical stimulation in control and capsaicin-treated tissues. In these studies a frequency-response curve was obtained in each preparation. After washout, tissues were treated with capsaicin (10 μM) for 30 min and washed for a further 30 min before re-constructing a frequency-response curve. For these experiments results were expressed as a percentage of the initial 8 Hz response in the first frequency-response curve.

In other experiments responses to both electrical stimulation and exogenous agents were assessed in the same tissue before and after antagonist treatment. In these experiments a frequency-response curve was obtained and, without washing, a cumulative concentration-effect curve to the test agonist was constructed. This procedure was then repeated after the addition of an antagonist. In these studies papaverine (200 μM) was added to the bathing fluid at the end of the experiments.

### *Drugs and solutions*

The following compounds were used in this study: L-arginine (Sigma), atropine sulphate (Sigma), calcitonin gene related peptide (CGRP, Cambridge Research Biochemicals), guanethidine hydrochloride (Ciba laboratories), L-$N^G$-monomethyl arginine methyl ester hydrochloride (L-NMMA, Sigma), senktide (Cambridge Research Biochemicals), substance P (Cambridge Research Biochemicals), substance P methyl ester (SPOMe, Cambridge Research Biochemicals), vasoactive intestinal polypeptide (VIP, Cambridge Research Biochemicals).

δ-Amino valeryl-[L-$Pro^9$, N-Me $Leu^{10}$]-$SP_{(7-11)}$ (GR73632), [$Lys^3$, $Gly^8$-R-γ-lactam, $Leu^9$]-neurokinin $A_{(3-10)}$ (GR64349), [D-$Pro^9$, [spiro-γ-lactam] $Leu^{10}$, $Trp^{11}$]-physalaemin$_{(1-11)}$ (GR-82334) and Ac-Leu-Asp-Gln-Trp-Phe-Gly-$NH_2$ (R396, See Pattachini *et al.* (1991)) were synthesized by the Chemical Research Department, Glaxo Group Research Ltd, Ware.

Stock solutions of peptides (approximately 1 mM) were prepared in acetic acid (1 mM) and frozen until use. Dilutions were made in modified Krebs solution and discarded at the end of the experiment.

### *Data analysis and statistics*

Unless otherwise stated, values are expressed as arithmetic mean ± s.e.mean or geometric mean (95% confidence limits). Comparisons between groups were made by Student's *t* test for paired or unpaired comparisons as appropriate.

Equi-effective concentration-ratios (C-R) were determined graphically by comparing $EC_{30}$ values for substance P in the presence and absence of antagonist. $Log_{10}$ (concentration-ratio − 1) were plotted against $log_{10}$ (concentration of antagonist) (Arunlakshana & Schild, 1959). If such plots were linear and had gradients not significantly different from unity, the affinity $pK_B$ was estimated from the mean of the individual values.

## Results

### *Effects of agonists*

Substance P caused concentration-dependent reductions of prostaglandin $F_{2\alpha}$-induced tone. In most preparations the response was rapid in onset and maximal within approximately 30 s of drug administration (Figure 1). Substance P was a very potent agonist in causing relaxation with an $EC_{30}$ value of 0.20 (0.09–0.43) nM ($n = 13$) and a maximum effect of 65 ± 5% of the relaxation induced by papaverine (200 μM). Consecutive concentration-effect curves were reproducible (<2 fold shift in control concentration-effect curves). After removal of the endothelium by perfusion with Triton X-100 solution (see Methods) substance P had little or no effect on prostaglandin $F_{2\alpha}$-induced tone.

The selective $NK_1$ receptor agonists, SPOMe and GR73632, also caused relaxations of dog middle cerebral artery with a time course and maximum effect similar to that observed with substance P (Figure 2). However, the selective $NK_2$ receptor agonist, GR64349, and the selective $NK_3$ receptor agonist, senktide, had no effect on tone at concentrations up to 30 nM: higher concentrations (0.1–1 μM) caused modest relaxations compared to those of substance P. Although a maximum effect of GR64349 and senktide was not obtained, the highest concentrations of these agents caused a relaxation of only 23 ± 5% and 16 ± 6% ($n = 6$) respectively of the papaverine (200 μM)-induced relaxation (Figure 2). The relative potency values for the selective agonists compared to substance P are shown in Table 1.

### *Effects of neurokinin antagonists*

GR82334 caused a concentration-dependent rightward displacement of the substance P concentration-effect curve with no apparent supression of the maximum response (Figure 3a). Analysis of the antagonist action of GR82334 yielded a Schild plot which approximated to a straight line with a slope of 0.84 (0.62–1.06). This slope was not significantly different from unity, indicating competitive antagonism. The affinity, expressed as a $pK_B$ value was 7.4 ± 0.2 ($n = 10$). In marked contrast, the effects of substance P were resistant to antagonism by R396 (10 μM, Figure 3b). The concentration-ratio for substance P in the presence of this antagonist was 0.74 (0.09–6.70, $n = 3$).

### *Electrical field stimulation studies*

In the presence of atropine (1 μM) and guanethidine (1 μM) electrical stimulation of dog middle cerebral artery, precontracted with prostaglandin $F_{2\alpha}$ (1–10 μM) caused frequency-dependent relaxations (Figure 4) which were neurogenically mediated since they were abolished by tetrodotoxin (1 μM). In approximately 90% of tissues, responses

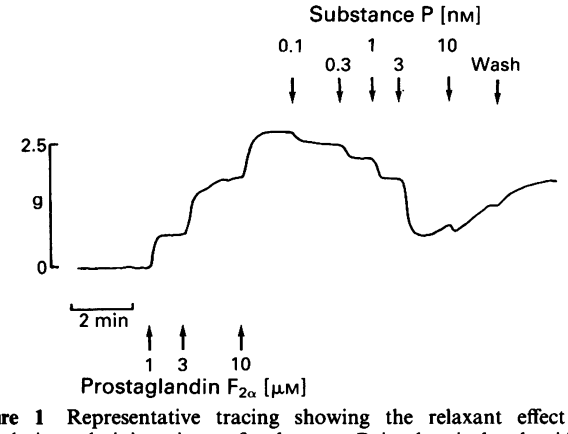


**Figure 1** Representative tracing showing the relaxant effect of cumulative administrations of substance P in dog isolated middle cerebral artery contracted with prostaglandin $F_{2\alpha}$ (1–10 μM).

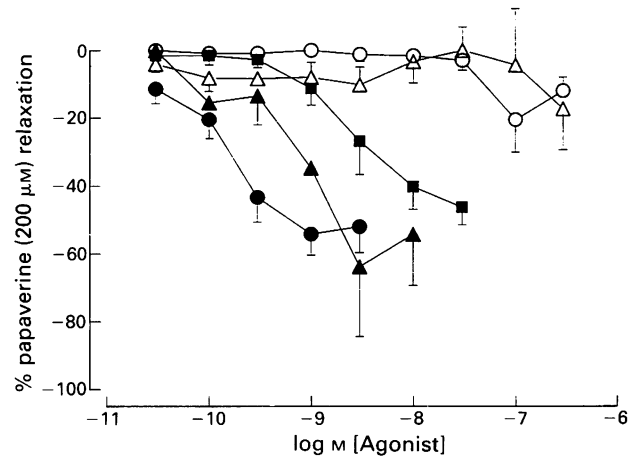


**Figure 2** Concentration-effect curves to substance P (2nd control curve, ●), substance P methyl ester (▲), GR73632 (■), GR64349 (○) and senktide (△) in dog middle cerebral artery contracted with prostaglandin $F_{2\alpha}$ (1–10 μM). Responses are expressed as a 9% papaverine (200 μM)-induced relaxation. Values are means with s.e.mean shown by vertical bars, $n = 3$–7 except for substance P where $n = 13$.

**Table 1** Relative potencies and maximum responses of neurokinin receptor agonists in dog isolated middle cerebral artery

| *Agonist* | *Relative potency* | *% papaverine max* |
|---|---|---|
| Substance P | 1 | 65 ± 5 |
| GR73632 | 20.7(6.7–63.9) | 52 ± 4 |
| SPOMe | 5.9(0.8–41.5) | 63 ± 16 |
| GR64349 | >425(211–857) | 24 ± 7 |
| Senktide | >245( 65–922) | 16 ± 6 |

Values are geometric means (95% confidence limits) or arithmetic mean ± s.e.mean from 3–7 experiments except for substance P where $n = 13$. Relative potencies were calculated at the level of 30% of the papaverine (200 μM)-induced relaxation. For abbreviations, see Methods.

were monophasic with tension returning to pre-response levels after stimulation had ceased. Up to three frequency-response curves could be obtained and in untreated tissues responses were reproducible. The magnitude of the relaxation caused by electrical stimulation was 0.73 ± 0.14 g at 8 Hz ($n = 13$).

### *Effect of antagonists on responses to electrical stimulation*

Neurogenic relaxations were unaffected by a high concentration of the $NK_1$ receptor antagonist, GR82334 (10 μM, Figure 5a). In view of recent reports suggesting that nitric oxide may be involved in NANC neurotransmission (Li & Rand, 1989; Toda & Okamara, 1990), we decided to examine the effects of the inhibitor of nitric oxide formation, L-NMMA (Moncada *et al.*, 1989) on the response to electrical stimulation. Addition of L-NMMA (100 μM) caused a further increase in tone of 0.81 ± 0.31 g ($n = 16$) in tissues precontracted with prostaglandin $F_{2\alpha}$. This contractile effect of L-NMMA was transient and had subsided before the construction of the subsequent frequency-response curve. In the presence of L-NMMA, neurogenic relaxations were markedly, and significantly attenuated ($P<0.05$ paired Student's $t$ test, Figure 5b). However, in the same tissues, L-NMMA (100 μM) did not modify relaxant responses produced by CGRP (0.1–30 nM, concentration-ratio 2.1 (0.4–4.3), geometric mean (range), $n = 3$) or VIP (0.1–100 nM, concentration-ratio 2.6 (0.9–9.5), geometric mean (range), $n = 3$).

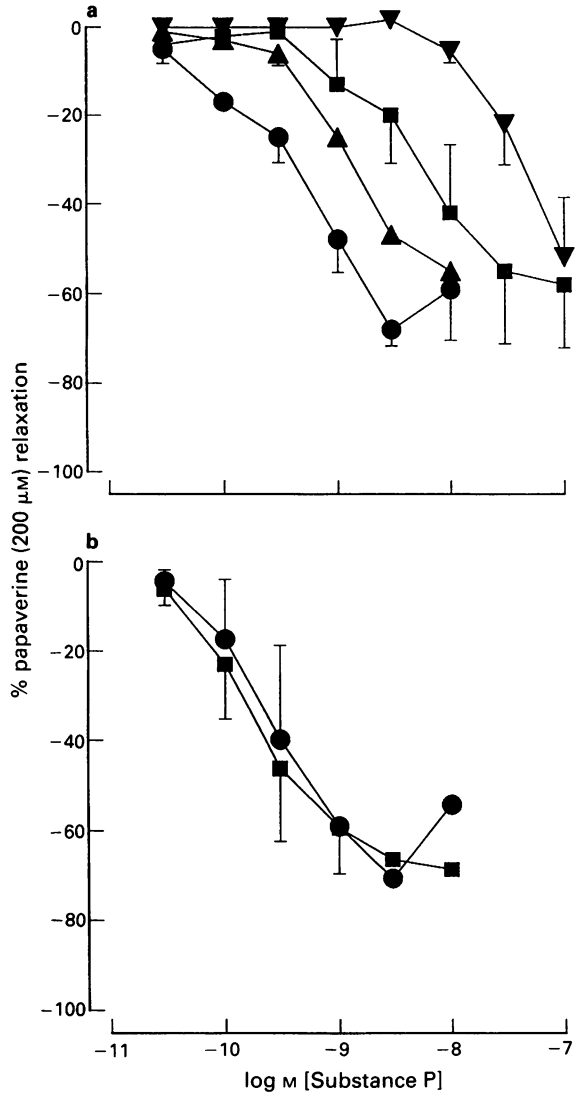


**Figure 3** Concentration-effect curves to substance P in dog middle cerebral artery in the absence (●) and presence of (a) GR82334, 0.1 μM (▲), 1 μM (■), 10 μM (▼) or (b) R396 (10 μM, ■). Responses are expressed as a % papaverine (200 μM)-induced relaxation. Values are means with s.e.mean shown by vertical bars, $n = 3$–4 except for control (●) where $n = 10$.

### *Effect of endothelium removal*

Perfusion with Triton X-100 to remove the endothelium did not affect the contraction produced by 3 μM prostaglandin $F_{2\alpha}$ (0.76 ± 0.12 g and 0.89 ± 0.21 g in control and Triton X-100-treated preparations respectively, $n = 11$). However, substance P (10 nM) caused relaxation only in untreated tissues. In endothelium-denuded tissues, electrical field stimulation caused frequency-dependent neurogenic relaxations which were similar in magnitude and appearance to those obtained in untreated tissues. Furthermore, responses to electrical stimulation in Triton X-100 treated preparations were also significantly reduced in the presence of L-NMMA (100 μM, $P<0.05$ paired Student's $t$ test, Figure 5c).

### *Effect of capsaicin on neurogenic dilatations*

Application of capsaicin (10 μM) had variable effects on resting tone in the dog middle cerebral artery. In some tissues capsaicin caused a transient contraction of 0.49 ± 0.19 g ($n = 8$) which returned to baseline within 10 min. In other tissues capsaicin caused a small reduction in resting tone of 0.19 ± 0.05 g ($n = 12$) which was slower in both onset and offset than the contractile effect. Incubation for 30 min with capsaicin followed by 30 min washing had no effect on the magnitude of the contractile response to prostaglandin $F_{2\alpha}$ (3 μM, 1.30 ± 0.36 g and 1.28 ± 0.49 g before and after capsaicin-treatment respectively, $n = 3$). Furthermore, capsaicin-treatment had no significant effect on neurogenically mediated relaxations (Figure 5d).

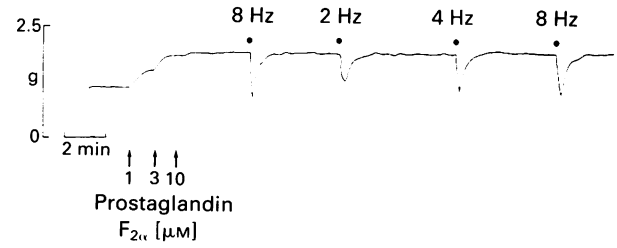


**Figure 4** Representative tracing showing neurogenic relaxations of dog middle cerebral artery in the presence of atropine (1 μM) and guanethidine (1 μM). Tension was increased with prostaglandin $F_{2\alpha}$. When the contraction had stabilized an initial 8 Hz stimulation was applied followed by a frequency response relationship using 2, 4 and 8 Hz (0.3 ms, 10V and 80 pulses). For antagonist studies, drugs were added between the initial 8 Hz stimulation and the construction of the frequency-response relationship.

## Discussion

The aims of this study were two fold. First, to characterize the receptor mediating relaxation to substance P in the dog middle cerebral artery and secondly to assess whether substance P is involved in non-adrenergic, non-cholinergic (NANC) neurogenic relaxation in this tissue. To date, three neurokinin receptor subtypes have been identified on the basis of the relative potency of natural tachykinins with substance P, neurokinin A and neurokinin B being more potent at $NK_1$, $NK_2$ and $NK_3$ receptors respectively (Regoli *et al.*, 1988). Recently, selective, synthetic peptide agonists and antagonists have been identified (see below) and in this study their effect in the dog middle cerebral artery has been studied. The results obtained are consistent with the view that $NK_1$ receptors mediate relaxation to substance P in this tissue. Thus the $NK_1$ receptor selective agonists SPOMe (Watson *et al.*, 1983) and GR73632 (Hagan *et al.*, 1989), were potent relaxant agents, compared to substance P, whilst the selective $NK_2$ and $NK_3$ receptor agonists GR64349 (Hagan *et al.*, 1989) and senktide (Wormser *et al.*, 1986), caused only modest relaxations at high concentrations compared to those of substance P. Differential degradation by peptidases is unlikely to account for these effects since GR73632, GR64349 and senktide are reported to be metabolically stable (Hagan *et al.*, 1989; Guard *et al.*, 1990) and the peptidase inhibitors phosphoramidon (1 μM) and bestatin (10 μM, Littlewood *et al.*, 1988) were without effect on the response to substance P itself (C.M. Stubbs, unpublished observation). The relaxant response to substance P was unaffected by the selective $NK_2$ receptor antagonist, R396 (Pattachini *et al.*, 1991). This compound has been reported to have a $pA_2$ value of 7.7 against neurokinin A-induced contractions of the hamster trachea and a value of 5.7 against neurokinin A-induced contractions of the rabbit pulmonary artery, both responses reported to be mediated via $NK_2$ receptor activation (Maggi *et al.*, 1990). However, at the concentration used in the present study (10 μM) the lack of effect of this antagonist rules out an action of substance P at either of the putative $NK_2$ receptor subtypes. In contrast to the effect of the $NK_2$ receptor antagonist, the selective $NK_1$ receptor antagonist, GR82334, blocked the relaxant effects of substance P with a $pK_B$ value of 7.4. This value is similar to the $pK_B$ value reported for GR82334 against SPOMe-induced

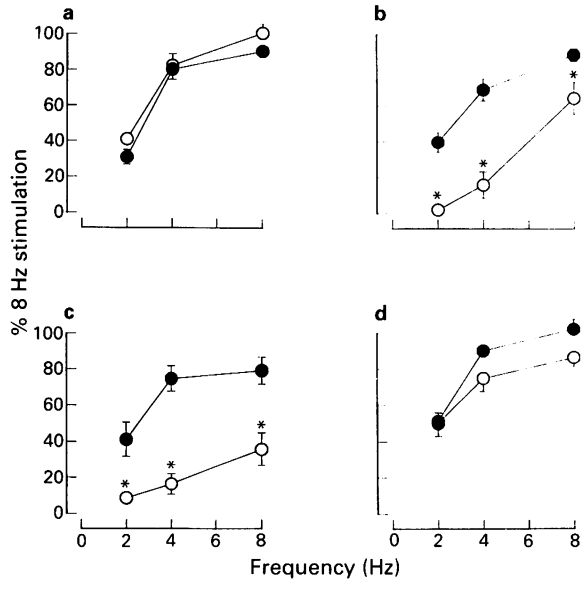


**Figure 5** Effect of (a) GR82334 (10 μM), (b) L-$N^G$-monomethyl arginine methyl ester L-NMMA (100 μM), (c) L-NMMA (100 μM), Triton X-100 tissues) and (d) capsaicin (10 μM) on NANC neurogenic relaxations of dog middle cerebral artery contracted with prostaglandin $F_{2\alpha}$ (1– 10 μM). Responses produced by 2, 4 and 8 Hz are expressed as a % of the response to 8 Hz elicited immediately prior to the construction of a frequency-response relationship in the absence (●) and presence (○) of the antagonist (see Figure 4). For capsaicin, responses are expressed as a % of the initial 8 Hz response of the frequency-response curve in the absence of capsaicin. Values are means with s.e.mean shown by vertical bars, $n = 3$–5. *$P<0.05$.

contractions of the guinea-pig isolated ileum preparation and GR73632-induced dilatations of the rabbit isolated aorta. GR82334 is a highly selective antagonist at $NK_1$ receptors, having no significant effect at a concentration of 10 μM at $NK_2$ or $NK_3$ receptors (Hagan *et al.*, 1991). Furthermore its specificity for neurokinin receptors is displayed by the observation that GR82334 (10 μM) has no significant effect on relaxations to CGRP or VIP in dog middle cerebral artery (C.M. Stubbs, unpublished observation). Thus the results of both agonist and antagonist studies are suggestive of $NK_1$ receptors mediating substance P-induced relaxations of dog isolated middle cerebral artery. Additionally, receptors mediating contraction to substance P (Nantel *et al.*, 1990) do not appear to be present in this preparation since substance P did not cause contraction in the absence of active tone, after endothelium removal or in the presence of GR82334.

Immunohistochemical studies have shown a dense innervation of substance P and CGRP containing sensory nerve fibres within the adventitial-medial border of cerebral arteries of several species including man (Uddman & Edvinsson, 1989). Indeed CGRP and substance P have been shown to be co-localized in certain populations of sensory neurones in cerebral blood vessels (Uddman *et al.*, 1985). In the presence of atropine and guanethidine, electrical stimulation of the dog middle cerebral artery resulted in a marked, neurogenically mediated, relaxation. We were interested to determine the mediators involved in this non-adrenergic, non-cholinergic (NANC) relaxation and in particular whether substance P or indeed other sensory neuropeptides were implicated. However, neither GR82334 nor removal of the endothelium had any effect on the response to electrical stimulation. Since we have shown in this study that both treatments markedly attenuate the response to exogenously applied substance P, it is unlikely that substance P is involved in the NANC response.

Capsaicin, a specific sensory neurotoxin, has been shown to deplete neuropeptides, including substance P and CGRP from sensory nerves in cerebral arteries, heart and spinal cord (Duckles & Buck, 1982; Miyauchi *et al.*, 1987; Saria *et al.*, 1986). Addition of capsaicin had variable effects on resting tone in the middle cerebral artery; both contractile and relaxant responses were observed. This dual effect of capsaicin has been reported previously and appears to be dependent on both the species and portion of vessel used (Duckles, 1986). Capsaicin-induced contractions have been attributed to a direct effect of capsaicin on the vascular smooth muscle whereas relaxation is presumed to be a consequence of a prejunctional action causing the release of relaxant transmitter substances (Duckles, 1986). However, in the present study capsaicin treatment caused only a small, non-significant reduction of the response to electrical stimulation. Since neurogenically mediated relaxations were not affected by capsaicin pretreatment, the transmitter(s) mediating NANC neurotransmission in the middle cerebral artery do not appear to be neuropeptides released from sensory nerves. It is unlikely that the protocol adopted for the capsaicin studies was insufficient to cause depletion of transmitters from sensory nerves since the same procedure caused a marked reduction (52 ± 15%, $n = 6$) of the neurogenically mediated contractile response of the rabbit iris sphincter muscle (C.M. Stubbs unpublished observation), a preparation in which substance P is known to be implicated in neurotransmission (Ueda *et al.*, 1981). Furthermore, it is also unlikely that the stimulation parameters used in the present study could not activate sensory neurones since similar stimulation parameters cause activation of capsaicin-sensitive primary afferents in the guinea-pig isolated pulmonary artery (Maggi *et al.*, 1990). The modest reduction in the response to electrical stimulation after capsaicin treatment may reflect a capsaicin-induced depletion of neuropeptides from sensory nerves, as has been observed in other blood vessels (Duckles & Buck, 1982; Furness *et al.*, 1982; Maggi *et al.*, 1990). However, if this is the case, it is clear that any transmitter released from sensory nerves provides only a marginal contribution to neurogenically mediated relaxations in the dog middle cerebral artery.

Recent evidence suggests a role for nitric oxide in NANC neurogenic relaxations in a number of isolated tissue preparations (Toda *et al.*, 1990; Tucker & Okamura, 1990; Hobbs & Gibson, 1990). In the present study L-NMMA, a specific inhibitor of the formation of nitric oxide from L-arginine (Moncada *et al.*, 1989) markedly attenuated the response to electrical stimulation in both endothelium intact and denuded preparations. A recent report has suggested that neurogenically mediated relaxation in sheep middle cerebral artery involves the release of VIP which causes the formation of nitric oxide (Gaw *et al.*, 1991). In the present study, the inhibitory effect of L-NMMA on NANC relaxations was specific since L-NMMA did not affect the response to CGRP or VIP. This latter observation provides further evidence to rule out a role for these neuropeptides as relaxant neurotransmitters in this preparation. However, our findings strongly suggest that nitric oxide is released upon nerve stimulation and contributes to NANC neurogenic relaxations of dog middle cerebral artery. The lack of effect of endothelium removal on the L-NMMA-induced inhibition of the response to electrical stimulation suggests that the released nitric oxide acts directly on the smooth muscle cells to cause relaxation. The results of the present study cannot determine whether nitric oxide itself or a nitric oxide liberating substance is released upon nerve stimulation. However, since nitric oxide is very reactive (Palmer *et al.*, 1987), it cannot be stored pre-formed in synaptic vesicles, unless bound to a stabilising molecule.

In conclusion, the results of the present study show that $NK_1$ receptors mediate the relaxation to substance P in the dog middle cerebral artery although substance P does not appear to be involved in NANC neurotransmission. The results suggest, however, that NANC neurogenic relaxations in this vessel involve the release of nitric oxide or a nitric oxide generating substance.

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# Effect of treatment with vitamin $D_3$ on the responses of the duodenum of spontaneously hypertensive rats to bradykinin and to potassium

Teresa Feres, Lucia M. Vianna, Antonio C.M. Paiva & [1]Therezinha, B. Paiva

Department of Biophysics, Escola Paulista de Medicina, 04034, São Paulo, SP, Brazil

**1** The diet of spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) and Wistar (NWR) rats was supplemented with either 2% calcium lactate in the drinking water or 12.5 μg vitamin $D_3$ 100 $g^{-1}$ body weight daily by gavage, for 14 days.

**2** The blood pressure of the SHR treated with either calcium or vitamin D decreased to the same levels as that of WKY and NWR.

**3** The response to bradykinin of the SHR isolated duodenum, which is predominantly contractile, upon treatment with vitamin D (but not with calcium), became predominantly relaxant, approaching the normal behaviour of the WKY and NWR duodenum.

**4** The relaxant responses of the SHR and WKY duodenum to potassium were smaller than those of NWR, but treatment with vitamin D increased the response in all three rat strains.

**5** It is concluded that, besides sharing the hypotensive effect of calcium, vitamin D treatment of SHR has an effect on the duodenum smooth muscle which might be due to calmodulin-dependent activation of calcium-dependent potassium channels.

**Keywords:** Bradykinin; duodenum; intestinal smooth muscle; spontaneously hypertensive rats; vitamin D

## Introduction

Several studies have shown that an altered calcium metabolism may play a significant role in the development and maintenance of hypertension both in man and in spontaneously hypertensive animals (SHR) (for a review, see McCarron, 1989). In the SHR, vitamin D metabolism was reported to be abnormal (Lucas *et al.*, 1986) and calcium supplementation in the diet caused blood pressure reduction (Ayashi, 1979; Bukoski & McCarron, 1986). More recently, dietary supplementation with vitamin $D_3$ was shown to reduce arterial blood pressure in the SHR (Vianna *et al.*, 1992). Apparently, a disturbance in vitamin $D_3$ and calcium metabolism is linked to the hypertensive mechanism in SHR (Lucas *et al.*, 1986).

Calcium is also known to be associated with the electrophysiological stability of the cell membrane, being important for the relaxation mechanism in smooth muscle (Rinaldi & Bohr, 1989). However, in spite of numerous studies on the vascular reactivity in SHR, controversial results have been obtained, depending on the kind of vascular smooth muscle used (for a review see Bohr & Webb, 1988).

An interesting model for studying smooth muscle relaxation in the rat is the response of the duodenum to bradykinin. In normal rats, the response to low concentrations of this agonist consists only of a relaxation, whereas higher concentrations elicit relaxation followed by contraction (Boschcov *et al.*, 1984). Previous work from this laboratory showed that the response of the SHR to bradykinin is predominantly contractile, with suppression of its relaxant component (Miasiro *et al.*, 1985). We have, therefore, investigated whether dietary supplementation with vitamin $D_3$, under conditions that cause reversion of the hypertension in SHR, is also able to reverse the abnormal behaviour of the duodenum of these animals towards bradykinin. In this paper, we present the results of a study on the effect of treatment with vitamin $D_3$ on the responses of SHR and of normotensive Wistar Kyoto rats (WKY), as well as normotensive Wistar rats (NWR).

## Methods

Experiments were carried out on the Okamoto-Aoki strain of SHR, their normotensive controls (WKY), and normotensive Wistar rats (NWR). The animals were female aged 20 weeks and weighed 200 ± 5 (SHR), 220 ± 7 (WKY), 198 ± 3 g (NWR). They were fed a standard diet (Labina rat chow, Purina), containing 6,600 i.u. vitamin $D_3$ $kg^{-1}$. After a basal period of 10 days, one group of animals were submitted to a daily supplementation, by gavage, of 12.5 μg (500 i.u.) vitamin $D_3$ per 100 g body weight, dissolved in 0.35 ml coconut oil. In another group the drinking water was replaced by a 2.0% calcium lactate aqueous solution. Control groups had access to normal drinking water, and one of them received 0.35 ml coconut oil by gavage, daily.

Systolic blood pressure was measured twice weekly from the tail of prewarmed unanesthetized rats by a plethysmographic method. An average of three readings was recorded for each animal.

The rat duodenum preparation was set up as previously described (Boschcov *et al.*, 1984). Briefly, the duodenum was suspended in a 5 ml chamber containing a salt solution of the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.36, $MgCl_2$ 0.49, $NaH_2PO_4$ 0.36, $NaHCO_3$ 11.9, D-glucose 5.0. The bath solution was maintained at 37°C and bubbled with a mixture of $CO_2$ (5%) and $O_2$ (95%). The organs were submitted to a 1 g load and their isotonic contractions were recorded (with a 6 fold magnification) after a 60 min equilibration period.

The concentration-response curves were obtained within the first 90 min after the end of the equilibration period. The drugs, in volumes not exceeding 0.2 ml, were added directly

[1] Author for correspondence.

to the organ bath at 5 min intervals for KCl, at 15 min intervals for low bradykinin concentrations (which produced relaxation) and at 30 min intervals for higher bradykinin concentrations (which caused also contractile responses, with a slower recovery of the normal responsive state). The preparation was washed after a 90 s contact time with either agent.

The relaxant component of the response was measured from the baseline to the deepest point of the relaxation and the contractile component from the baseline to the highest point of the response. The dose-response curves were analyzed by linear regression of the double-reciprocal plot, from which $ED_{50}$ values were obtained.

Bradykinin was a synthetic product made in this laboratory (Sabia *et al.*, 1977). The inorganic salts were products of the highest analytical grade from Merck Darmstadt. Vitamin $D_3$ (cholecalciferol) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

All data were expressed as means ± s.e.means and were analyzed by Student's *t* test.

## Results

In the SHR, low bradykinin concentrations elicited only small relaxations that did not show concentration-dependence in the range 0.4–2 nM (Figure 1a). At bradykinin concentrations of 4 nM and above, the SHR duodenum responded with a concentration-dependent contraction preceded by a small relaxation. This predominantly contractile response of the SHR duodenum contrasted with the predominantly relaxant response of the NWR and WKY duodenum, in agreement with a previous report (Miasiro *et al.*, 1985). The typical responses shown in Figures 1b (WKY) and 1c (NWR) show a concentration-dependent relaxation in the range 0.4 nM, above which the responses consisted of a maximum relaxation followed by a concentration-dependent contraction. Concentration-response curves obtained in these experiments yielded the $ED_{50}$ values listed in Table 1.

The SHR which received 2% calcium lactate in the drinking water, or a daily diet supplementation with 12.5 μg vitamin $D_3$, had their blood pressure reduced to normal levels, whereas no significant changes were observed in the blood pressure of WKY animals submitted to the same treatment (Figure 2).

In the calcium-treated SHR, the responses of the duodenum to bradykinin remained predominantly contractile, with a minor relaxant component which was not dose-dependent, similar to that seen in non-treated SHR (compare Figures 1a and 3b). However, a marked change was observed in the behaviour of the duodenum preparations of vitamin $D_3$-treated SHR (Figure 3a), which became similar to those of the NWR and WKY preparations, with concentration-dependent relaxation in the range 0.4–40 nM, and appearance of the contractile component of the response only at concentrations above 4 nM. These changes were evident by a comparison of the concentration-response curves in the treated and control groups, summarized by the $ED_{50}$ values shown in Table 1.

It is interesting to note that the vitamin $D_3$ treatment also changed the responses of the duodenum from the normotensive controls. The $ED_{50}$ values for the relaxant component of the responses of both NWR and WKY rats were significantly decreased after the treatment (Table 1). As for the contractile component, the duodenum of the vitamin $D_3$-treated WKY, but not that of the NWR, presented a shift to the right on

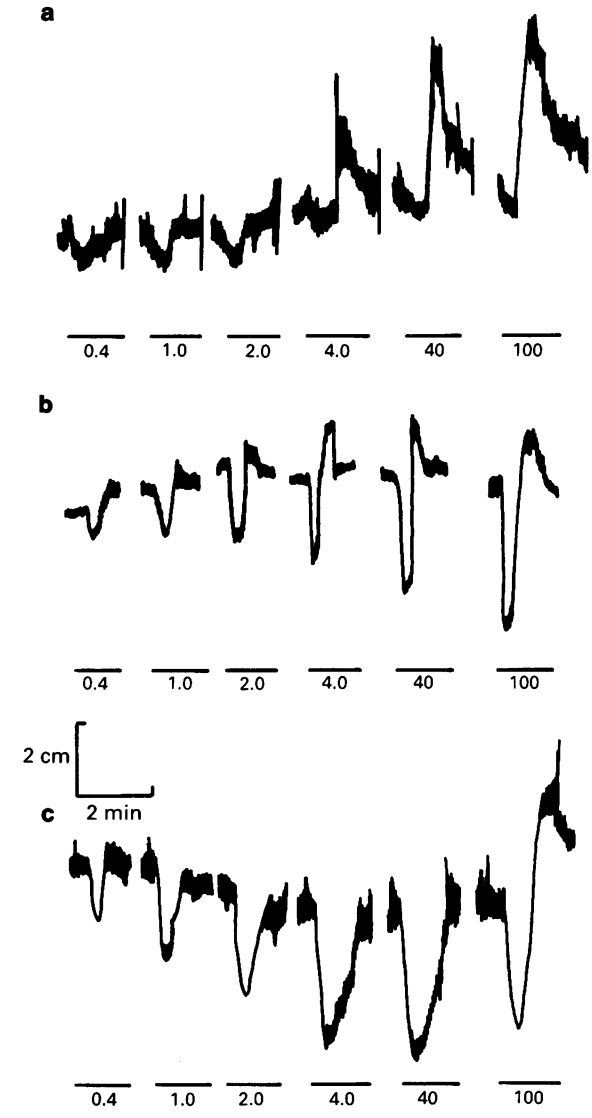


**Figure 1** Responses of three rat duodenum preparations from SHR (a), WKY (b), and NWR (c) to different concentrations (in nM) of bradykinin. Each treatment lasted for 90 s (horizontal bar) and was followed by washing and a resting period of 13.5 min (indicated by the interruptions in the tracings). The amplitude of the isotonic responses was magnified 6 fold. These results are representative of those obtained in at least 8 experiments.

**Table 1** $ED_{50}$ values for the relaxant and contractile components of the responses of rat duodenum before and after treatment with vitamin $D_3$

| | *Relaxation* | | *Contraction* | |
|---|---|---|---|---|
| | *Before treatment* | *After treatment* | *Before treatment* | *After treatment* |
| NWR | $6.76 \times 10^{-9} \pm 0.6$ | $5.88 \times 10^{-10} \pm 0.16$* | $1.58 \times 10^{-7} \pm 0.9$ | $1.00 \times 10^{-7} \pm 0.87$ |
| SHR | ND | $4.89 \times 10^{-9} \pm 0.79$ | $3.02 \times 10^{-9} \pm 0.81$ | $5.01 \times 10^{-8} \pm 0.81$* |
| WKY | $1.62 \times 10^{-8} \pm 0.79$ | $5.89 \times 10^{-10} \pm 0.32$* | $5.13 \times 10^{-9} \pm 0.76$ | $1.58 \times 10^{-8} \pm 0.76$* |

Values are means ± s.e.means of 6–8 experiments. *Significantly different from values before treatment ($P < 0.5$); ND indicates that $ED_{50}$ was not determined because the effect was not concentration-dependent.

the bradykinin dose-response curves after the treatment.

Duodenum preparations from SHR and WKY presented an impaired relaxant response to $K^+$ that was interpreted as a result of the inhibition of the $Na^+/K^+$ pump activity in these animal strains, as compared with the NWR (Miasiro *et al.*, 1985). After vitamin $D_3$ treatment, duodenum from all three strains presented a significant increase of the relaxant response to $K^+$ (Figure 3).

## Discussion

The blood pressure of SHR was shown to be lowered by chronic dietary supplementation with either calcium (Ayachi, 1979; Bukoski *et al.*, 1986) or vitamin $D_3$ (Vianna *et al.*, 1992). The mechanism involved in the hypotensive effect of calcium supplementation in the SHR has been the subject of much investigation (for a review, see Bukoski *et al.*, 1986), but more recent evidence suggests that the lowering of the blood viscosity due to a decreased haematocrit may play a major role in this mechanism (Susic *et al.*, 1984).

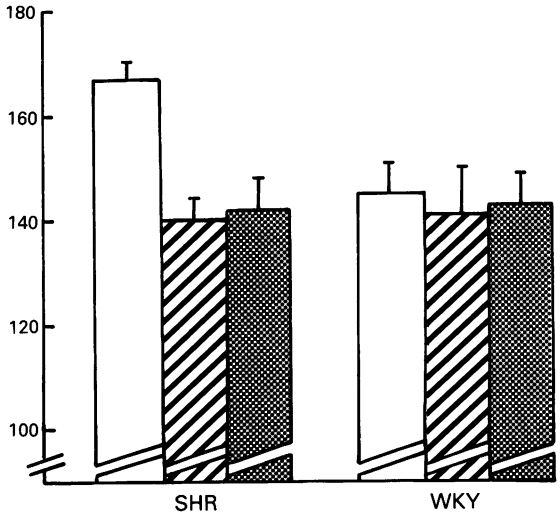


**Figure 2** Effect of treatment with vitamin $D_3$ or calcium on the blood pressure of SHR and WKY rats. Open columns, controls; hatched columns after supplementation with 12.5 μg vitamin $D_3$ 100 $g^{-1}$ body weight daily; stippled columns, after 14 days receiving 2% calcium lactate in the drinking water. Data are means of 6 animals and the s.e.mean is indicated by vertical bars.

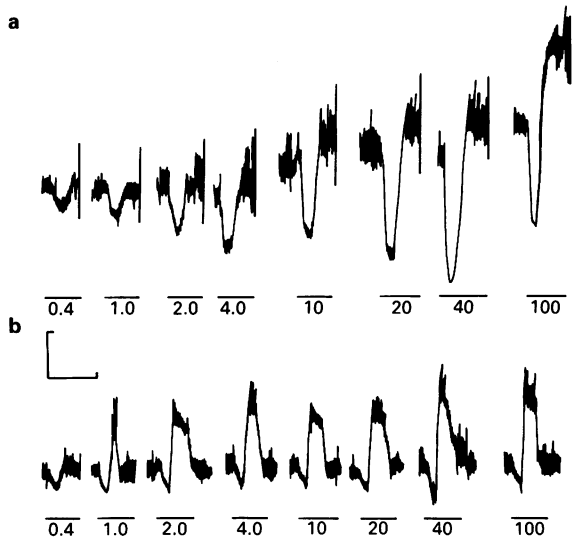


**Figure 3** Responses of two rat duodenum preparations from SHR after treatment for two weeks with: (a) 12.5 μg vitamin $D_3$ 100 $g^{-1}$ body weight daily, by gavage; (b) 2% calcium lactate in the drinking water. The indicated concentrations of bradykinin (in nM) were applied for 90 s (horizontal bars), followed by washing and a resting period of 13.5 min (interruptions in the tracings). The amplitude of the isotonic responses was magnified 6 fold.

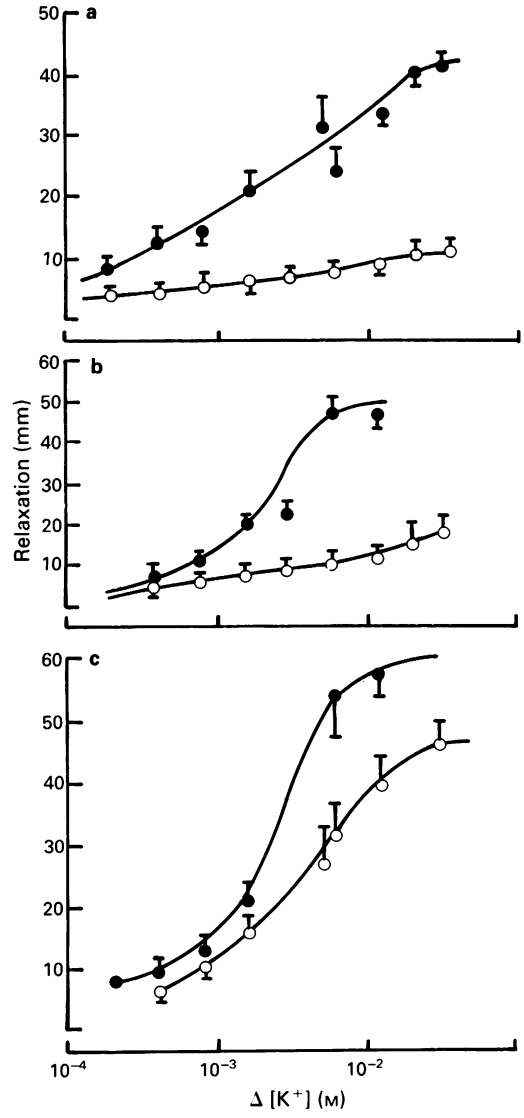


**Figure 4** Concentration-response curves for the relaxant component of the duodenum response to KCl in duodenum preparations isolated from SHR (a), WKY (b) and NWR (c): (●) treated with 12.5 μg vitamin $D_3$; (○) controls.

In the present work we have investigated how dietary supplementation with calcium or vitamin D affects the abnormal behaviour of the SHR duodenum towards the effects of bradykinin. We have found that, although both treatments are equally effective in lowering the blood pressure in these animals, only the supplementation with vitamin $D_3$ changed the duodenum response from predominantly contractile to predominantly relaxant, making the behaviour of preparations from vitamin $D_3$-treated SHR indistinguishable from those of the normotensive controls. It may be concluded that the vitamin treatment besides having a mechanism in common with that of calcium supplementation with respect to blood pressure lowering, affects the duodenum responses through a different mechanism.

In duodenum preparations of normotensive rats, bradykinin has a predominantly relaxant effect, a contractile

component becoming evident only at high concentrations, in which a biphasic response is observed, namely, a maximum relaxation followed by contraction. Previous work has indicated that the relaxant and contractile components of the response are respectively due to activation of $B_2$ and $B_1$ receptor populations (Paiva *et al.*, 1989).

The mechanism underlying the relaxant effect induced by bradykinin in several smooth muscle preparations has been shown to involve hyperpolarization due to the opening of apamin-sensitive calcium-activated $K^+$ channels (Carter *et al.*, 1986; Hall *et al.*, 1991). The activation of these channels depends on calmodulin levels (Pershadsingh *et al.*, 1986; Okada *et al.*, 1987), which are depressed in several tissues of the SHR (Baba *et al.*, 1987). Since vitamin D promotes the synthesis of several calcium-binding proteins, including calmodulin (Gross & Kumar, 1990; Fernandez *et al.*, 1990), it is conceivable that its effect on the relaxant component of the bradykinin response may be due to an increase in the calmodulin content in the SHR duodenum and consequent increase in the responsivity of calcium-activated $K^+$ channels.

Concerning the contractile component of the response, Boschcov *et al.* (1984) have proposed that a deficient calcium handling by the cell membrane in SHR favours both an increased $Na^+$ conductance and the duodenum contraction induced by high bradykinin concentrations. Vitamin $D_3$ treatment, by increasing the binding of calcium to the cell membrane promotes membrane stability (Bukoski *et al.*, 1986) decreasing $Na^+$ conductance and consequently might be responsible for the decrease in the contractile component of the responses to bradykinin in the vitamin D-treated SHR. In addition, the decrease of the contractile component of the response of the duodenum of vitamin D-treated SHR may in part be due to the increase in the relaxant component, since the biphasic response of gastrointestinal smooth muscle preparations appears to reflect a balance between two opposing actions (Carter *et al.*, 1985).

The relaxation induced by KCl, which is impaired in SHR, was also normalized after the vitamin $D_3$ treatment. However, this was a generic effect, since the duodenum of NWR and WKY also showed a significant increase in the relaxant component of the response to potassium. The relaxation induced by the increase of extracellular potassium is attributed to hyperpolarization due to the stimulation of the $Na^+/K^+$ pump (Rinaldi *et al.*, 1989). The decreased relaxant response of the SHR and WKY duodenum to potassium confirmed previous findings from our laboratory which showed an inhibition of the $Na^+/K^+$ pump in this strain (Miasiro *et al.*, 1985). Our results therefore suggest that the normalization of the relaxant response of the SHR by chronic treatment with vitamin $D_3$ may be due to an activation of this pump by vitamin D. The normalization of the $Na^+/K^+$ pump activity could also contribute to the hypotensive effect of vitamin $D_3$, since Bukosi *et al.* (1986) has already suggested a link between $Na^+/K^+$ pump activity normalization and the hypotensive effect of dietary calcium.

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# Effects of purines on the longitudinal muscle of the rat colon

S.J. Bailey & [1]S.M.O. Hourani

Receptors and Cellular Regulation Research Group, School of Biological Sciences, University of Surrey, Guildford, Surrey GU2 5XH

**1** Adenosine and adenosine 5'-triphosphate (ATP) have been reported to cause relaxation of the rat colon longitudinal muscle preparation; the purinoceptors mediating this effect were investigated by use of a series of agonists and antagonists.

**2** The tissue was precontracted with carbachol (1 μM), and the purines induced reversible relaxations with a potency order of 5'-N-ethylcarboxamidoadenosine (NECA) >$N^6$-cyclopentyladenosine (CPA)=adenosine 5'-(α,β-methylene) triphosphonate (AMPCPP) >adenosine=adenylyl 5'-(β,γ-methylene) disphosphonate (AMPPCP)=ATP. The $P_1$-selective antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (3 μM) shifted to the right the log concentration-response curves of all these agonists except for AMPCPP, indicating that they all act via $P_1$-purinoceptors. The order of potency of the adenosine analogues and the relatively high concentrations of the antagonist required indicated that these receptors are of the $A_2$ subtype. The $P_2$-selective antagonist suramin (300 μM) inhibited responses to AMPCPP, but not to the other agonists.

**3** The dephosphorylation of the nucleotides was studied by high performance liquid chromatography following incubation with the longitudinal muscle preparation for up to 30 min. ATP was rapidly degraded, largely to adenosine, and AMPPCP and AMPCPP were also degraded, although more slowly, to adenosine and adenosine 5'-(α,β-methylene) diphosphonate (AMPCP) respectively. AMPCP, like AMPCPP, caused relaxations by acting on $P_2$-purinoceptors, as it was also inhibited by suramin (300 μM). Incubation of the tissue with adenosine deaminase abolished responses to adenosine, reduced those to ATP and AMPPCP, but had no effect on those to AMPCPP. ATP and AMPPCP therefore appear to be acting on the $A_2$ receptors in this tissue largely via their degradation product adenosine.

**4** The longitudinal muscle of the rat colon therefore contains both $P_1$- and $P_2$-purinoceptors, which both mediate relaxation. The $P_1$-purinoceptors are of the $A_2$ subtype and the $P_2$-purinoceptors are probably of the $P_{2Y}$ subtype, although the rapid degradation of the nucleotides means that it is difficult to classify them with certainty.

**Keywords:** Rat colon longitudinal muscle; purinoceptors; ATP; adenosine; ectonucleotidases; suramin

## Introduction

The purinoceptors which mediate the many and varied pharmacological responses to adenosine and adenine nucleotides have been clearly subdivided into $P_1$, recognising adenosine, and $P_2$, recognising adenosine 5'-triphosphate (ATP) (Burnstock, 1978), and selective antagonists for each of these receptor types exist. Xanthine derivatives such as theophylline and its analogues are $P_1$-purinoceptor antagonists, having no effect on the actions of ATP in most tissues, and the trypanocidal drug suramin has recently been shown to act as a $P_2$-purinoceptor antagonist in a number of tissues (Dunn & Blakely, 1988; Stone, 1989; Den Hertog *et al.*, 1989a; b; Hoyle *et al.*, 1990; Burnstock, 1990; Kennedy, 1990; Leff *et al.*, 1990; Bailey *et al.*, 1992). As well as acting at $P_2$-purinoceptors, ATP is rapidly and sequentially dephosphorylated by ectonucleotidases present on the surface of cells, ultimately to adenosine, which is more slowly taken up into cells or deaminated to the inactive inosine (Pearson & Slakey, 1990). This transformation of ATP into adenosine may affect the observed responses to ATP, as not only will it reduce its effects at $P_2$-purinoceptors but it may also result in indirect effects at $P_1$-purinoceptors. Unfortunately, no selective inhibitors of this breakdown are available (Hourani & Chown, 1989), but the contribution of adenosine to the effects of ATP can be investigated using adenosine deaminase (ADA) to remove any adenosine produced, or inhibitors of adenosine uptake such as S-(4-nitrobenzyl)-6-thioguanosine (NBTG) to enhance the effects of adenosine.

$P_1$-purinoceptors have been subdivided into $A_1$ and $A_2$, and these can be distinguished in functional studies by the order of potency of adenosine analogues, with $N^6$-substituted compounds such as $N^6$-cyclopentyladenosine (CPA) being more potent than 5'-substituted compounds such as 5'-N-ethylcarboxamidoadenosine (NECA) on $A_1$ receptors, but less potent on $A_2$ receptors. In addition, the antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) has nanomolar affinity for $A_1$ receptors and micromolar affinity for $A_2$ receptors, and clearly distinguishes between the two receptor subtypes in isolated tissue preparations (Collis *et al.*, 1989; Collis, 1990; Bruns, 1990; Jacobson, 1990). In general, $A_2$ receptors have been found to mediate the inhibitory effects of adenosine on smooth muscle, causing either relaxation of precontracted muscle or reduction in the contractile effects of other agonists, whereas $A_1$ receptors mediate contractile effects and the presynaptic inhibition of transmitter release (White, 1988; Olsson & Pearson, 1990).

$P_2$-purinoceptors on smooth muscle have also been subdivided into $P_{2X}$ and $P_{2Y}$ (Burnstock & Kennedy, 1985; Gordon, 1986) and these may be distinguished by the use of ATP analogues (Cusack & Hourani, 1990), although this subdivision is not as firmly based as that for $P_1$-purinoceptors as no selective competitive antagonists exist, suramin being equally effective on each subtype (Dunn & Blakely, 1988; Den Hertog *et al.*, 1989a; b; Hoyle *et al.*, 1990; Leff *et al.*, 1990). On $P_{2Y}$ receptors, 2-substituted analogues such as 2-methylthioadenosine 5'-triphosphate (2-MeSATP) are more potent than ATP which is more potent than its methylene phosphonate analogues such as adenosine 5'-(α,β-methylene) triphosphonate (AMPCPP) and adenylyl 5'-(β,γ-methylene)

[1] Author for correspondence

diphosphonate (AMPPCP), whereas on $P_{2X}$ receptors AMPPCP and AMPCPP are more potent than ATP which is equipotent with 2-MeSATP (Burnstock & Kennedy, 1985). Although the structure-activity relationships for ATP and its analogues in some tissues may be affected by the rapid degradation of ATP and its 2-substituted analogues and the relative stability of the methylene phosphonate analogues (Welford *et al.*, 1986; 1987), the different structure-activity relationships for $P_{2X}$ and $P_{2Y}$ receptors have been confirmed by use of a series of stable isopolar phosphonate ATP analogues (Cusack *et al.*, 1987). In addition, two nucleotide analogues, L-adenylyl 5'-(β,γ-methylene) diphosphonate (L-AMPPCP) and adenosine 5'-(2-fluorodiphosphate) (ADP-β-F) have been developed as selective agonists for $P_{2X}$ and $P_{2Y}$ receptors respectively (Hourani *et al.*, 1986; 1988). Although in general, $P_{2X}$ receptors mediate contraction of smooth muscle and $P_{2Y}$ receptors mediate relaxation and this was a basis for the original subdivision (Burnstock & Kennedy, 1985), this is not a secure criterion for receptor classification and indeed in two tissues, neonatal rat duodenum and the rat colon muscularis mucosae, ATP induces contraction not relaxation via receptors whose structure-activity relationships are indicative of the $P_{2Y}$ subtype (Nicholls *et al.*, 1990; Bailey & Hourani, 1990).

A question which has been a matter of continuing controversy is the extent to which ATP may interact directly with $P_1$ receptors (Stone, 1989; Bruns, 1990). There have been a number of reports of the effects of ATP being blocked by $P_1$ antagonists in some tissues, although it is not always clear whether this is a direct effect of ATP or due to its degradation to adenosine. In some cases the more stable analogue AMPPCP has also been shown to have $P_1$ effects, which has generally been interpreted as indicating that the $P_1$ effects of ATP itself are direct, not via its breakdown to adenosine (Dahlen & Hedqvist, 1980; Collis & Pettinger, 1982; Wiklund *et al.*, 1985), although it has also been taken as evidence that AMPPCP is itself degraded (Moody & Burnstock, 1982), or that a third class of purinoceptor, called $P_3$, exists at which both adenosine and adenine nucleotides are active (Shinozuka *et al.*, 1988). This controversy may be resolved by our recent finding that in some tissues AMPPCP itself can have direct effects on $P_1$-purinoceptors whereas ATP, 2-MeSATP and AMPCPP do not, in spite of the fact that ATP is much more rapidly degraded to adenosine than is AMPPCP. In the rat colon muscularis mucosae and the rat duodenum, AMPPCP appears to act almost entirely via $P_1$ receptors rather than by the $P_{2Y}$ receptors which are also present, as its effects can be completely inhibited by the $P_1$ antagonist, 8-sulphophenyltheophylline (8-SPT) in the same way as those of adenosine, whereas in the guinea-pig taenia caeci, 8-SPT causes only a roughly two fold shift in the concentration-response curve to AMPPCP, indicating that it acts largely via the $P_{2Y}$ receptors here (Bailey & Hourani, 1990; Hourani *et al.*, 1991). Somewhat different $pA_2$ values were obtained for 8-SPT against AMPPCP in these tissues (approximately 5 in the taenia caeci, approximately 6 in the duodenum and colon), indicating that different $P_1$ receptors might be present, and that AMPPCP might have selectivity for $A_1$ receptors. However, as 8-SPT has been reported not to be selective for $A_1$ receptors in isolated tissues (Collis *et al.*, 1987) although it does show some selectivity in binding studies (Bruns *et al.*, 1986), these small differences were not easy to interpret. Another problem is that the affinity of $P_1$ receptors for agonists and antagonists is known to be dependent on the species used, with rat tissues in general having a higher affinity than guinea-pig tissues (Collis, 1990), which could also explain the differences we observed with 8-SPT. However, using a series of agonists and the highly $A_1$-selective antagonist DPCPX we have shown that on the rat colon muscularis mucosae the $P_1$ receptors are of the $A_1$ subtype whereas those on the rat duodenum are a mixture of $A_1$ and $A_2$, with AMPPCP apparently acting on the $A_1$ population although adenosine acts on the $A_2$ (Nicholls *et al.*, 1992; Bailey *et al.*, 1992). As the $P_1$ receptors on the taenia caeci are known to be of the $A_2$ subtype (Burnstock *et al.*, 1984), this would be consistent with the hypothesis that AMPPCP has selectivity for $A_1$ receptors, although species differences could also play a role. In addition, in the rat urinary bladder in which AMPPCP causes contractions via $P_{2X}$ receptors, its effects were not enhanced by a concentration of DPCPX which inhibited the effect of adenosine, suggesting that it had no action on the inhibitory $A_2$ receptors present in this tissue (Nicholls *et al.*, 1992).

To clarify this matter we wished to use a rat tissue similar to the guinea-pig taenia caeci, which relaxes to purines via $P_{2Y}$ and $A_2$ receptors, and to investigate whether AMPPCP would have any $P_1$ effect in this tissue. Rather than use a vascular tissue such as the rat aorta in which there is the complication that any observed relaxation to adenosine or to ATP may be indirect, via receptors on the endothelium rather than on the smooth muscle (Rose'Meyer & Hope, 1990; Olsson & Pearson, 1990), we chose to investigate the longitudinal muscle of the rat colon. This tissue has not been widely used but has been reported to relax in response to adenosine and to ATP when precontracted with the muscarinic antagonist oxotremorine (Romano, 1981), and therefore appeared likely to be pharmacologically equivalent to the guinea-pig taenia caeci.

## Methods

### *Pharmacological studies*

Male Wistar rats (150–250 g) were killed by cervical dislocation and the distal colon removed and placed in warm (32°C) Tyrode buffer (ionic composition (mM): $Na^+$ 149.1, $K^+$ 2.8, $Ca^{2+}$ 1.8, $Mg^{2+}$ 2.1, $Cl^-$ 147.5, $H_2PO_4^-$ 0.3, $HCO_3^-$ 11.9, glucose 5.6) pregassed with 95%$O_2$/5% $CO_2$. The dissection of the longitudinal muscle of the colon was carried out as described by Romano (1981) and Bailey & Jordan (1984), with minor modifications. Briefly, a glass pipette, external diameter 5 mm, was placed inside the colon and the outer tubular layer of longitudinal muscle was removed by gentle rubbing with wet cotton wool, leaving a thick walled tube, the muscularis mucosae. The longitudinal muscle was suspended in a 3 ml organ bath at 32°C in gassed Tyrode solution, and contractions were recorded isometrically under a resting tension of 1 g with a Grass FT03 strain gauge and displayed on a Grass 79C polygraph. The tone of the tissue was raised with carbachol (1 μM for 3 min, which caused 50–70% of the maximal contraction) and relaxations to adenosine and adenine nucleotides were expressed as % relaxation of this carbachol-induced contraction. The longitudinal muscle was allowed to equilibrate for 90 min before control concentration-response curves to purinoceptor agonists were determined, followed by incubation with an antagonist, ADA or NBTG for 40 min before the concentration-response curves were repeated in the presence of these substances. Recovery of responses to the agonists was established following washout of these substances for up to 40 min; 12–20 min were allowed between doses of the purinoceptor agonists, which were left in contact with the tissue for 2–5 min until a maximal relaxation had been observed.

### *Degradation studies*

Segments of rat colon longitudinal muscle (approximately 30 mg wet weight) were suspended in 3 ml organ baths as described for the pharmacological studies. Following preincubation for 90–120 min with frequent washing, each was exposed to ATP, AMPPCP and AMPCPP (100 μM at 30 min intervals), and samples of the bathing medium were taken at 2, 5, 10 and 20 min and frozen for later analysis by high performance liquid chromatography as described by Welford *et al.* (1986, 1987).

*Materials*

ATP, AMPPCP, AMPCPP, AMPCP, adenosine, ADA (Type VI) and NBTG were obtained from Sigma UK. Ltd, DPCPX, 2-MeSATP, CPA and NECA were obtained from Research Biochemicals, and suramin was a generous gift from Bayer, UK. Other purine analogues were kindly provided by Dr Noel J. Cusack, Whitby Research Inc, Richmond, Virginia, U.S.A. CPA (10 mM) was dissolved in 20% ethanol, DPCPX (1 mM) was dissolved in 2% aqueous dimethylsulphoxide (DMSO) containing 6 mM NaOH and NBTG (50 mM) was dissolved in DMSO. After dilution corresponding to the final bath concentration of the substances used these solvents had no effect on the responses of the tissue. The ADA solution was supplied in 50% glycerol-0.01 M potassium phosphate and was diluted in distilled water to give a stock solution of 200 units $ml^{-1}$.

## Results

Adenosine, ATP, AMPPCP, AMPCPP, NECA and CPA each relaxed the carbachol-contracted rat colon longitudinal muscle, and the order of potency was NECA> CPA = AMPCPP> adenosine = AMPPCP = ATP (Figure 1). DPCPX

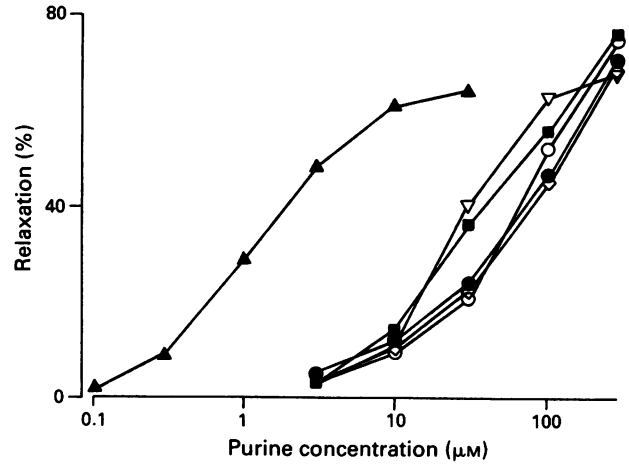


**Figure 1** Relaxation of the rat colon longitudinal muscle by adenosine (●), NECA (▲), CPA (■), ATP (○), AMPPCP (◇) or AMPCPP (▽). Each point is the mean of at least 15 determinations, and the error bars, which never exceeded 5%, have been omitted for clarity. For abbreviations, see text.

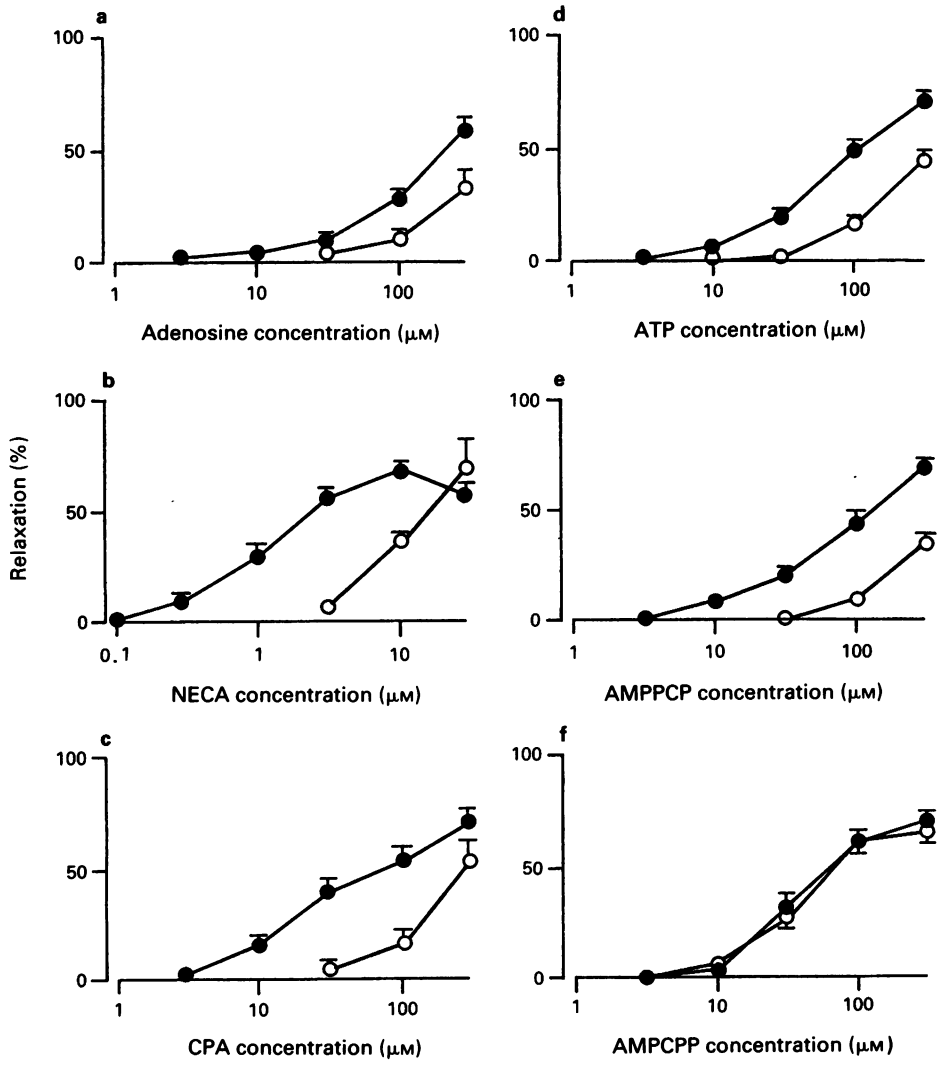


**Figure 2** Relaxation of the rat colon longitudinal muscle by (a) adenosine, (b) NECA, (c) CPA, (d) ATP, (e) AMPPCP or (f) AMPCPP alone (●) or in the presence of DPCPX (3 μM) (○). Each point is the mean of at least 4 determinations and the vertical bars show s.e.mean. For abbreviations, see text.

(3 μM) inhibited the responses to NECA, CPA, adenosine, AMPPCP and ATP, but did not affect the relaxations induced by AMPCPP (Figure 2), whereas suramin (300 μM) inhibited the responses to AMPCPP but not those of the other agonists (Figure 3). DPCPX (1 μM) caused smaller shifts to the right of the log concentration-response curves to all the agonists except for AMPCPP (results not shown), but Schild analysis was not performed as concentrations of DPCPX above 3 μM could not be achieved. L-AMPPCP (100 μM) and 2-MeSATP (100 μM) were almost inactive, ADP-β-F(100 μM) induced a relaxation similar to that of ATP (100 μM), while adenosine 5'-(α,β-methylene) diphosphonate (AMPCP) (100 μM) induced a relaxation similar to that of AMPCPP (100 μM) (Figure 4). Representative traces showing the relaxations induced by the agonists are shown in Figure 4. The responses to AMPCP were inhibited by suramin (300 μM) in a similar manner to those of AMPCPP (results not shown).

Incubation of the tissues with ADA (2 units $ml^{-1}$) abolished the relaxations induced by adenosine and inhibited those induced by ATP and AMPPCP, but had no effect on those induced by AMPCPP (Figure 5). Incubation of the tissues with NBTG (50 μM) potentiated responses to adenosine, causing a roughly three fold shift to the left of the log concentration-response curve, and also potentiated responses to AMPCPP but to a somewhat lesser extent (results not shown).

*Degradation studies*

Dephosphorylation of ATP by the rat longitudinal muscle preparation was rapid, with approximately 80% being degraded, largely to adenosine and AMP, during 30 min incubation. AMPPCP was degraded more slowly to adenosine, with approximately 20% conversion during 30 min incubation, and AMPCPP was degraded to AMPCP, with approximately 30% conversion over 30 min (Figure 6).

## Discussion

These results show that the outer longitudinal muscle layer of the rat colon contains $P_1$- and $P_2$-purinoceptors which both mediate relaxation, and that the responses of this tissue to purines are qualitatively similar to the responses seen in the guinea-pig taenia caeci, the longitudinal muscle of the guinea-pig caecum. For the $P_1$-purinoceptor in the longitudinal muscle the potency order of agonists is NECA> CPA> adenosine, and DPCPX has an apparent dissociation constant in the micromolar range, suggesting that the receptor is of the $A_2$ subtype (Bruns, 1990; Collis, 1990; Daly, 1990). DPCPX (3 μM) caused a somewhat smaller shift to the right of the log concentration-response curve to adenosine compared with NECA and CPA, which may suggest that it also interfered with adenosine uptake. In general the post-

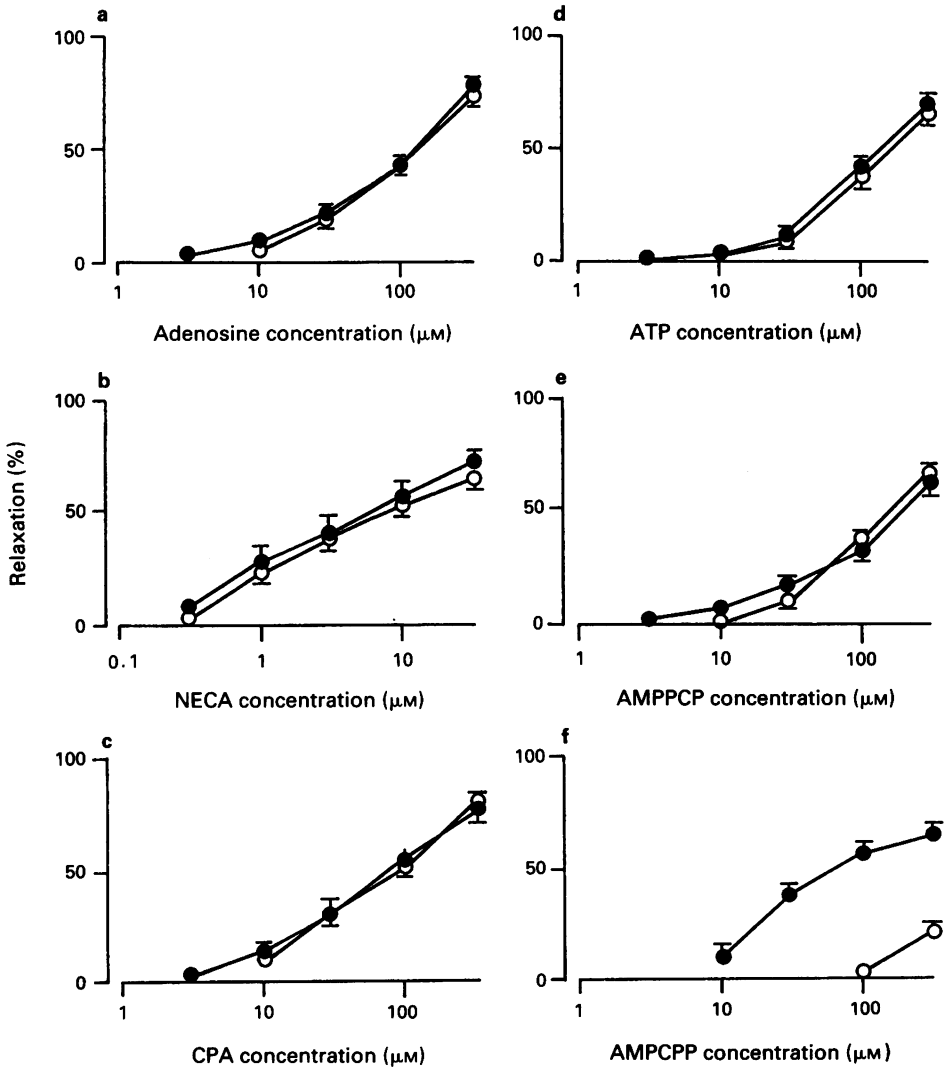


**Figure 3** Relaxation of the rat colon longitudinal muscle by (a) adenosine, (b) NECA, (c) CPA, (d) ATP, (e) AMPPCP or (f) AMPCPP alone (●) or in the presence of suramin (300 μM) (○). Each point is the mean of at least 4 determinations and the vertical bars show s.e.mean. For abbreviations, see text.

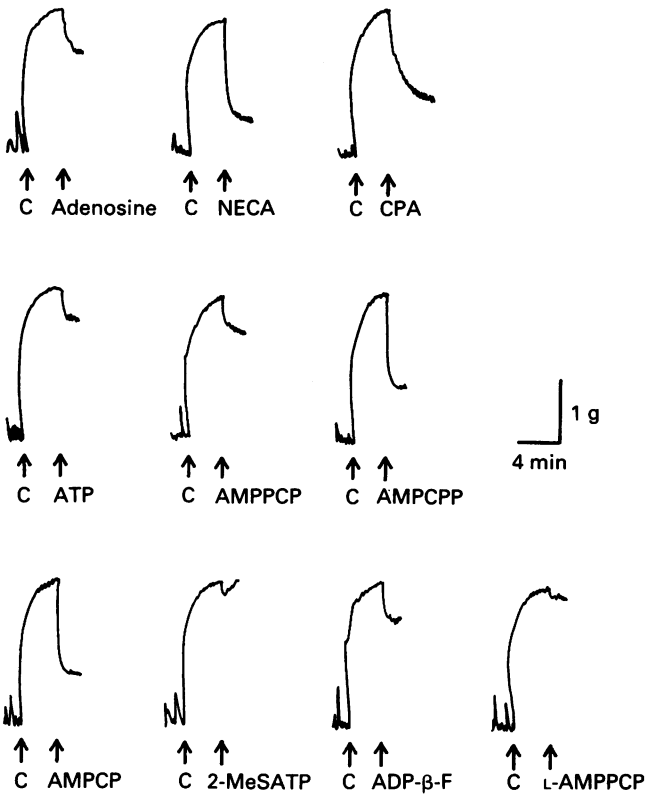


**Figure 4** Representative traces showing relaxations of the rat colon longitudinal muscle to purines after precontraction of the tissue with carbachol (1 μM) (C). The concentration of purine added in each case was 100 μM, except for NECA which was 10 μM. For abbreviations used in figure, see text.

synaptic inhibitory effects of adenosine in smooth muscle including the guinea-pig taenia caeci are mediated by $A_2$ receptors, so the finding that the adenosine receptor here is of the $A_2$ subtype is consistent with results in other tissues (White, 1988; Stone, 1989; Kennedy, 1990; Olsson & Pearson, 1990). In those tissues in which adenosine causes contraction, such as the renal vasculature (Kenakin & Pike, 1987) and the guinea-pig myometrium (Smith *et al.*, 1988), this appears to be via $A_1$ receptors, and this is also the case in the rat colon muscularis mucosae (Bailey *et al.*, 1992). It has also been reported that the guinea-pig aorta and trachea each possess both $A_1$ and $A_2$ receptors which mediate contraction and relaxation respectively, although for adenosine the $A_2$-mediated relaxation is dominant (Farmer *et al.*, 1988; Stoggall & Shaw, 1990). The existence in the rat colon of excitatory $A_1$ receptors (on the muscularis mucosae) and inhibitory $A_2$ receptors (on the longitudinal muscle) is therefore consistent with this general pattern, although the two receptor types are clearly at anatomically distinct locations within the colon, a possibility which has not been investigated in the other tissues.

The rat colon longitudinal muscle also possesses $P_2$-purinoceptors, as AMPCPP caused a relaxation which was inhibited by the $P_2$-purinoceptor antagonist suramin but not by DPCPX. The actions of ATP and AMPPCP appeared however to be mediated via the $A_2$ receptor, as they were not inhibited by suramin but were inhibited by DPCPX at the same concentration as was adenosine. It seems likely that this $A_2$ effect was at least partially indirect and a result of the degradation of ATP and AMPPCP to adenosine, as the responses to ATP and AMPPCP were reduced by ADA, which had no effect on the responses to AMPCPP. The adenosine uptake inhibitor NBTG potentiated responses to adenosine as expected, but also potentiated responses to AMPCPP which does not act on $A_2$ receptors here, and therefore was not useful for resolving the question as to whether the actions of ATP and AMPPCP were direct or via adenosine. The potentiation by NBTG of responses to AMP-

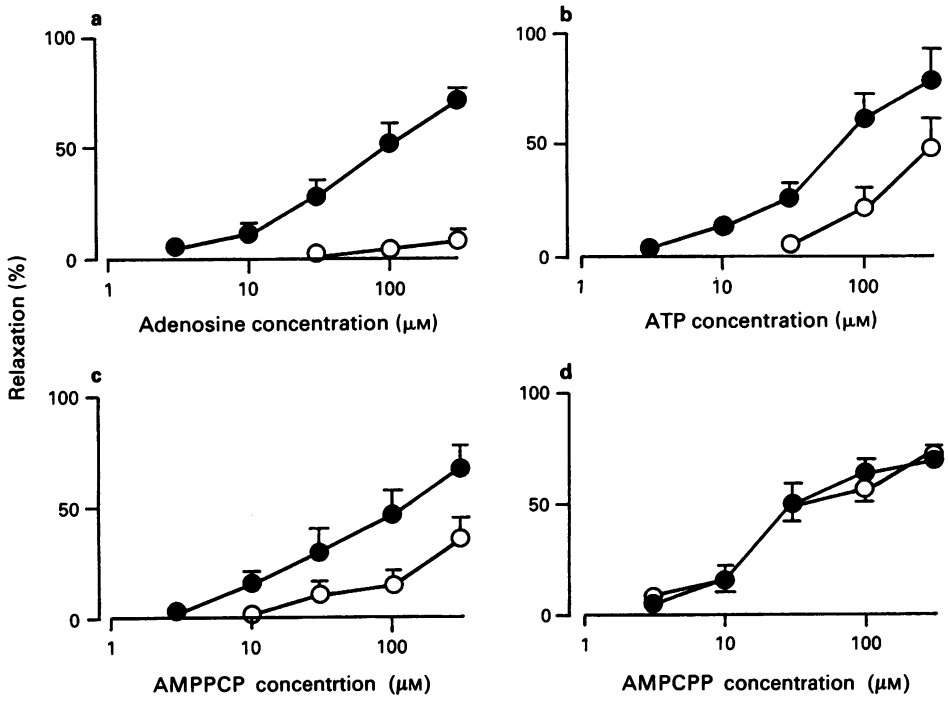


**Figure 5** Relaxation of the rat colon longitudinal muscle by (a) adenosine, (b) ATP, (c) AMPPCP or (d) AMPCPP alone (●) or in the presence of adenosine deaminase (2 units $ml^{-1}$) (○). Each point is the mean of at least 5 determinations and the vertical bars show s.e.mean. For abbreviations, see text.

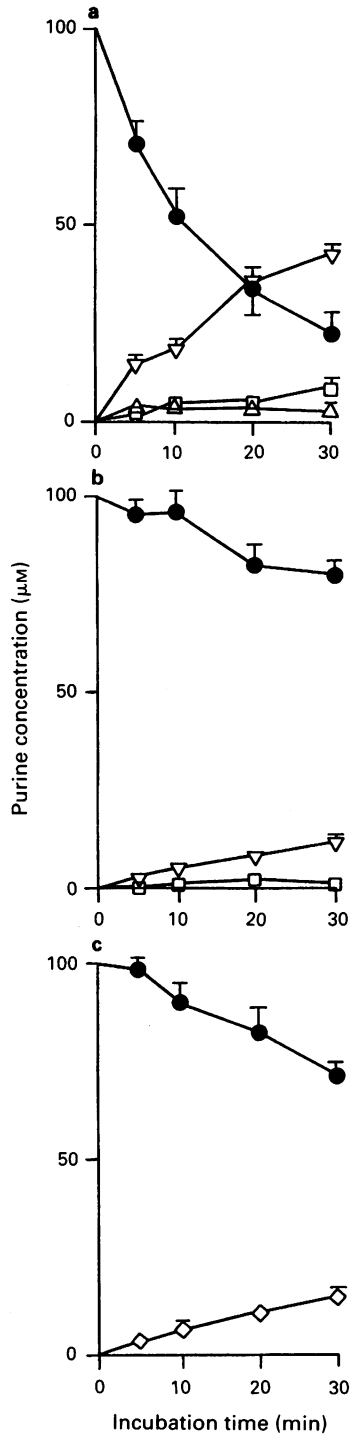


**Figure 6** Degradation of purines by the rat colon longitudinal muscle preparation. Removal of (a) ATP, (b) AMPPCP or (c) AMP-CPP (●) and appearance of ADP (△), AMP (□), adenosine (▽) or AMPCP (◇). Each point is the mean of at least 4 determinations and the vertical bars show s.e. mean. For abbreviations, see text.

CPP was presumably due to increased endogenous adenosine levels enhancing all relaxant responses, but this was not investigated further. It is probable that AMPCPP retained its $P_2$-purinoceptor activity even though it is also significantly degraded, to at least the same extent as AMPPCP, because its breakdown product was not adenosine but AMPCP, which was inhibited by suramin and therefore acts on $P_2$-purinoceptors. These findings are in contrast to our results with the rat colon muscularis mucosae and the rat duodenum, where it is clear that despite its rapid degradation, ATP has little if any $P_1$-purinoceptor activity whereas AMPPCP acts almost entirely and directly via $P_1$-purinoceptors (Bailey & Hourani, 1990; Hourani *et al.*, 1991). Unfortunately the uncertainty regarding the direct $A_2$ receptor activity of AMPPCP itself in the longitudinal muscle means that the use of this tissue has not resolved our initial hypothesis that AMPPCP might have $A_1$-selective $P_1$-purinoceptor activity, as had been suggested by our previous studies (Bailey & Hourani, 1990; Nicholls *et al.*, 1990; Hourani *et al.*, 1991; Bailey *et al.*, 1992; Nicholls *et al.*, 1992).

Although the $P_2$-purinoceptor which mediates relaxation in the guinea-pig taenia caeci is clearly of the $P_{2Y}$ subtype, with an order of potency of 2-MeSATP>ATP>AMPCPP (Burnstock *et al.*, 1984; Burnstock & Kennedy, 1985), the $P_2$-purinoceptor in the rat colon longitudinal muscle is not so easy to define by use of these agonists as ATP itself had no $P_2$-purinoceptor activity in this tissue and 2-MeSATP was almost inactive. The weak activity of 2-MeSATP may be due to its rapid breakdown to 2-methylthioadenosine, which is somewhat less active than adenosine on the $A_2$ receptors in the guinea-pig taenia caeci (Satchell & Maguire, 1975) and is therefore likely to be less active in the longitudinal muscle preparation. Indeed the very rapid degradation of the nucleotides, including the normally resistant AMPPCP and AMPCPP, by this preparation makes analysis of the structure-activity relationships of the $P_2$-purinoceptor very difficult. The response mediated by this receptor is a relaxation which is qualitatively similar to that seen in the guinea-pig taenia caeci, which might suggest involvement of a $P_{2Y}$-purinoceptor, but this is not a reliable method for receptor classification, particularly in view of the existence of $P_{2Y}$-purinoceptors which mediate contraction on the muscularis mucosae and the neonatal rat duodenum (Bailey & Hourani, 1990; Nicholls *et al.*, 1990). The lack of activity of L-AMPPCP and the activity of ADP-β-F in the longitudinal muscle would appear to confirm that the $P_2$-purinoceptor here is of the $P_{2Y}$ subtype, as L-AMPPCP and ADP-β-F are selective agonists at $P_{2X}$- and $P_{2Y}$-purinoceptors respectively (Hourani *et al.*, 1986; 1988). However, the same argument could in theory apply to L-AMPPCP as to 2-MeSATP, as its potential breakdown product, L-adenosine, is inactive on $P_1$-purinoceptors (Brown *et al.*, 1982), and similarly ADP-β-F could be acting via its probable breakdown product, adenosine, so this evidence cannot be regarded as conclusive. What is needed, both to define the $P_2$-purinoceptor in this tissue and to resolve the question of whether ATP and AMPPCP act directly or indirectly on the $P_1$-purinoceptor, is a selective inhibitor of the ectonucleotidases which are responsible for the breakdown.

Notwithstanding these uncertainties, it is clear from the work presented here together with our previous work (Bailey & Hourani, 1990; Bailey *et al.*, 1992), that the two preparations from the rat colon, the muscularis mucosae and the longitudinal muscle, have different $P_1$-purinoceptor subtypes, $A_1$ mediating contraction and $A_2$ mediating relaxation, and that they also possess $P_2$ purinoceptors which mediate the same effect in each case as the $P_1$ purinoceptors. The physiological significance of these receptors is unknown, but probably reflects the widespread roles of purines as transmitters and modulators in the autonomic nervous system. The nature of the $P_2$-purinoceptor on the longitudinal muscle is still undefined, but it is likely to be of the $P_{2Y}$ subtype as in the muscularis mucosae, although the response observed is different in the two preparations. In the longitudinal muscle preparation the breakdown of nucleotides is extremely rapid, and is considerably faster than that previously observed in the muscularis mucosae in spite of the fact that the longitudinal muscle is a much smaller tissue, with a wet weight of about 30 mg compared to around 200 mg for the muscularis mucosae. This very rapid degradation undoubtedly affects the responses to the nucleotides, and this makes the study of the pharmacology of the $P_2$-purinoceptors in this

tissue somewhat problematical, and shows clearly the care which must be taken when attempting to use nucleotide agonist potencies to define receptor subtypes.

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# Effectiveness of $GABA_B$ antagonists in inhibiting baclofen-induced reductions in cytosolic free Ca concentration in isolated melanotrophs of rat

I. Shibuya, [2]S. Kongsamut & [1]W.W. Douglas

Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut, 06510, U.S.A.

**1** The purpose of the present experiments was to assess the activities of $GABA_B$ receptor antagonists in mammalian isolated melanotrophs.

**2** Cytosolic free Ca concentration ($[Ca^{2+}]_i$) in rat melanotrophs in primary culture was monitored with the fluorescent probe, fura-2.

**3** (−)-Baclofen lowered $[Ca^{2+}]_i$ in a concentration-dependent manner with an $EC_{50}$ of 0.96 μM. The reduction in $[Ca^{2+}]_i$ produced by (−)-baclofen at a maximally effective concentration (100 μM) was similar to that produced by the classic transmitter inhibitory to melanotroph secretion, dopamine, at a corresponding concentration (100 nM), or by perifusion with a nominally Ca-free solution.

**4** The $GABA_B$ receptor antagonists, 3-aminopropyl(diethoxymethyl)phosphinic acid (CGP 35348), 2-hydroxy saclofen, phaclofen and 4-amino-3-(5-methoxybenzo[b]furan-2-yl) butanoic acid (9H), had inhibitory effects on the reduction in $[Ca^{2+}]_i$ produced by (−)-baclofen (3 μM). Of the antagonists tested, CGP 35348 was the most potent with an $IC_{50}$ of 60 μM, compared to 120 to 400 μM for the others. CGP 35348 acted competitively.

**5** CGP 35348 alone had no effect on basal $[Ca^{2+}]_i$, or on the changes in $[Ca^{2+}]_i$ produced by dopamine (10 nM) or the specific $GABA_A$ receptor agonist, muscimol (10 μM).

**6** The evidence indicates that of the antagonists tested, CGP 35348 offers the greatest promise for pharmacological analysis of the functional significance of the $GABA_B$ receptors in melanotrophs.

**Keywords:** Melanotroph; $GABA_B$ receptors; $GABA_B$ antagonists; CGP 35348; 2-hydroxy saclofen; phaclofen; baclofen; $GABA_A$ receptors; cytosolic calcium; fura-2

## Introduction

Melanotrophs, present as a virtually homogeneous population in the intermediate lobe of the pituitary gland, differ from other adenohypophyseal cells in being directly innervated. The nerves, which course from the hypothalamus through the infundibular stalk to form synaptic-like contacts with the melanotrophs, exert a tonic inhibitory influence on melanotroph secretion which otherwise occurs at a high spontaneous rate. The classic inhibitory transmitter is dopamine (see Holzbauer & Racké, 1985), but γ-aminobutyric acid (GABA) has also been detected in the nerves by immunohistochemical methods (Oertel *et al.*, 1982; Vincent *et al.*, 1982; Sakaue *et al.*, 1988), seemingly as a co-stored transmitter (Stoeckel *et al.*, 1985; Vuillez *et al.*, 1987; Schimchowitsch *et al.*, 1991).

The physiological function of this GABA is not clear (see Kongsamut *et al.*, 1991) but *in vitro* experiments have shown that application of GABA to melanotrophs directly affects electrical activity (Taraskevich & Douglas, 1982; 1985), output of hormones (Tomiko *et al.*, 1983; Demeneix *et al.*, 1984) and the concentration of cytosolic free Ca ($[Ca^{2+}]_i$) (Nemeth *et al.*, 1988; Taraskevich & Douglas, 1990). Moreover, release of endogeneous GABA has been demonstrated, albeit indirectly, by a 'postsynaptic' effect recorded in melanotrophs upon stimulation of the pituitary stalk (MacVicar & Pittman, 1986; Williams *et al.*, 1989).

One factor that has hampered progress in understanding the physiological relevance of GABA in the control of melanotroph function is that receptors of both $GABA_A$ and $GABA_B$ type are present on the cells (see discussion), and although the former are readily blocked by bicuculline, the classic $GABA_A$ antagonist, it is only recently that a $GABA_B$ antagonist effective in melanotrophs has been found; this is CGP 34348, a novel compound previously shown to possess effective $GABA_B$ antagonist activity on a variety of central nervous system preparations (Olpe *et al.*, 1990). The effectiveness of this compound in melanotrophs was detected in experiments conducted on isolated neurointermediate lobes of the toad, *Xenopus laevis* (Shibuya *et al.*, 1991), a species with remarkably powerful, bicuculline-resistant inhibitory effects of GABA on melanotroph secretion (Verburg van Kemenade *et al.*, 1987). In this preparation, CGP 35348 possessed useful antagonist activity, which contrasted with the lack of activity of other $GABA_B$ antagonists, namely, phaclofen, 2-hydroxy saclofen (Kerr *et al.*, 1987; 1988) and 9H (Beattie *et al.*, 1989). Thus CGP 35348, but not the other compounds, blocked the intense secreto-inhibitory effects of the $GABA_B$ agonist, baclofen, and of GABA given in the presence of bicuculline (to eliminate any $GABA_A$ component). Moreover, this block was selective: responses to dopamine or to $GABA_A$-receptor activation were unimpaired (Shibuya *et al.*, 1991). Observations on rat neurointermediate lobes indicated that CGP 35348 was also effective in countering baclofen-induced inhibition of melanotroph secretion in a mammalian species (Shibuya *et al.*, 1991).

The purpose of the present experiments was to obtain evidence on the direct effects of various $GABA_B$ antagonists in mammalian melanotrophs to define more fully the actions of such drugs on these endocrine cells and thus their possible usefulness as pharmacological agents with which to analyse physiological function. We have used isolated melanotrophs of rat in primary culture, and have assessed the activity of CGP 35348 and several other $GABA_B$ antagonists in counter-

[1] Author for correspondence.
[2] Present address: Department of Biological Research, Hoechst Roussel Pharmaceuticals Inc., Somerville, NJ 08876, U.S.A.

ing $GABA_B$-induced reductions in $[Ca^{2+}]_i$ using the fluorescent Ca probe, fura-2. There are several indications that the inhibitory effects on melanotroph secretion of both dopamine (acting through $D_2$ receptors) and GABA (acting through $GABA_B$ receptors) are the consequence of such reductions, which have been interpreted as likely to arise from actions of the transmitters impeding Ca influx through voltage-dependent Ca channels of low threshold (Nemeth *et al.*, 1988; 1990; Taraskevich & Douglas, 1990). Furthermore, by studying the quenching effect of the Ca surrogate, Mn, on the fura-2 signal, we have obtained evidence for a brisk influx of Ca through channels of this type in isolated melanotrophs of rat in basal (unstimulated) conditions, and moreover, have shown that dopamine acting through $D_2$ receptors or GABA acting through $GABA_B$ receptors closes these channels as witnessed by arrest of quenching (Shibuya *et al.*, 1992). Our decision to assess the effects of the $GABA_B$ antagonists by measurements of $[Ca^{2+}]_i$ was influenced by two factors: firstly, this parameter is an early event following receptor occupancy; and secondly, it is more easily obtained and quantified than secretory responses.

## Methods

### *Dissociation and culture of melanotrophs*

Male Sprague-Dawley rats (weighing 200–350 g) were decapitated under ether anesthesia and neurointermediate lobes of the pituitary gland were isolated and incubated in standard solution containing (mM): NaCl 135, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 15 and glucose 11 with 0.1% bovine serum albumin, and pH adjusted to 7.4 with NaOH. Melanotrophs were dissociated and cultured for 1 to 4 days according to the procedures previously described (Tomiko *et al.*, 1984).

### *Fura-2 loading and cell perifusion*

Culture medium was removed and the cells were washed with standard solution and then loaded, in the same solution, with acetoxymethyl esters of fura-2 (fura-2/AM, 1 μM) at 35°C for 1 h. The cells were then washed once with dye-free standard solution and kept at room temperature until used. A portion of the cell suspension amounting to about 1/30 of the cells obtained from one rat was transferred to a perifusion chamber, the bottom of which consisted of a glass coverslip. This was placed on the stage of an inverted microscope (Nikon, Diaphot) and perifusion with standard solution was begun 5 to 10 min later when the cells had settled and attached to the cover glass. The volume of the perifusion solution in the chamber was kept constant (about 130 μl) by adding and withdrawing the solution at the same rate (1.5 ml $min^{-1}$) with a roller pump (Rainin, Rabbit) and the temperature was maintained at 37°C by circulating water around the chamber. Different solutions were selected by tap and the half time for a complete change of the solution in the chamber was about 7 s.

### *Measurement of fluorescence*

This was carried out on clumps of 5 to 30 cells by epi-illumination using optical filters and a dichroic mirror (Omega Optical, VT, U.S.A.). Light emitted from a 150 W Xenon lamp (Oriel, CT, U.S.A.) was introduced into the inverted microscope after passing through excitation filters (380 and 340 nm, with 10 nm band width) held in a filter wheel (Oriel, CT, U.S.A.). The excitation filters were alternated every 1.5 s electro-mechanically and a shutter (Vincent Assoc., NY, U.S.A.) placed just before the excitation filters was opened for about 50 ms after one or other of the filters came to a stop in the light path. The beam then was passed through an appropriate neutral density filter, and reflected by a dichroic mirror (reflecting wavelengths $<400$ nm) onto the melanotrophs in the perifusion chamber via an objective lens (CF Fluor 20×, Nikon). The fluorescence emitted by the cells traversed the dichroic mirror, an emission filter (540 nm with 40 nm band pass) and an iris diaphragm before it was introduced into a photomultiplier tube (Hamamatsu photonics, 1P21). The output voltage from the photomultiplier was digitized with a Labmaster DMA (Scientific Solutions, OH, U.S.A.). Control of the various components and aquisition of digitized data were accomplished by a computer programme developed by one of us (I.S.). The fluorescence intensities after background subtraction at 340 and at 380 nm (F340 and F380, respectively) were recorded together with the ratio of F340 over F380 (R), displayed on a monitor screen, stored on a hard disk, and plotted on an xy-plotter. $[Ca^{2+}]_i$ was calculated from R by use of the following equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_i = K_d \times (R - R_{min})/(R_{max} - R) \times \beta,$$

where $K_d$ is the dissociation constant for fura-2 (224 nM); $R_{max}$ and $R_{min}$ are the ratios for unbound and bound forms of the fura-2/$Ca^{2+}$ complex, respectively; and $\beta$ is the ratio between maximum and minimum fluorescence intensities of fura-2 at 380 nm excitation. $R_{max}$ and $R_{min}$ were estimated with the fluorescence intensities of fura-2 solution (5 μM) containing 10 mM $CaCl_2$ and 1 mM EDTA, respectively. Autofluorescence in melanotrophs was negligible ($<0.1\%$) compared with the fluorescence in the fura-2 loaded cells.

### *Drugs*

The sources of the drugs used were as follows: 3-aminopropyl (diethoxymethyl)phosphinic acid (CGP 35348), phaclofen, (−)-baclofen, and (+)-baclofen (Dr H. Bittiger, Ciba-Geigy, Basel, Switzerland); 2-hydroxy saclofen (2OH saclofen; Research Biochemicals Inc., Natick, MA, U.S.A.), and 4-amino-3-(5-methoxybenzo[b]furan-2-yl) butanoic acid (9H; Dr P. Berthelot, Laboratoire de Pharmacie Chimique, Lille, France).

## Results

### *(−)-Baclofen lowers $[Ca^{2+}]_i$ in a concentration-dependent manner*

Repetitive exposure to the same concentration of (−)-baclofen given at 5 min intervals had similar effects on $[Ca^{2+}]_i$ (examples are seen in Figure 3). The fall in $[Ca^{2+}]_i$ induced by (−)-baclofen was concentration-dependent (Figure 1a) with threshold effects appearing between 100 nM and 1 μM, maximum effects between 10 and 100 μM, and $EC_{50}$ around 1 μM. The fall in $[Ca^{2+}]_i$ began within a few seconds of introducing (−)-baclofen to the chamber and reached the maximum within 30 s. Upon removing (−)-baclofen, $[Ca^{2+}]_i$ rebounded rapidly and, with the exception of the lowest concentrations of the drug, consistently overshot the pre-existing levels. These spike-like overshoots in $[Ca^{2+}]_i$ subsided, in turn, to around the control levels in 3–5 min. The changes in $[Ca^{2+}]_i$ obtained with dopamine (Figure 1b) closely resembled those obtained with (−)-baclofen and, moreover, the amplitude of the reductions in $[Ca^{2+}]_i$ in response to maximally effective concentrations of the two drugs were also similar. Furthermore, the reductions in $[Ca^{2+}]_i$ obtained with maximally effective concentrations of the two drugs were comparable with those obtained during perifusion with a nominally Ca-free solution ($CaCl_2$ replaced with $MgCl_2$). The reintroduction of Ca caused an overshooting transient increase in $[Ca^{2+}]_i$ much like that seen after exposure to (−)-baclofen or dopamine (Figure 1). The (+)-isomer of baclofen, which lacks the $GABA_B$ agonistic activity (its relative potency to (−)-baclofen is 0.001, Hill & Bowery, 1981), was without effect on $[Ca^{2+}]_i$ at concentrations below 100 μM, although it caused a small fall in $[Ca^{2+}]_i$ at 1 mM (Figure 2). The con-

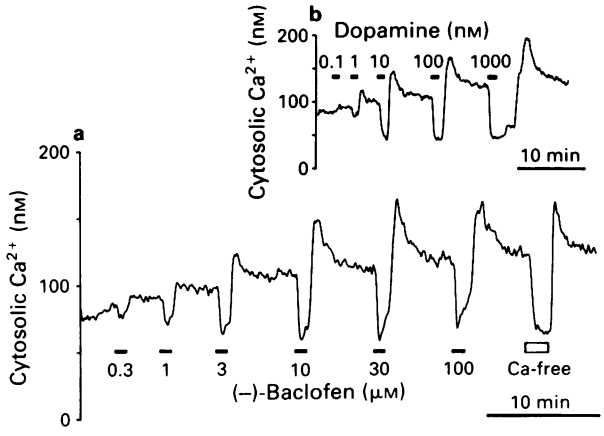


**Figure 1** Representative traces of cytosolic free Ca concentration ($[Ca^{2+}]_i$) in isolated and perifused rat melanotrophs showing responses to increasing concentrations of (−)-baclofen (a) and dopamine (b), and for comparison (in a), exposure to a (nominally) Ca-free solution ($CaCl_2$ replaced with $MgCl_2$). The periods of exposure to the various solutions are indicated by the bars. Note that the concentration-dependence of the responses to (−)-baclofen and to dopamine and that maximally effective concentrations lower $[Ca^{2+}]_i$ to the same extent as is obtained with the Ca-free solution, and also the overshooting elevation in $[Ca^{2+}]_i$ on withdrawing the drugs or restoring Ca.

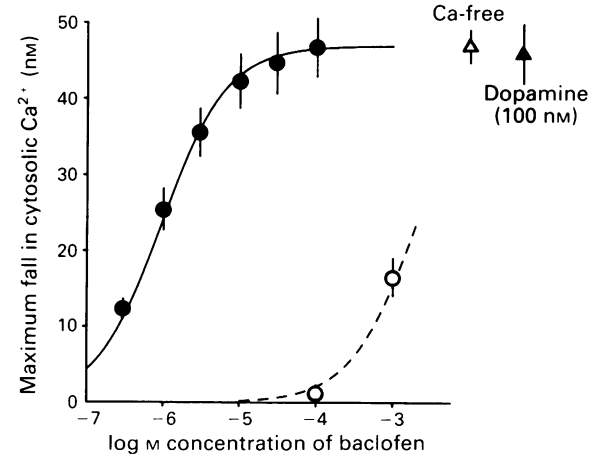


**Figure 2** Concentration-response curve for (−)-baclofen (●), vertical lines show s.e.mean. Each value is the mean of the maximum reduction in $[Ca^{2+}]_i$ obtained in 5 to 8 tests; vertical lines show s.e.mean. The sigmoid curve shown for (−)-baclofen was obtained by the method of least squares. The $EC_{50}$ of (−)-baclofen was 0.96 μM. For comparison are shown the values obtained with dopamine in a maximally effective concentration ($n = 7$) and with Ca-free solution ($n = 12$). Note that (+)-baclofen (○, $n = 5$) had little activity.

centration-response curve for (−)-baclofen yielded an $EC_{50}$ of 0.94 μM.

### *Effects of CGP 35348 and other GABA$_B$ antagonists on the fall in $[Ca^{2+}]_i$ induced by (−)-baclofen*

To assess the effects of the $GABA_B$ antagonists against responses to (−)-baclofen, the latter was used at a concentration of 3 μM which consistently produced a substantial yet submaximal fall in $[Ca^{2+}]_i$. Prior addition of CGP 35348 inhibited, in a concentration-dependent manner, the effects of (−)-baclofen, both the decrease and the overshoot in $[Ca^{2+}]_i$. The inhibitory effect, which was reversible, was evident at 30 μM CGP 35348 and complete at 300 μM (Figure 3a–c). By itself, CGP 35348 did not affect $[Ca^{2+}]_i$ at any concentration tested, the highest being 1 mM (data not shown). For comparison with CGP 35348, similar experiments were performed with several other $GABA_B$ antagonists. Among these, 2OH saclofen (300 μM), albeit less potent than CGP 35348, also substantially reduced the fall in $[Ca^{2+}]_i$ caused by (−)-baclofen (3 μM, Figure 3d). Both phaclofen (300 μM) and 9H (300 μM) were weaker antagonists and only partially inhibited the effect of (−)-baclofen (3 μM) (Figure 3e and f). We did not attempt to assess the baclofen-antagonist activity of 2OH saclofen or phaclofen at concentrations higher than 300 μM, since each of these drugs at higher concentrations (0.5 to 1 mM) itself affected basal levels of $[Ca^{2+}]_i$. The concentration-response curves for the several $GABA_B$ antagonists at different concentrations are presented in Figure 4. The $IC_{50}$

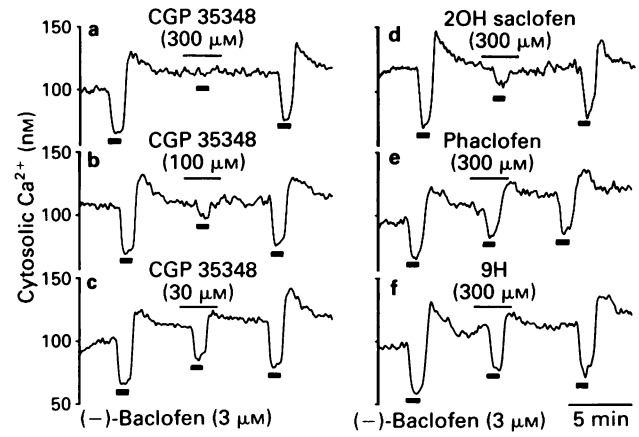


**Figure 3** The effects, on baclofen-induced lowering of $[Ca^{2+}]_i$, of various $GABA_B$ antagonists (CGP 35348, a-c; 2OH saclofen, d; phaclofen, e; and 9H, f). All the substances tested had some inhibitory effect (which was in each instance reversible) but at the highest concentration tested (300 μM) only CGP 35348 blocked completely. The period of exposure to (−)-baclofen is indicated by the thick bars under each response and that to the antagonists by the thin bars above the response.

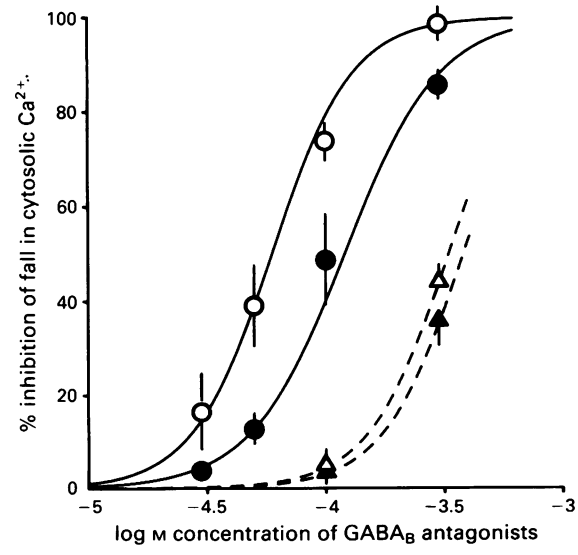


**Figure 4** Concentration-response curves for the inhibitory effects of several $GABA_B$ antagonists on the fall in $[Ca^{2+}]_i$ induced by (−)-baclofen (3 μM). The %inhibition achieved with the antagonists was expressed as the value during exposure to baclofen plus antagonists as a % of the mean values obtained before and after with (−)-baclofen alone. Each value is the mean of 3 to 7 tests, vertical lines show s.e.mean. The curves were obtained by the method of least squares. $IC_{50}$ for CGP 35348 (○), 60 μM; for 2OH saclofen (●), 120 μM; for phaclofen (△), 330 μM; and for 9H (▲), 380 μM.

for CGP 35348 was about 60 μM and that of 2OH saclofen about 120 μM. A Schild plot (Arunlakshana & Schild, 1959) of the results obtained with CGP 35348 at three different concentrations (50, 100 and 300 μM) against (−)-baclofen yields a $pA_2$ of 4.8 and a slope of 1.08 (Figure 5), the latter indicating that CGP 35348 is a competitive antagonist.

### *Selectivity of CGP 35348: lack of effect on changes in $[Ca^{2+}]_i$ elicited by muscimol or dopamine*

CGP 35348 at a concentration (300 μM) which, as above noted, completely suppresses the effect of the $GABA_B$ agonist (−)-baclofen (3 μM) on $[Ca^{2+}]_i$, did not alter the corresponding effects of submaximal concentrations of the $GABA_A$ agonist muscimol (10 μM) or of dopamine (10 nM) (Figure 6). By contrast, the changes in $[Ca^{2+}]_i$ elicited by muscimol were abolished by the specific $GABA_A$ antagonist, bicuculline (bicuculline methiodide; BMI, 30 μM) and those elicited by dopamine were abolished by the specific $D_2$ antagonist, sulpiride (1 μM).

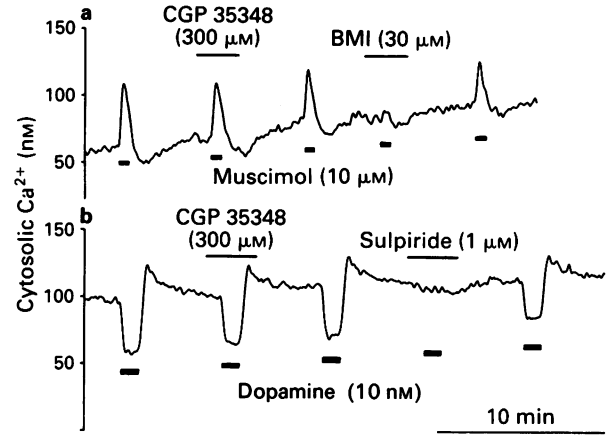


**Figure 6** Indications of selectivity: CGP 35348 in a concentration sufficient to block the response to (−)-baclofen (3 μM) does not affect responses to (a) muscimol (10 μM) or (b) dopamine (10 nM). These responses are, however, blocked by the specific $GABA_A$ antagonsist, bicuculline methiodide (BMI; 30 μM) and by the selective $D_2$ receptor antagonist, sulpiride (1 μM), respectively. Similar results were obtained in three other experiments.

## Discussion

### *GABA antagonists*

The initial studies on the direct effects of GABA on secretion in isolated melanotrophs, performed on rat, revealed that GABA has a biphasic effect; eliciting first a sharp transient increase in secretion followed by a more persistent reduction (Tomiko *et al.*, 1983). The first phase was susceptible to block by the specific $GABA_A$ receptor antagonist, bicuculline, and was attributed to the $GABA_A$-mediated depolarization observed in similar cell preparations (Taraskevich & Douglas, 1982). The second phase was resistant to block by bicuculline (Tomiko, 1983; Demeneix *et al.*, 1984). The latter authors found that the $GABA_B$ agonist, baclofen could reduce melanotroph secretion and suggested that the bicuculline-resistant inhibitory effect of GABA was probably mediated by $GABA_B$ receptors. Support for this interpretation has been provided by the recent demonstration, on isolated neurointermediate lobes of toads and rats, that the inhibitory effects on melanotroph secretion of baclofen and of GABA given in the presence of bicuculline were both suppressed by the specific $GABA_B$ antagonist, CGP 35348 (Shibuya *et al.*, 1991). The present results show that CGP 35348 acts directly on the melanotrophs to inhibit the effect of baclofen to lower $[Ca^{2+}]_i$. Our evidence indicates that the antagonism is competitive, as reported for the effects of CGP 35348 in brain slices (Seabrook *et al.*, 1990). In the isolated melanotrophs of the rat, inhibitory activity, albeit weaker than that of CGP 35348, was also noted in 2OH saclofen, phaclofen and 9H. By contrast, no antagonist activity was noted with these three drugs in the neurointermediate lobes of the toad, the preparation on which these $GABA_B$ antagonists were previously compared (Shibuya *et al.*, 1991). This disparity may result from species differences. Alternatively, the lack of activity of the $GABA_B$ antagonists other than CGP 35348 in the toad lobes may reflect a poor ability to penetrate the tissue (cf. results on brain obtained by Olpe *et al.*, 1990 and Malcangio *et al.*, 1991).

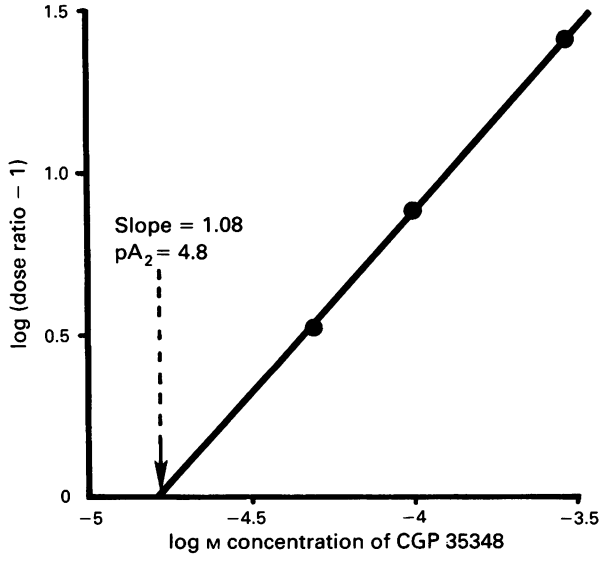


**Figure 5** A Schild plot for CGP 35348 against (−)-baclofen. Dose-ratios were calculated from the $EC_{50}$ values estimated from concentration-response curves for (−)-baclofen obtained in the absence of CGP 35348 (shown in Figure 2), or in the presence of CGP 35348 (50, 100 and 300 μM). $pA_2$ was estimated to be 4.8 from the x-intercept. The slope of 1.08 indicates that CGP 35348 is a competitive antagonist.

### *Efficacy of $GABA_B$ antagonists*

The molar ratio of $GABA_B$ antagonist to agonist (baclofen) required for adequate block in the isolated melanotrophs was high: approximately 100: 1 for CGP 35348. This reinforces our previously stated view (Shibuya *et al.*, 1991a), based also on the need for a great excess of CGP 35348 to inhibit the responses to baclofen in the toad lobes, that the $GABA_B$ receptors in melanotrophs may differ from those in the central or peripheral nervous systems in which effective antagonism against baclofen has been obtained with much lower molar ratios; for example, 3: 1 for CGP 35348 in hippocampal CA1 pyramidal neurones (Olpe *et al.*, 1990); and 5: 1 for 2OH saclofen in cat spinal cord (Curtis *et al.*, 1988). Bowery (1989) has mentioned the possible existence of subtypes of the $GABA_B$ receptors. In any event, CGP 35348 is effective in melanotrophs and is the most potent of the $GABA_B$ antagonists we tested. This, along with its selectivity, rapid reversibility, and effectiveness on neurointermediate lobes, indicates that CGP 35348 is the most promising of the pharmacological antagonists with which to assess involvement of $GABA_B$ receptor activation in the physiological control of melanotrophs. Effectiveness in tissue preparations is an essential attribute of a pharmacological tool, and in this regard, it may be noted that Olpe *et al.* (1990) have empha-

sized that CGP 35348 readily penetrates the brain following systemic administration. Furthermore, Malcangio *et al.* (1991) found that CGP 35348 administered systemically antagonized baclofen-induced antinociception, whereas 2OH saclofen and phaclofen given by the same route were inactive.

In summary, the present experiments support the view that bicuculline-insensitive GABA receptors on melanotrophs belong to the class of $GABA_B$ receptors, although they are perhaps somewhat different from those described in neuronal tissues. They also show that of the $GABA_B$ antagonists tested, CGP 35348, which has by itself no effect on basal $[Ca^{2+}]_i$, is the most potent and is without effect on responses mediated through dopamine $D_2$ receptors or $GABA_A$ receptors. These properties, taken in conjunction with the previously demonstrated effectiveness of CGP 35348 as a $GABA_B$ antagonist in whole neurointermediate lobes, indicate that CGP 35348 may be a useful pharmacological tool with which to study the participation of $GABA_B$-mediated events in the physiological control of melanotroph function.

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# Direct and indirect stimulations of cyclic AMP formation in human brain

[1]D.A. Kendall, P.J. Millns & *J.L. Firth

Departments of Physiology and Pharmacology and *Neurosurgery, Queens' Medical Centre, Nottingham

**1** Adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation, by use of an [$^3$H]-adenine prelabelling assay, was measured in fragments of human cerebral cortex, taken in the course of various neurosurgical procedures.

**2** Large accumulations of [$^3$H]-cyclic AMP due to isoprenaline, noradrenaline and adenosine and small effects due to forskolin were observed. Histamine, 5-hydroxytryptamine (5-HT) and the excitatory amino acids glutamate and quisqualate were ineffective.

**3** The response to noradrenaline consisted of two components; a direct β-adrenoceptor response and an enhancement mediated by an α-adrenoceptor which appears to be similar to that in rat cerebral cortex.

**4** The response to isoprenaline was also potentiated by histamine $H_1$ receptor stimulation but the direct effect of 2-chloroadenosine was not altered by histamine, 5-HT or quisqualate.

**5** It is concluded that some, but not all, of the indirect modulations of cyclic AMP formation previously observed in experimental animal brain exist in human cerebral cortex.

**Keywords:** Cyclic AMP; human brain; neuromodulators; receptors

## Introduction

Many receptors for hormones and neurotransmitters are positively coupled to adenylate cyclase and mediate the formation of the intracellular messenger adenosine 3':5'-cyclic monophosphate (cyclic AMP). Other receptors having no direct linkage to the cyclase e.g. $\alpha_1$-adrenoceptors, histamine $H_1$, $GABA_B$, somatostatin receptors (Magistretti & Schoreret, 1984; Sherer *et al.*, 1988; Rougon *et al.*, 1983) can act synergistically with directly-coupled receptors to enhance cyclic nucleotide formation and thereby act as positive neuromodulators.

These potentiations of the formation of cyclic AMP (and other second messengers) are notably species-selective (Hill & Kendall, 1989) and the existence of a particular modulation in human brain cannot be assumed on the basis of data gathered from experimental animals. Although there have been a very limited number of previous studies of cyclic AMP formation in human brain (e.g. Shimizu *et al.*, 1971; Fumagalli *et al.*, 1971) only direct responses in a few brain samples were analysed. Therefore, we have examined some direct and indirect stimulations of cyclic AMP production *in vitro* in fragments of human cerebral cortex taken during the course of neurosurgical procedures.

A preliminary account of some of these results has been communicated to the British Pharmacological Society (Kendall & Firth, 1991).

## Methods

### *Tissue preparation*

Pieces of brain ranging in size from about 100 mg to 1 g were taken from different regions of the cerebral cortex during various trans-cortical neurosurgical procedures including subcortical tumour removal or biopsy and cerebral decompression. The mean age of the donors was 56 years (range 4–74) and there were approximately equal numbers of male and female donors.

The tissue was placed immediately in ice-cold Krebs-Henseleit buffer equilibrated with 95%$O_2$/$CO_2$ for transportation to the laboratory where it was cut into slices (350 by 350 μM) with a McIlwain tissue chopper. The slices were then pre-incubated in Krebs buffer at 37°C for 60 min with three intermediate changes of medium. The tissues were treated as being potentially infectious and all transfer procedures and gassing were done in a microbiological safety hood in a designated human tissue laboratory. Cyclic AMP was measured by use of an [$^3$H]-adenine prelabelling method as described by Robinson & Kendall (1989).

### *Incorporation of [$^3$H]-adenine and agonist stimulation of cyclic AMP formation*

Slices were incubated with [$^3$H]-adenine (20 μCi 10 ml$^{-1}$ buffer) for 45 min after which they were washed three times with 100 ml buffer to remove free [$^3$H]-adenine; 10 μl aliquots of packed slices were then dispensed into flat-bottomed plastic insert vials containing Krebs buffer, receptor antagonists when appropriate, and in some experiments, 10 μM rolipram (to inhibit phosphodiesterase). After 15 min equilibration, receptor agonists were added in volumes of 10 μl. The final volume of the incubations was 300 μl. After 10 min the incubations were terminated by the addition of 200 μl 1 M HCl.

### *Separation of [$^3$H]-cyclic AMP*

Samples were diluted by the addition of 750 μl water and were centrifuged for 10 min at 2000 *g*. Aliquots of the medium were then taken for estimation of [$^3$H]-adenine uptake into the slices ('totals'). Samples (900 μl) of the remaining supernatants were then added to test tubes containing [$^{14}$C]-cyclic AMP recovery marker (about 2000 d.p.m.). The samples were applied to columns containing Dowex-50 (in the $H^+$ form) and the eluant dripped onto

[1] Author for correspondence.

neutral alumina columns previously equilibrated with imidazole (100 mM). [$^3$H]-cyclic AMP was then eluted from the alumina with 5 ml imidazole. [$^3$H]-cyclic AMP in the samples was quantified by use of a dual label ($^3$H/$^{14}$C) programme in an LKB beta-counter and the data were corrected for recovery. 'Totals' were counted on a single label $^3$H programme to allow calculation of % conversion (adenine to cyclic AMP).

### *Data analysis*

Dose-response curves were analysed by the graphical and non-linear curve fitting programme GraphPad InPlot (ISI). $EC_{50}$s were calculated for noradrenaline and histamine from experiments such as that shown in Figure 3. $IC_{50}$s were the concentrations of prazosin, yohimbine or mepyramine that reduced the noradrenaline-potentiations of the responses to isoprenaline by 50%. Results are shown as mean ± s.e.mean.

### *Drugs*

All drugs and chemicals were purchased from Sigma Chemical Co. Ltd or Fisons P.L.C.

## Results

### *Accumulation of [$^3$H]-adenine*

The human brain slices accumulated [$^3$H]-adenine avidly with approximately 500,000 d.p.m. of water-soluble radioactivity having become associated with 10 µl of packed slices by the end of the incubation period. This is comparable with the amount incorporated by 25 µl samples of rat or guinea-pig cerebro-cortical slices. The basal activity of adenylate cyclase would appear to be similar in human and other mammalian brains with between 0.3 and 0.5% conversion of total radioactivity to [$^3$H]-cyclic AMP in unstimulated slices (in the absence of phosphodiesterase inhibition) over the period of incubation. In the presence of the phosphodiesterase inhibitor, rolipram (50 µM), both basal and stimulated [$^3$H]-cyclic AMP accumulation were approximately doubled (data not shown). However, the levels in the absence of rolipram were sufficient to omit the drug for all successive experiments.

### *Agonist stimulation of [$^3$H]-cyclic AMP*

Figure 1 shows the effects of a number of known stimulators of cyclic AMP at concentrations which have previously been found to be maximally effective in rodent brains. Adenosine and the β-adrenoceptor agonist, isoprenaline, produced large stimulations, the effect of the latter being exceeded by the mixed α-/β-adrenoceptor agonist, noradrenaline. The response to forskolin, a direct activator of cyclase, was relatively small, but significant. Histamine (1 mM), 5-hydroxytryptamine (5-HT, 300 µM), glutamate (3 mM) and quisqualic acid (100 µM) were ineffective (data not shown).

### *Potentiation of β-adrenoceptor-stimulated cyclic AMP formation*

As illustrated by the data in Figure 1, the response to noradrenaline exceeded that to isoprenaline suggesting the existence of a non-β-adrenoceptor potentiation similar to that previously found in rat brain (Robinson & Kendall, 1989). The degree of potentiation varied between samples (2.0 ± 0.5 fold, $n=10$) but the augmentation was present in every one that was examined. In the presence of a maximally effective concentration of isoprenaline (Figure 2) noradrenaline enhanced the accumulation of [$^3$H]-cyclic AMP in a concentration-related fashion (Figure 3) with an $EC_{50}$ of 31 ± 5 µM ($n=3$). The difference between maximally effective concentrations of isoprenaline and noradrenaline was abolished by the selective $\alpha_1$-adrenoceptor antagonist, prazosin ($IC_{50}$ = 33 ± 11 nM, $n=3$; Figure 4) and by the $\alpha_2$-antagonist, yohimbine, albeit with lower affinity ($IC_{50}$ = 165 ± 7 nM, $n=3$)

Although histamine alone was without effect on [$^3$H]-cyclic AMP formation it significantly enhanced the response to isoprenaline in a concentration-dependent fashion ($EC_{50}$ = 5 ± 2 µM, $n=3$) and this enhancement was completely reversed by the selective histamine $H_1$-receptor antagonist, mepyramine, with an $IC_{50}$ of 35 ± 6 nM, ($n=3$) (Figure 5).

### *Effects of potential modulators on the responses to 2-chloroadenosine*

In guinea-pig brain, cyclic AMP stimulation due to adenosine or its analogues can be potentiated by 5-HT, histamine, and excitatory amino acids (Sattin & Rall, 1970; Schultz & Daly, 1973; Donaldson *et al.*, 1990). However, in human cerebral cortex the responses to 2-chloroadenosine were unaffected by 5-HT and histamine (Figure 6) or by quisqualic acid (data not shown).

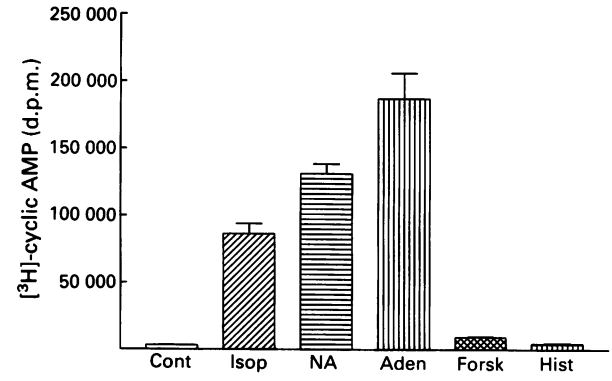


**Figure 1** Accumulation of [$^3$H]-cyclic AMP (expressed as disintegrations per minute (d.p.m.) per 10 µl packed slices in 10 min) in response to isoprenaline (Isop, 1 µM); noradrenaline (NA, 300 µM); adenosine (Aden, 300 µM); forskolin (Forsk, 10 µM); histamine (Hist, 1 mM); no stimulation (Cont.). The figure represents data from a single experiment with triplicate incubations. The mean responses in 3 experiments (expressed as percentage of control ± s.e.mean) were; isoprenaline, 2010 ± 200; noradrenaline, 3300 ± 320; adenosine, 4500 ± 490; forskolin, 229 ± 25: histamine, 115 ± 15. All responses apart from that to histamine were significantly different from control ($P<0.05$, unpaired $t$ test).

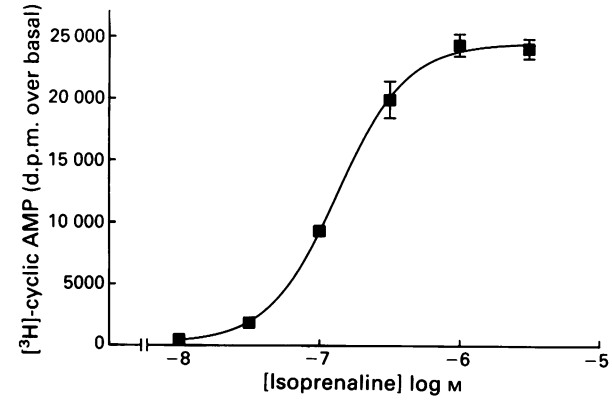


**Figure 2** Accumulation of [$^3$H]-cyclic AMP in response to increasing concentrations of isoprenaline. The basal levels have been subtracted. The $EC_{50}$ was 150 nM. The data are from a single experiment conducted in triplicate. The mean $EC_{50}$ from three determinations was 185 ± 25 nM.

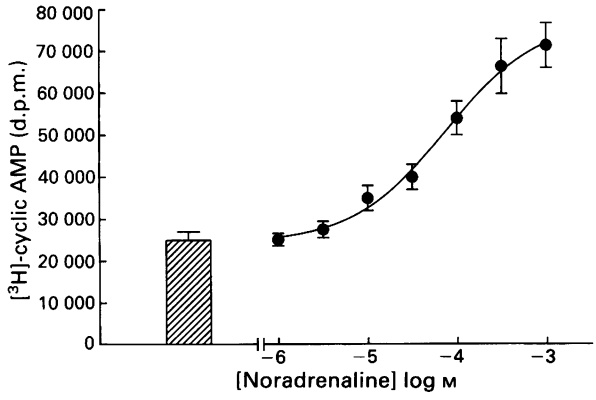


**Figure 3** Potentiation of the maximum cyclic AMP response to isoprenaline (1 μM) by increasing concentrations of noradrenaline. The basal accumulations have been subtracted. The data are from a single experiment conducted in triplicate. The mean $EC_{50}$ calculated from three experiments was 31 ± 18 μM.

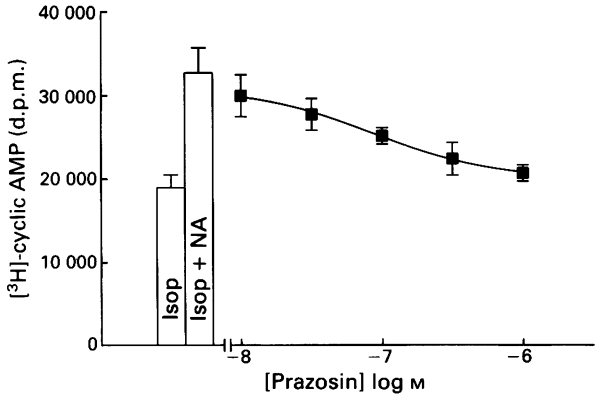


**Figure 4** Inhibition of the noradrenaline potentiation of isoprenaline-stimulated [$^3$H]-cyclic AMP accumulation by prazosin. The data are from a single experiment conducted in triplicate in the presence of 1 μM isoprenaline (Isop) and 300 μM noradrenaline (NA). The mean $IC_{50}$s for prazosin and yohimbine (which were determined with an identical protocol) were 33 ± 11 and 165 ± 7 nM respectively ($n = 3$).

## Discussion

The [$^3$H]-adenine prelabelling assay has proved to be particularly useful for measurement of cyclic AMP formation in human brain slices due to the large uptake of adenine (and its incorporation into the ATP pool) with the result that only small fragments of tissue are needed for each assay.

Stimulation of certain receptors, notably adenosine (probably $A_2$) and β-adrenoceptors, produced large increases in cyclic AMP formation (Figure 1). The response to forskolin on the other hand was smaller than would have been predicted on the basis of experiments on rat brain slices (Daly *et al.*, 1982) although in some other mammalian brain slices e.g. from the mouse, the effects of forskolin are also less marked (Kendall, unpublished). The reasons for this lack of effectiveness are not clear. Forskolin can both directly activate adenylate cyclase and enhance the coupling of receptor-mediated responses (Daly *et al.*, 1982) and since, on the basis of the large responses to adenosine and isoprenaline, the cyclase in human brain would appear to be reasonably active, the minor effect of forskolin could, for instance, be due to a relative lack of endogenous, cyclase-activating transmitters remaining in the human tissue.

The response to histamine in other mammalian species is a combination of direct activation of adenylate cyclase due to

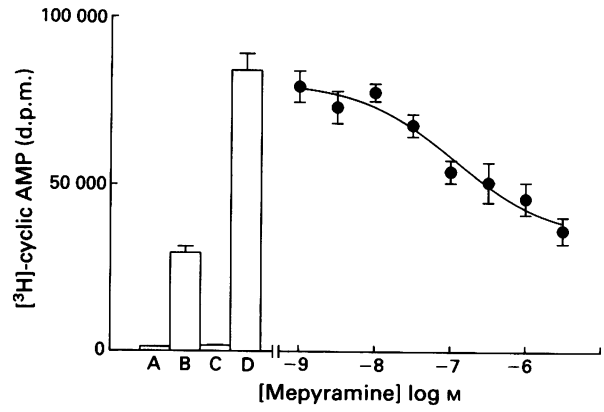


**Figure 5** Potentation of isoprenaline-stimulated [$^3$H]-cyclic AMP by histamine and reversal due to mepyramine. The response to a maximally effective concentration of isoprenaline (1μM) was significantly enhanced ($P<0.01$, unpaired $t$ test) by histamine (1 mM) added 5 min beforehand. Inclusion of increasing concentrations of mepyramine 10 min before histamine progressively antagonized the enhancement. In the presence of 1 μM mepyramine, the response to isoprenaline plus histamine was no longer significantly different ($P>0.05$, unpaired $t$ test) from that due to isoprenaline alone. The figure represents a single experiment using quadruplicate tissue samples: (A) control; (B) isoprenaline (1 μM); (C) histamine (1 mM); (D) isoprenaline plus histamine. The mean $IC_{50}$ calculated from 3 experiments was 35 ± 6 nM.

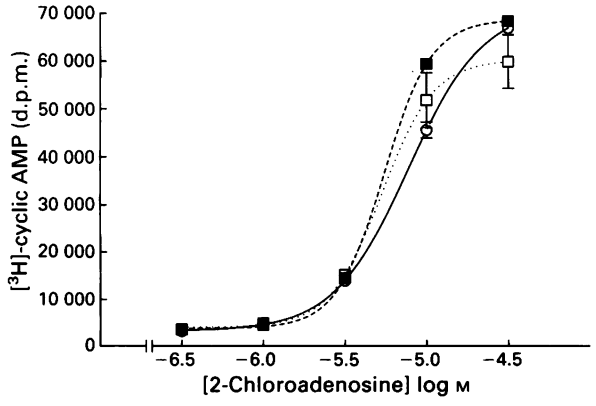


**Figure 6** Lack of effect of histamine and 5-hydroxytryptamine (5-HT) on 2-chloroadenosine (2-CA) stimulated [$^3$H]-cyclic AMP formation: □····□ 2-CA alone; ■----■ 2-CA in the presence of 300 μM 5-HT (added 5 min previously); ○——○ 2-CA in the presence of 1 mM histamine. The data are from a single experiment conducted in triplicate. On a further occasion there were no effects of histamine (1 mM) or 5-HT (300 μM) on the responses to 30 μM 2-CA.

$H_2$-receptors and a potentiation of that response by $H_1$-receptors (Baudry *et al.*, 1975). We have previously shown that phospholipase C-linked $H_1$-receptors are active in human brain slices (Kendall & Firth, 1990) and, as demonstrated in this study, $H_1$-receptors potentiate the effects of β-adrenoceptor activation, so the ineffectiveness of histamine alone is probably due to a low density of directly coupled $H_2$-receptors in human cortex.

The direct response to isoprenaline (Figure 2) is assumed to be mediated via β-adrenoceptors since it was completely abolished in the presence of 1 μM propranolol (data not shown) but the effect of noradrenaline would appear to involve both β- and α-adrenoceptors. In the presence of a maximally effective concentration of isoprenaline, noradrenaline stimulated the formation of an additional amount of cyclic AMP in a concentration-related fashion (Figure 3).

This non-β-receptor effect seems to be due to $\alpha_1$-adrenoceptors since it was inhibited by the selective $\alpha_1$-antagonist, prazosin (Figure 4) more potently than by the selective $\alpha_2$-antagonist, yohimbine. However, there was only a 5 fold difference between the affinities of the two antagonists whereas results from radioligand binding studies and determinations of noradrenaline-stimulated inositol phospholipid hydrolysis (Kendall *et al.*, 1985) would predict a much greater ratio if an $\alpha_1$-like adrenoceptor was involved. This affinity ratio is very close to that found in rat cerebral cortex slices (Robinson & Kendall, 1989) so it is probable that similar recognition sites exist in the two species.

The response to isoprenaline was also potentiated by histamine acting, on the evidence of the affinity for mepyramine, through an $H_1$-receptor. The $IC_{50}$ for this effect of mepyramine (35 nM) was very similar to that found previously for the inhibition of histamine-stimulated phosphoinositide hydrolysis in human brain (Kendall & Firth, 1990) and it is possible that the same $H_1$ receptors are involved. This particular example of potentiation would appear to be unique to human brain, at least amongst those species examined to date, and might simply reflect the co-location of β-adrenoceptors and $H_1$-receptors.

The molecular mechanisms underlying these potentiations in human brain are unknown and it cannot even be assumed that they are the same as those subserving similar effects in other mammalian brains (Robinson & Kendall, 1989 and references therein).

Some other examples of receptor cross-talk that have been previously observed in animal experiments do not appear to exist in human brain. In guinea-pig brain, responses to adenosine or its analogue 2-chloroadenosine are greatly enhanced by both 5-HT and by histamine $H_1$-receptor activation (Schultz & Daly, 1973; Donaldson *et al.*, 1990) but in the human cortical slices the 2-chloroadenosine dose-response curve was not significantly affected by the inclusion of either 5-HT or histamine (Figure 6). Excitatory amino acids also potentiate the cyclic AMP response to 2-chloroadenosine in guinea-pig (Baba *et al.*, 1988; Donaldson *et al.*, 1990) but in the human slices, quisqualic acid and glutamate were without effect (data not shown).

However, before true species differences are proposed, the differences in experimental protocol have to be considered. Although, there was close agreement between the data gathered from different subjects, relatively small fragments of brain from different cortical areas were used, compared with the whole cortices in animal experiments, so that the potentiations that were apparently absent may exist in relatively small sub-regions. Also, the donors were anaesthetized (compared with experimental animals whose brains are taken post mortem) and residual effects of anaesthetic (Miller, 1985) and other drug treatments or of pathological changes in the donors cannot be disregarded. Finally, the donors comprised a heterogeneous group in terms of age, sex, and general state of health so that the relatively close agreement between the data from individual donors is perhaps more surprising than the differences.

In conclusion, we have been able to measure very significant cyclic AMP responses in human brain slices and have observed that some, but by no means all, of the positive modulations of direct receptor responses previously observed in the brains of experimental animals exist in human cerebral cortex.

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# Multiple effects of ryanodine on intracellular free $Ca^{2+}$ in smooth muscle cells from bovine and porcine coronary artery: modulation of sarcoplasmic reticulum function

Colette Wagner-Mann, Qicheng Hu & [1]Michael Sturek

Department of Physiology, School of Medicine and Dalton Research Center, University of Missouri, Columbia, Missouri 65211, U.S.A.

**1** The effects of ryanodine and caffeine on intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) were studied by use of fura-2 microfluorometry in single smooth muscle cells freshly dispersed from bovine and porcine coronary artery.

**2** Bovine and porcine cells demonstrated similar sensitivities to 10 min of exposure to ryanodine in physiological salt solution (PSS), as determined by comparable dose-dependent decreases in the subsequent $[Ca^{2+}]_i$ transient induced by 5 mM caffeine.

**3** Ryanodine (10 μM) caused a significant increase in $[Ca^{2+}]_i$ to a plateau level 27 ± 3% and 38 ± 4% above baseline $[Ca^{2+}]_i$ (baseline $[Ca^{2+}]_i$ = $[Ca^{2+}]_i$ at 0 min) in porcine and bovine cells, respectively, when bathed in PSS. In bovine cells the time required to reach ½ the plateau level was only 3 min versus 6 min for porcine cells.

**4** The ryanodine-induced plateau increase in $[Ca^{2+}]_i$ was 35 ± 5% above baseline for bovine cells bathed in 0 Ca PSS (PSS including 10 μM EGTA with no added $Ca^{2+}$), but only 7 ± 3% above baseline in porcine cells during 10 min exposure to 10 μM ryanodine. In bovine cells $[Ca^{2+}]_i$ showed proportional increases when extracellular $Ca^{2+}$ was increased from the normal 2 mM $Ca^{2+}$ PSS to 5 and 10 mM.

**5** Cells pretreated with caffeine in 0 Ca PSS, which depleted the caffeine-sensitive sarcoplasmic reticulum $Ca^{2+}$ store, showed no increase in $[Ca^{2+}]_i$ when challenged with 10 μM ryanodine. The ryanodine-associated increase in $[Ca^{2+}]_i$, which was sustained in 0 Ca PSS during the 10 min ryanodine exposure in cells not pretreated with caffeine, suggests that ryanodine releases $Ca^{2+}$ from the sarcoplasmic reticulum, but also inhibits $Ca^{2+}$ efflux.

**6** Intracellular free $Ba^{2+}$ ($[Ba^{2+}]_i$) was measured with fura-2 microfluorometry to define further the $Ca^{2+}$ efflux pathway inhibited by ryanodine; specifically, $Ba^{2+}$ is not transported by the $Ca^{2+}$ pump, but will substitute for $Ca^{2+}$ in $Na^+$-$Ca^{2+}$ exchange. In porcine cells pretreated with caffeine in 0 Ca PSS to deplete the caffeine-sensitive sarcoplasmic reticulum $Ca^{2+}$ store, depolarization with 80 mM $K^+$ in 2 mM external $Ba^{2+}$ caused a 100 ± 6% increase in fura-2 fluorescence ($[Ba^{2+}]_i$). During the 17.5 min 0 Ca PSS recovery from depolarization, exposure to 10 μM ryanodine inhibited the removal of $[Ba^{2+}]_i$ by 69 ± 3% when compared with control (0 Ca PSS without ryanodine).

**7** It was concluded that in bovine and porcine smooth muscle cells: (a) ryanodine (≥ 10 μM) releases $Ca^{2+}$ from the sarcoplasmic reticulum; (b) ryanodine (≥ 10 μM) decreases $Ca^{2+}$ efflux, probably by inhibition of $Na^+$-$Ca^{2+}$ exchange; (c) the sarcoplasmic reticulum $Ca^{2+}$ store may be larger in bovine than in porcine smooth muscle cells; thus, porcine cells have a relatively greater reliance on $Ca^{2+}$ influx to increase $[Ca^{2+}]_i$.

**Keywords:** Caffeine; $Ca^{2+}$; ryanodine; sarcoplasmic reticulum; vascular smooth muscle; $Ba^{2+}$; $Na^+$-$Ca^{2+}$ exchange, $Ca^{2+}$ efflux

## Introduction

Intracellular free $Ca^{2+}$ ($[Ca^{2+}]_i$ and its regulation are crucial determinants of smooth muscle contractility. The sarcoplasmic reticulum (SR) has been identified as the primary intracellular organelle which modulates or buffers $[Ca^{2+}]_i$ through $Ca^{2+}$ sequestration/storage or agonist-induced $Ca^{2+}$ release (Van Breemen *et al.*, 1988; Van Breeman & Saida, 1989; Wagner-Mann *et al.*, 1991). A thorough understanding of the SR and its roles in vascular smooth muscle function, however, is yet to be attained.

Pharmacological intervention, with agents such as caffeine, is a useful approach to a better understanding of the SR and its functions (Palade *et al.*, 1989). While much information can be acquired regarding the release of $Ca^{2+}$ stored in the SR by use of the SR $Ca^{2+}$ releasing property of caffeine, there are limitations to this use. Reportedly, caffeine interferes with $Ca^{2+}$ influx (Leijten & Van Breemen, 1984) and may increase adenosine 3':5'-cyclic monophosphate (cyclic AMP) and promote SR $Ca^{2+}$ pump activation (Van Breemen & Saida, 1989), thereby potentially confounding the interpretation of $[Ca^{2+}]_i$ regulation by the SR.

Another, seemingly more SR-specific agent is ryanodine, a neutral alkaloid of plant origin (Jenden & Fairhurst, 1969; Sutko *et al.*, 1985). It appears to deplete the SR $Ca^{2+}$ store in smooth muscle, thereby inhibiting or attenuating agonist-induced contraction dependent on $Ca^{2+}$ release (Kanmura *et al.*, 1988; Ashida *et al.*, 1988; Nishimura *et al.*, 1989). The use of ryanodine in smooth muscle experimentation is not without controversy, however, as Ito *et al.* (1986) reported that in guinea-pig aorta, ryanodine inhibited release of intracellular stores of $Ca^{2+}$. Furthermore, in this laboratory, with the use of ryanodine as a tool for the study of SR function in vascular smooth muscle, species (bovine versus porcine) differences have been noted in response to 10 μM ryanodine (Wagner-Mann & Sturek, 1991; Wagner-Mann *et al.*, 1991). Planar lipid bilayer experiments on SR membranes derived from cardiac and skeletal muscle have focused on examination of ryanodine modulation of single $Ca^{2+}$ release

[1] Author for correspondence: Dalton Research Center, University of Missouri, Columbia, MO 65211, U.S.A.

channel activity (Palade *et al.*, 1989; Nagasaki & Fleischer, 1989; Chu *et al.*, 1990). These studies, while providing valuable detailed information regarding pharmacology and biophysics of SR $Ca^{2+}$ release channels, do not assess integrated effects of ryanodine in the intact cell. These considerations may profoundly influence the use of ryanodine as a pharmacological tool (Sutko *et al.*, 1985; Sutko & Kenyon, 1990).

The purpose of this study, therefore, was to characterize more fully the actions of ryanodine on $[Ca^{2+}]_i$ regulation in single vascular smooth muscle cells. Specifically we determined whether ryanodine: (1) depletes the SR and/or blocks $Ca^{2+}$ release from the SR; and (2) alters $[Ca^{2+}]_i$ buffering by interfering with $Ca^{2+}$ efflux. Also, we found that differences in responses to ryanodine exist between porcine and bovine coronary smooth muscle. The information gained will increase the value of ryanodine as a pharmacological tool to study SR function in $[Ca^{2+}]_i$ buffering/release in vascular smooth muscle.

## Methods

### *Smooth muscle cell dispersion*

Bovine and porcine hearts were obtained fresh from local abattoirs. The left circumflex and right (bovine), or only right (porcine), coronary arteries were dissected from the hearts by sterile techniques less than 30 min following the humane stunning and exsanguination of the animals. The arteries were immediately placed in the following iced medium (in mM): $CaCl_2$ 2, NaCl 135, $MgCl_2$ 1, $KH_2PO_4$ 0.44, $Na_2HPO_4$ 0.34, $NaHCO_3$ 2.6, HEPES 20, glucose 10; 2% horse serum (Hazelton, Lenexa, KS); dilutions (vol:vol) of amino acids (0.02), vitamins (0.01), phenol red (0.002), penicillin/streptomycin (0.01; Gibco, Grand Island, NY, U.S.A.); pH 7.4 with NaOH. Attached cardiac muscle and connective tissue were dissected free by sterile technique in a modification of the above solution, containing low Ca (0.5 mM $CaCl_2$) and no horse serum. Single smooth muscle cells were obtained by enzymatic dispersion techniques described in detail previously (Wagner-Mann *et al.*, 1991; Sturek *et al.*, 1991c). A 1.5 cm long segment was cut longitudinally and pinned lumen side up in a 30 ml jar which contained 2 ml of the dispersion solution (low $Ca^{2+}$ solution also containing 294 $uml^{-1}$ collagenase (CLS II, Worthington), 2 mg $ml^{-1}$ bovine serum albumin (Fraction V, Sigma), 1 mg $ml^{-1}$ soybean trypsin inhibitor (SI, Worthington), and 0.4 mg $ml^{-1}$ DNase I (Type IV, Sigma)). The jar was then placed in a heated (37°C) shaking water bath for 1 h per dispersion fraction. As cells of the first fraction are predominantly endothelial cells, identifiable by their distinct morphology and uptake of acetylated low density lipoprotein (Sturek *et al.*, 1991b), cells from the second or third fraction were used for the experiments. These cells were compared morphologically to cultured cardiac fibroblasts derived from primary biopsy explant cultures. The smooth muscle cells themselves, besides being characteristically oblong in shape, were more readily identified by their contractile responses to agonists such as caffeine (see Results below for more details) (Wagner-Mann *et al.*, 1991; Sturek *et al.*, 1991c).

### *Microfluorometry*

Whole single cells were studied with fura-2 to measure $[Ca^{2+}]_i$ and $[Ba^{2+}]_i$ (Grynkiewicz *et al.*, 1985; Schilling *et al.*, 1989) by microfluorometry methods similar to those described by Thayer *et al.* (1988) and more fully described in other papers from this laboratory (Wagner-Mann *et al.*, 1991; Sturek *et al.*, 1991b,c). Briefly, sample-and-hold analog circuitry amplifiers were used to feed the separate analog signals corresponding to the 340 and 380 nm excitation wavelengths into separate channels of an A-D converter (ADAC Corp., Woburn, MA) and microcomputer equipped with Labtech Acquire (ADAC Corp.) data acquisition system. The fluorescence ratios ($F_{340}/F_{380}$) were calculated from select fluorescence values, which were transferred into a spreadsheet programme (Quattro, Borland, Inc.). The superfusion chamber, a machined plexiglass block having a glass coverslip bottom, allowed for complete exchange of solutions in 15 s by the solutions flowing in a thin sheet across the cells in the chamber.

Due to the uncertainty of the $Ca^{2+}$-sensitivity of intracellular fura-2 loaded into the cells by the membrane permeant acetoxymethylester form and the difficulty of *in situ* calibrations on single cells required for accurate absolute $[Ca^{2+}]_i$ determinations, $[Ca^{2+}]_i$ is reported as the ratio of 340 nm to 380 nm fluorescence signals ($F_{340}/F_{380}$). *In vitro* and 'myoplasmic' calibrations of the fura-2 ratio to $[Ca^{2+}]$ using $Ca^{2+}$-EGTA buffers are described in detail elsewhere (Wagner-Mann *et al.*, 1991; Sturek *et al.*, 1991c) and briefly presented here. The validity and reliability of the relationship between the fluorescence ratio and $[Ca^{2+}]$ in this laboratory are also described elsewhere (Wagner-Mann *et al.*, 1991) and demonstrated in the present paper (see Results, Figure 2). Briefly, the *in vitro* calibration was generated by adding the fura-2 pentapotassium salt (0.1 mM) to mock intracellular solutions comprised of (in mM) KCl 126, NaCl 10, HEPES 20, $MgCl_2$ $H_2O$ 1 and fura-2 salt 0.1, pH 7.1 with KOH. These solutions were adjusted to 4 different free ionized $[Ca^{2+}]$ with appropriate ratios of EGTA and $CaK_2$-EGTA (the final [EGTA] was always 10 mM). Using the microfluorometry system described above, fluorescence ratios were measured from 2 µl droplets of the solutions placed on coverslips. For myoplasmic calibrations, smooth muscle cells from bovine and porcine coronary arteries were dispersed separately. For each species, the cells were loaded with 2.5 µM fura-2 acetoxymethyl ester (AM) by incubating them for 15 min at 37°C in a physiological salt solution (PSS, described below), then rinsing the cells for 30 min at 37°C in PSS free of fura-2. The cells were then divided into four fractions for each species, centrifuged, then resuspended in 0.5 ml of calibration solution containing 100, 400, 1000 or 2000 nM free $[Ca^{2+}]$. These fractions were then treated with saponin (100 µg $ml^{-1}$) to permeabilize selectively the sarcolemma, thereby releasing myoplasmic fura-2 into the solution (van Breemen *et al.*, 1988). Fluorescence ratios were measured from 5 µl droplets of each fraction placed on coverslips. The terms fluorescence ratio and $[Ca^{2+}]_i$ have been used interchangeably throughout the text.

Freshly dispersed smooth muscle cells were loaded with fura-2 according to the protocol described in Wagner-Mann *et al.* (1991). The physiological salt solution (PSS) consisted of (in mM): $CaCl_2$ 2, glucose 10, HEPES 10, KCl 5, $MgCl_2$ 1, NaCl 138, pH 7.4. This solution was used in the experiments as the superfusing fluid and for the direct additions of drugs. Unless otherwise indicated, the cells were constantly superfused with PSS. When the superfusate was changed to e.g. 0 Ca PSS, or ryanodine or caffeine added to the superfusate, the duration of the exposure within a protocol was indicated in the figures by horizontal lines. The $Ca^{2+}$-free medium (0 Ca PSS) consisted of the PSS solution modified to contain no $CaCl_2$, and to which 2 mM NaCl and 10 µM EGTA were added. All experiments were conducted with cells at room temperature (22–25°C) unless otherwise indicated.

Cells were allowed to equilibrate in PSS for at least 5 min or until the fluorescence ratio ($F_{340}/F_{380}$) stabilized (baseline). This was followed by a 5 min depolarization with PSS in which 80 mM KCl (80 K) replaced equimolar amounts of NaCl. The depolarization was conducted to verify the viability of each cell by the resulting increase in $[Ca^{2+}]_i$ and contraction observed by video monitoring. Also, the depolarization loaded the SR with $Ca^{2+}$ before the cells were challenged with an agonist. Concentrations of ryanodine ranging from 0.001 µM to 100 µM (Calbiochem, San Diego, CA, U.S.A.) were used. The only concentration of caffeine used

was 5 mM (Sigma, St. Louis, MO, U.S.A.). Cells were randomly assigned to the experimental protocols.

### *Statistical analysis*

Data are expressed as mean ± s.e.mean. When comparing two groups only, Students' *t* test (independent/paired as indicated by the data) was applied. For comparison of multiple groups, analysis of variance with repeated measures was used. When a significant *F* ratio was calculated, an appropriate *post hoc* analysis was applied to compare specific pairs. In all instances, baseline refers to the resting ratio ($F_{340}/F_{380}$) at the onset of any experimental protocol (0 min), before challenges with any agonist. When expressing any change in $[Ca^{2+}]_i$ over time as the result of a specific agonist's action(s), Δ ratio was calculated by subtracting the ratio value at the onset of the exposure from the highest (or lowest, if appropriate) ratio value attained with that agonist, and % Δ ratio was calculated by dividing Δ ratio by the ratio value at the onset of the exposure to the agonist then multiplying by 100. The significance level for all analyses was $P<0.05$.

## Results

Smooth muscle cells from the porcine right coronary artery (RCA) and from the bovine RCA and left circumflex coronary artery (LCFX) were compared regarding baseline $[Ca^{2+}]_i$, and change in $[Ca^{2+}]_i$ in response to 80 mM $K^+$ (80 K) depolarization and caffeine (5 mM). Table 1 summarizes these data, which indicated that bovine RCA and LCFX were not different and, thus, the data were pooled throughout the remainder of the experiments for comparison with porcine RCA. Representative light micrographs of the cell types that could be seen in the dispersions are pictured in Figure 1. These cell types include a smooth muscle cell (Figure 1a), a cluster of endothelial cells (Figure 1b), a cluster of fibroblasts (Figure 1c), and typical smooth muscle cells compared to adjacent endothelial cells (Figure 1d) or adjacent fibroblasts (Figure 1e). Immunocytochemical methods have been useful for determining the percentage of cells in a population that are smooth muscle versus endothelial or fibroblasts (e.g. Loeb *et al.*, 1985). Unfortunately, the detection of smooth muscle specific actin with antibodies requires dehydration of the cell with methanol and thus, the cell would be useless for fura-2 microfluorometric measurements of $[Ca^{2+}]_i$. Instead, morphological identification (Figure 1), and evidence of contraction (their primary function and the more appropriate and widely used method for identification of smooth muscle cells in freshly dispersed preparations for experiments on single cells) were used. Typically, reports of detailed methods for freshly dispersing smooth muscle cells have relied totally on functional characterization, rather than immunocytochemistry (e.g. Van Dijk & Laird, 1984; DeFeo & Morgan, 1985; Warshaw *et al.*, 1986). The clear advantage of using contraction for smooth muscle cell identification is that every cell studied is identified as a functional smooth muscle cell by simultaneous video monitoring, as shown in our previous papers (Wagner-Mann *et al.*, 1991; Sturek *et al.*, 1991a). This method does not rely on percentages of cells in a population being smooth muscle cells. Similarly, other studies using single cells for voltage-clamp indicate morphological identification of smooth muscle cells (Amédée *et al.*, 1990).

Routinely, in this laboratory, the resting fluorescence ratios in porcine cells are lower than the resting fluorescence ratios in bovine cells (Table 1). From the calibration data depicted in Figure 2 it can be seen that the fura-2 released upon permeabilization of the sarcolemmae of bovine and porcine cells respond comparably to $Ca^{2+}$. As shown previously (Wagner-Mann *et al.*, 1991; Sturek *et al.*, 1991c) the fura-2 trapped in the myoplasm when loaded via fura-2/AM shows depressed $Ca^{2+}$ sensitivity compared to the *in vitro* fura-2 pentapotassium salt. These data support the contention that differences are the result of species-specific $[Ca^{2+}]_i$ regulation, not differences in fura-2 sensitivity or handling. Temperature controlled studies were conducted in porcine cells to determine whether the action of ryanodine on smooth muscle cells in this experimental setup were significantly affected by temperature. Ryanodine induced a 28.2 ± 4.2% increase in $[Ca^{2+}]_i$ in cells ($n = 9$) maintained at 34°C, a value which was not significantly different from cells maintained at room temperature of 22–25°C (data not shown).

**Table 1** Comparison of fura-2 fluorescence ratios ($F_{340}/F_{380}$) from bovine left circumflex (LCFX) and right coronary artery (RCA) and porcine RCA

| | N | *Baseline* | *80 K* | *Caffeine* |
|---|---|---|---|---|
| Bovine LCFX | 34 | 0.96 ± 0.03 | 1.44 ± 0.10 | 2.55 ± 0.21 |
| Bovine RCA | 28 | 0.93 ± 0.03 | 1.45 ± 0.06 | 2.28 ± 0.17 |
| Porcine RCA | 10 | 0.75 ± 0.03 | 1.06 ± 0.04 | 1.33 ± 0.15 |

To identifiy the concentration(s) of ryanodine eliciting the greatest changes in $[Ca^{2+}]_i$ and the $Ca^{2+}$ stored in the sarcoplasmic reticulum (SR) in these cells, experiments were conducted in which cells were exposed for 10 min to one of six (seven in porcine) concentrations of ryanodine (0 μM (control), 0.001, 0.01, 0.1, 1.0, 10 μM; plus 100 μM in porcine). The 10 min ryanodine exposure was immediately followed with a 1 min caffeine challenge to assess the effect of ryanodine on the caffeine-sensitive $Ca^{2+}$ store. Figure 3a illustrates the time course of these experiments, for [ryanodine] = 0 (control) and 10 μM (porcine cells). In the porcine cells, only at ryanodine concentrations of 10 μM and 100 μM was there a ryanodine-induced (Figure 3a: Ry Δ ratio) increase in $[Ca^{2+}]_i$ to a plateau level during the 10 min ryanodine exposure, being 27 ± 3% and 25 ± 5%, respectively. In bovine cells during the 10 min ryanodine exposure, only at ryanodine 10 μM did $[Ca^{2+}]_i$ increase significantly, to 38 ± 4% above baseline (Figure 3b). Of particular interest was the subsequent peak caffeine-induced changes in $[Ca^{2+}]_i$. The peak response to caffeine was determined by calculating the % change in the fluorescence ratio from the maximum ryanodine-induced increase in $[Ca^{2+}]_i$ (taken to be the fluorescence ratio at time 19 min) to the peak caffeine-induced increase in $[Ca^{2+}]_i$ (Figure 3a: Caf Δ ratio). The summary of these Caf Δ ratio calculations from these experiments is depicted in Figure 3c. The points graphed represent the average caffeine-induced percentage change in the ratio for each cell at a given concentration of ryanodine. Using the Caf Δ ratio for each cell in these experiments, the caffeine-induced % Δ ratio was calculated, correcting for any gradual shift in ratio ($F_{340}/F_{380}$) from baseline over the 10 min ± Ry (ryanodine) exposure (see Figure 3a and b). In bovine cells, the caffeine-induced increase in $[Ca^{2+}]_i$ was significantly ($P<0.05$) lower than control for ryanodine concentrations ≥ 1 μM. Typically, porcine cells showed negligible drift. In porcine cells, for ryanodine concentrations ≥ 10 μM, the caffeine-induced increase in $[Ca^{2+}]_i$ was significantly ($P<0.05$) smaller than in cells exposed to ryanodine concentrations < 10 μM. These changes in caffeine-induced increases in $[Ca^{2+}]_i$ are consistent with a ryanodine-induced release of $Ca^{2+}$ from the SR with or without a measurable ryanodine-induced increase in $[Ca^{2+}]_i$ in bovine cells at ryanodine 1 μM.

The above described protocol was also conducted in 0 Ca PSS, in the presence of 10 μM ryanodine or absence of ryanodine (0 μM). These experiments were conducted to rule out an interaction of ryanodine (10 μM) with fura-2 as the cause of the above noted ryanodine-induced increase in the fluorescence ratio. Further, these experiments were designed to identify the contribution of $Ca^{2+}$ release from an internal store, separated from $Ca^{2+}$ influx, to the ryanodine-associated slow increase in $[Ca^{2+}]_i$. Figure 4a depicts the time course of the protocol and summarizes the responses of the

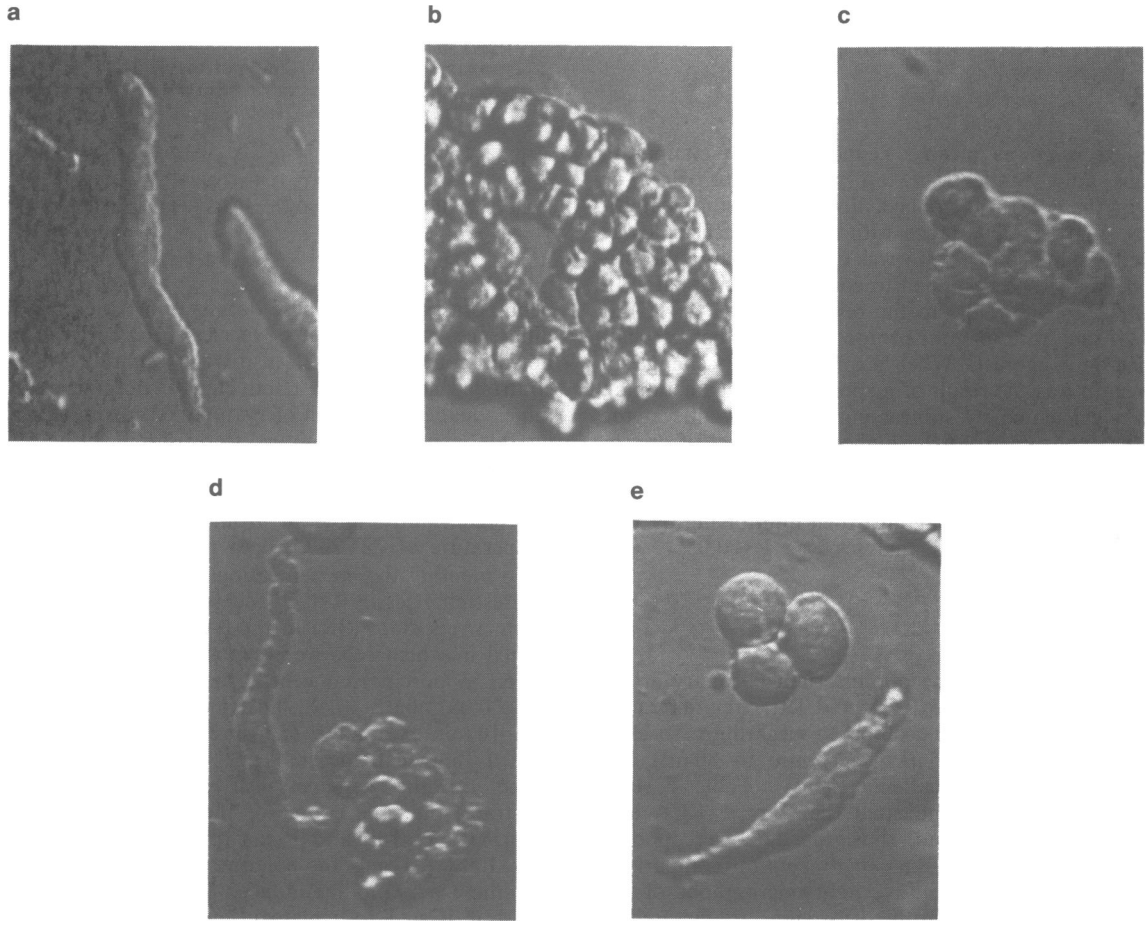



**Figure 1** Light micrographs (using Normarski optics) of the cell types seen in the dispersions (calibration bar is 20 μm). (a) In this panel is depicted a typical smooth muscle cell, the predominant cell type of second and third dispersion fractions. (b) Endothelial cells, the predominant cell type of the first dispersion fraction, are typically seen in characteristic grapelike clusters depicted here. (c) The other possible cell type to be found in the dispersion fractions of the coronary arteries is the fibroblast. It typically assumes a rounded configuration, frequently appearing in clusters. These fibroblasts are larger than the endothelial cells. (d) The smooth muscle and endothelial cells seen in close proximity in this panel are readily differentiated morphologically. (e) The smooth muscle cells and fibroblasts seen in close proximity are readily differentiated morphologically.

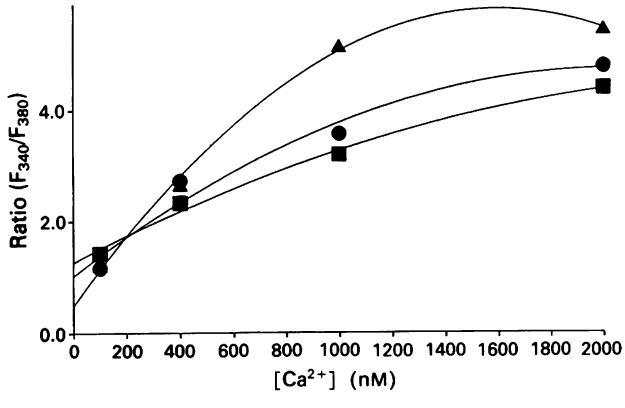



**Figure 2** Myoplasmic and *in vitro* calibration curves for bovine and porcine fura-2/AM loaded smooth muscle cells. Data points for the *in vitro* curve (▲) were generated by measuring the fluorescence ratio of specified concentrations of $Ca^{2+}$ (see below) set by $Ca^{2+}$-EGTA buffers in fura-2 pentapotassium salt (0.1 μM) in mock intracellular solutions (see Methods). For the myoplasmic curves, bovine (●) and porcine (■) smooth muscle cells were enzymatically dispersed then loaded with fura-2/AM. Cells were then divided into 4 approximately equal aliquots (4 for bovine and 4 for porcine), then permeabilized with saponin (100 μg $ml^{-1}$) and incubated with one of the following four [$Ca^{2+}$]: 100 nM, 400 nM, 1000 nM, 2000 nM. Ratios were recorded on a minimum of five different 5 μl droplets of the supernate from the saponin-permeabilized cells.

bovine tissue (+ Ry = 10 μM ryanodine, $n = 9$; − Ry = 0 μM, $n = 7$). In bovine cells, 10 μM ryanodine in 0 Ca PSS induced a 35 ± 5% ($n = 9$) increase in $[Ca^{2+}]_i$ above baseline, and $[Ca^{2+}]_i$ increased to 86 ± 5% above baseline with the subsequent caffeine challenge. These are in sharp contrast to the 0 Ca PSS cells exposed to 0 μM ryanodine which showed no change ($n = 7$) in $[Ca^{2+}]_i$ during the 10 min control period in the absence of ryanodine, then responded to the caffeine challenge with a 163 ± 9% increase in $[Ca^{2+}]_i$ above baseline. In porcine cells, the 10 μM ryanodine-induced increase in $[Ca^{2+}]_i$ was only 7 ± 3% ($n = 23$), while the subsequent caffeine-induced $[Ca^{2+}]_i$ increase was 63 ± 7% above baseline. In the corresponding porcine control experiment (ryanodine = 0 μM), there was an 8 ± 3% ($n = 11$) decrease in $[Ca^{2+}]_i$ from baseline during the 10 min 0 Ca PSS exposure and caffeine induced a 94 ± 13% increase in $[Ca^{2+}]_i$ (Figure 4b).

Evidence of ryanodine-associated response differences in bovine and porcine cells were manifest at this point. Specifically, the ryanodine-induced increase in $[Ca^{2+}]_i$ occurred at different rates. In bovine cells, the $[Ca^{2+}]_i$ increased and plateaued within the first 4 min of ryanodine exposure in both 0 Ca PSS and $Ca^{2+}$-containing PSS. The level of $[Ca^{2+}]_i$ reached was equivalent (Figure 5a). In porcine cells, however, the ryanodine-induced increase in $[Ca^{2+}]_i$ occurred more slowly, only plateauing at the conclusion of the 10 min ryanodine exposure. Furthermore, the ryanodine-induced rate of $[Ca^{2+}]_i$ increase in $Ca^{2+}$-containing PSS was signi-

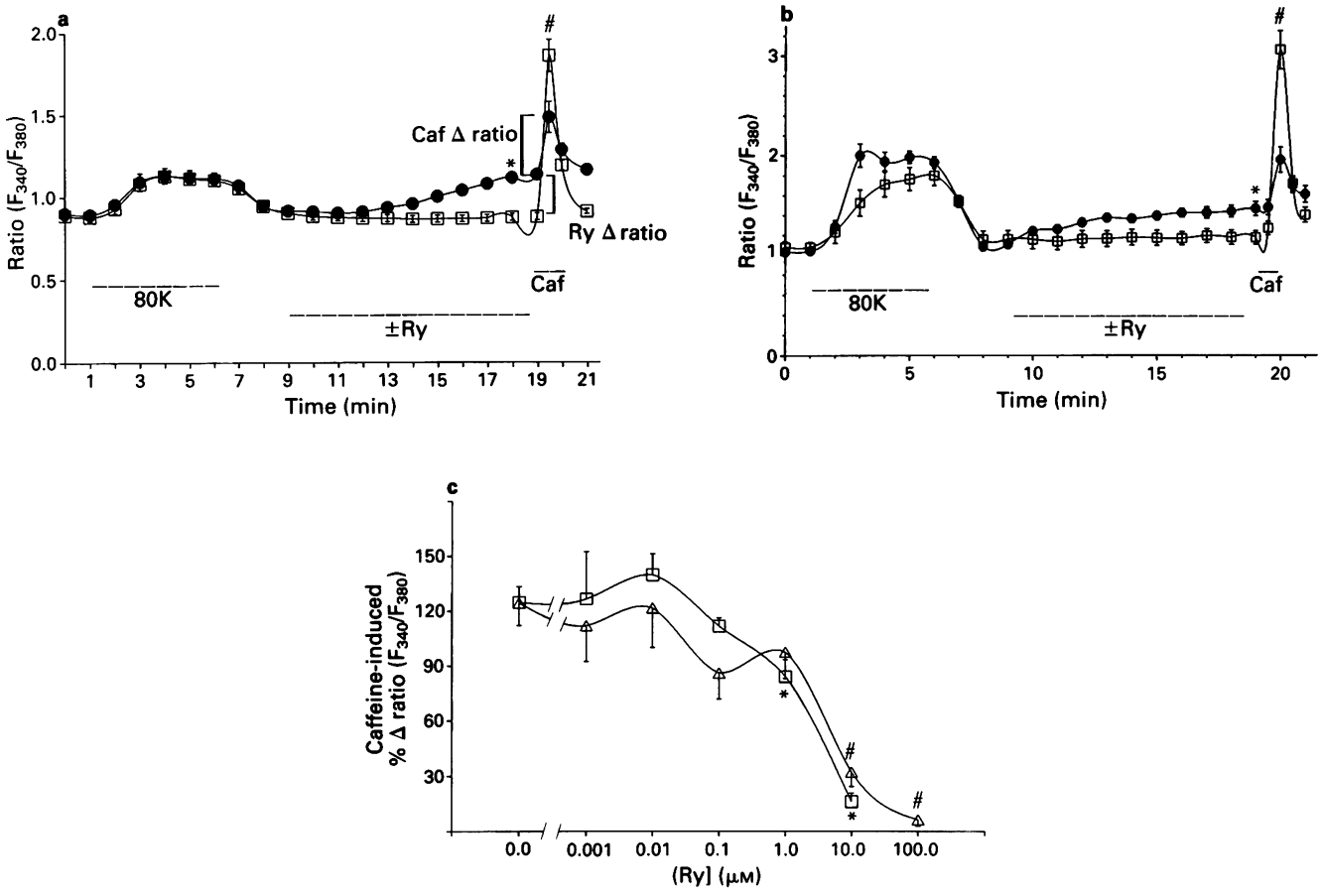
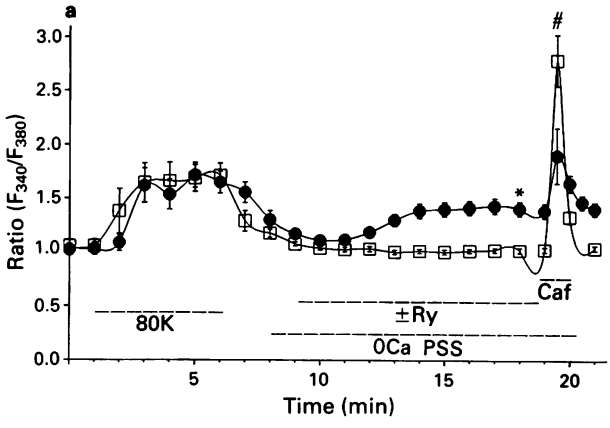



**Figure 3** Sustained increase in $[Ca^{2+}]_i$ and inhibition of the caffeine-induced $\Delta[Ca^{2+}]_i$ (Caf Δ ratio = [(peak caffeine-induced ratio ($F_{340}/F_{380}$)) − (ratio ($F_{340}/F_{380}$) at 19 min)]) by ryanodine. Freshly dispersed smooth muscle cells from porcine ($_p$) and bovine ($_b$) coronary artery were initially depolarized with 80 mM potassium (80 K, duration 5 min as indicated by the horizontal lines and approriate labels) then challenged with various concentrations of ryanodine (see (c) for details). Finally, all cells were exposed to caffeine (Caf; 5 mM) for 1 min. (a) Time course for single dose of ryanodine (+ Ry; 10 μM, $n = 12$; ●) and control (− Ry; 0.0 μM, $n = 29$; □) in porcine smooth muscle (* indicates + Ry fluorescence ratio is significantly greater than the − Ry fluorescence ratio at that data point; # indicates the fluorescence ratios are significantly different at that data point). (b) Time course for single dose of ryanodine (+ Ry; 10 μM, $n = 13$; ●) and control (− Ry; 0.0 μM, $n = 26$; □) in bovine smooth muscle cells (* indicates + Ry fluorescence ratio is significantly greater than the − Ry fluorescence ratio at that data point; # indicates the fluorescence ratios are significantly different at that data point). Contrast the rate of ryanodine-induced $[Ca^{2+}]_i$ increase in bovine versus porcine cells (Figures 3a and 3b). (c) Dose-response curves for ryanodine-induced inhibition of caffeine-induced $\Delta[Ca^{2+}]_i$. Points graphed represent the peak caffeine-induced percentage changes in $[Ca^{2+}]_i$ (% Δ ratio) after 10 min exposure to ryanodine, calculated from Caf Δ ratio (% Δ ratio = (Caf Δ ratio) × (ratio at onset of caffeine challenge)$^{-1}$ × 100) for each cell (see (a); Δ represent porcine data; □ represent bovine data; 0.0 μM (control): $n_b = 26$, $n_p = 29$; 0.001 μM: $n_b = 7$, $n_p = 16$; 0.01 μM: $n_b = 12$, $n_p = 13$; 0.1 μM: $n_b = 8$, $n_p = 6$; 1.0 μM: $n_b = 7$, $n_p = 13$: 10 μM: $n_b = 13$, $n_p = 12$; 100 μM: $n_p = 11$). # Significantly different from control in porcine cells; *significantly different from control in bovine cells.

ficantly ($P < 0.05$) greater than in 0 Ca PSS in porcine cells (Figure 5b).

Next, we identified the effect of releasing $Ca^{2+}$ from the SR with a known agonist (caffeine) on the ryanodine-induced changes in $[Ca^{2+}]_i$ to determine whether the $Ca^{2+}$ stores affected by caffeine and ryanodine overlapped or were in some way functionally linked. To accomplish this, bovine cells were challenged with 1 min of caffeine (5 mM) or 2.5 min caffeine (to more completely release $Ca^{2+}$ stored in the SR) plus ryanodine (10 μM) in 0 Ca PSS to release $Ca^{2+}$ stored in the SR. The exposure to ryanodine (10 μM) in 0 Ca PSS totalled 10 min and finally, cells were exposed for 1 min to caffeine (5 mM) (Figure 6a, open squares) to determine the relative content of the caffeine-sensitive $Ca^{2+}$ store. Even with the initial 1 min caffeine challenge, the total 10 min exposure to ryanodine in 0 Ca PSS induced a 17 ± 2% increase in $[Ca^{2+}]_i$ (relative to baseline). Similar experiments involving an initial 1 min caffeine exposure in 0 Ca PSS and in PSS, but with no ryanodine (control) for 10 min resulted in a 1 ± 2% ($n = 7$) and 12 ± 3% ($n = 9$) increase in $[Ca^{2+}]_i$, respectively after the 10 min control period (data not shown). In cells with the 1 min initial caffeine challenge prior to 10 min of ryanodine exposure in 0 Ca PSS, the second caffeine challenge induced only a 27 ± 4% increase in $[Ca^{2+}]_i$. In contrast, in cells not exposed to ryanodine, the second caffeine challenge induced an 89 ± 7% increase in $[Ca^{2+}]_i$ in PSS, and a 41 ± 4% increase in $[Ca^{2+}]_i$ in 0 Ca PSS (data not shown), thus indicating little depletion of the caffeine-sensitive $Ca^{2+}$ store in the control condition.

In bovine cells in 0 Ca PSS, following the initial 2.5 min caffeine, there was a slight (4 ± 2%) increase in $[Ca^{2+}]_i$ during the remaining 7.5 min of the 10 min exposure to ryanodine in 0 Ca PSS (Figure 6a, closed circles). The second caffeine challenge resulted in only an additional 4 ± 3% increase in $[Ca^{2+}]_i$ to 8 ± 3% above baseline. For cells exposed to caffeine for 2.5 min but not challenged with ryanodine (controls), the $[Ca^{2+}]_i$ dropped 10 ± 2% during the equivalent 7.5 min period in 0 Ca PSS; the second caffeine challenge in this group of cells resulted in a 17 ± 5% increase in $[Ca^{2+}]_i$ (when compared to 1 min before caffeine challenge) to 5 ± 2% above baseline (data not shown). The caffeine-induced change in $[Ca^{2+}]_i$ attained following the 10 min pre-



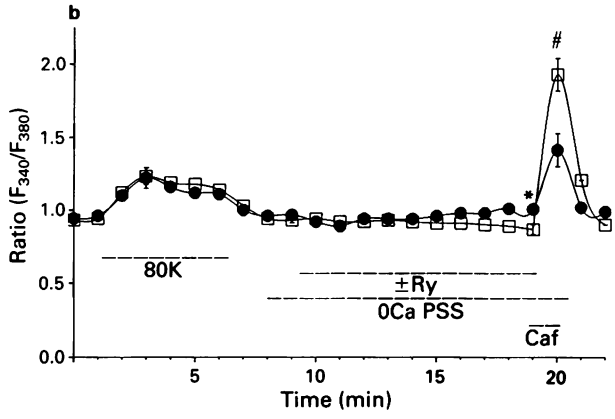
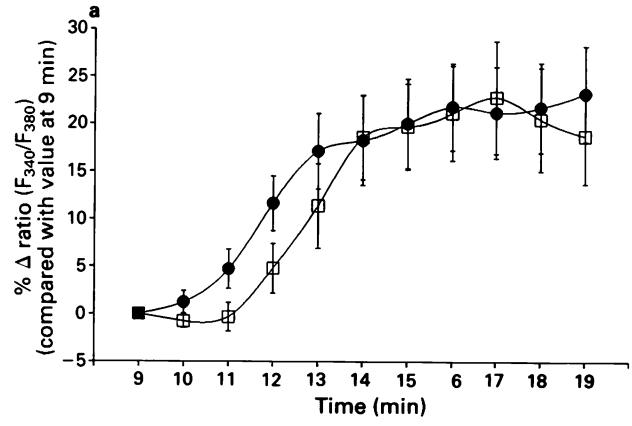




**Figure 4** Ryanodine-induced changes in $[Ca^{2+}]_i$ in bovine and porcine cells superfused with 0 Ca PSS. Cells were first depolarized with 80 mM potassium (80 K, duration 5 min, note horizontal line and associated label), then following a 1 min preconditioning in $Ca^{2+}$-free PSS (0 Ca PSS) cells were either challenged with 10 μM Ry (+ Ry) in 0 Ca PSS or without Ry (− Ry; [ryanodine] = 0.0 μM) in 0 Ca PSS. Finally, cells were exposed to caffeine (Caf; 5 mM) for 1 min. (a) These data were collected from bovine cells (+ Ry: $n = 9$, ●; − Ry: $n = 7$, □; * indicates + Ry fluorescence ratio is significantly greater than the − Ry fluorescence ratio at that data point; # indicates the fluorescence ratios are significantly different at that data point). (b) Ryanodine-induced changes in $[Ca^{2+}]_i$ and subsequent caffeine-induced $[Ca^{2+}]_i$ changes in porcine cells (+ Ry: $n = 23$, ●; − Ry: $n = 11$, □; * indicates + Ry fluorescence ratio is significantly greater than the − Ry fluorescence ratio at that data point; # indicates the fluorescence ratios are significantly different at that data point). Note the difference between the rate of ryanodine-induced changes in $[Ca^{2+}]_i$ increase in bovine compared with porcine cells.



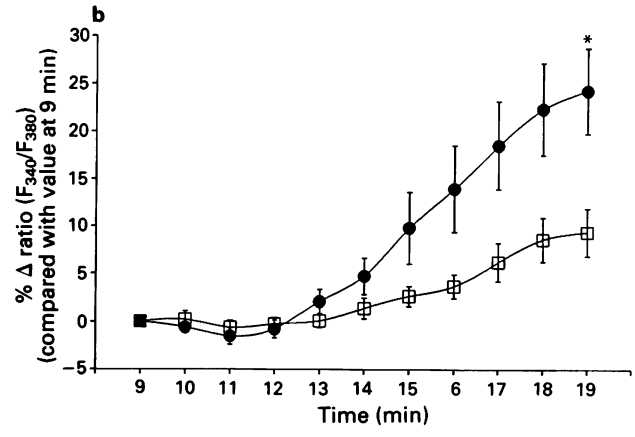




**Figure 5** Ryanodine-induced increases in $[Ca^{2+}]_i$ in $Ca^{2+}$-containing PSS and 0 Ca PSS. Relative rates of ryanodine-induced increases in $[Ca^{2+}]_i$ during the 10 min exposure to 10 μM ryanodine (from Figures 3a, 3b, 4a, and 4b) are shown as % Δ ratio = [ratio ($F_{340}/F_{380}$) at each of the indicated times (9 min to 19 min)]/[ratio ($F_{340}/F_{380}$) at 9 min] × 100−100) (Note: this equation is equivalent to that described in Figure 3c). (a) Bovine smooth muscle cells (0 Ca PSS, $n = 9$, □; $Ca^{2+}$-containing PSS, $n = 13$, ●). Note the equivalent peak Ry-induced increases in the ratio whether in 0 Ca PSS or $Ca^{2+}$-containing PSS, as well as the overlapping time courses for bovine cells. (b) Porcine smooth muscle cells (0 Ca PSS, $n = 22$, □; $Ca^{2+}$-containing PSS, $n = 12$, ●). In porcine tissue the peak Ry-induced increase in the ratio is significantly higher (*) in the group of cells superfused with $Ca^{2+}$-containing PSS than in 0 Ca PSS. Further, the rate of increase in ratio over the 10 min Ry exposure is greater in the $Ca^{2+}$-containing PSS superfused cells than the 0 Ca PSS superfused cells.

exposure to ryanodine was 59 ± 5% lower than the caffeine-induced change in $[Ca^{2+}]_i$ in the absence of 10 min exposure to ryanodine. The ryanodine-induced changes in $[Ca^{2+}]_i$ were more pronounced in protocols conducted in PSS ($Ca^{2+}$-containing) than in 0 Ca PSS. Following the initial 2.5 min caffeine plus ryanodine challenge, 10 min total exposure to ryanodine resulted in a 24 ± 3% increase in $[Ca^{2+}]_i$ (Figure 6c), in contrast to 7.5 min of PSS without ryanodine, which resulted in a 6 ± 2% increase in $[Ca^{2+}]_i$ (data not shown). The second caffeine challenge in cells treated with ryanodine resulted in an additional 16 ± 3% increase in $[Ca^{2+}]_i$, whereas the second caffeine challenge in the control cells (without ryanodine treatment) resulted in a 40 ± 5% increase in $[Ca^{2+}]_i$ (data not shown). Figures 6b (0 Ca PSS) and 6c ($Ca^{2+}$-containing PSS) summarize the effects of variable initial caffeine exposures on the subsequent ryanodine-induced change in fluorescence ratio. Clearly, with prolonged caffeine pretreatment and therefore, release of more stored $Ca^{2+}$ from the SR, the subsequent ryanodine exposure induced a smaller change in $[Ca^{2+}]_i$.

The evidence accumulated thus far supported a ryanodine-induced increase of $[Ca^{2+}]_i$ through release of $Ca^{2+}$ from an internal store. The question of the contribution of influx to the ryanodine-induced elevation in $[Ca^{2+}]_i$ in bovine tissue, while anticipated to be smaller than that of release, still required clarification. To this end Figure 7 is a summary of the results of the experiment evaluating the influence of increasing extracellular $[Ca^{2+}]$ on $[Ca^{2+}]_i$ of cells challenged with ryanodine. The increase in $[Ca^{2+}]_i$ associated with ryanodine rose further as the $[Ca^{2+}]$ increased in the medium bathing the cells. In 0 Ca PSS 10 μM ryanodine (6 min) did not induce a measurable change in $[Ca^{2+}]_i$. With each increase in $Ca^{2+}$ in the bathing medium to 2, 5 and 10 mM $Ca^{2+}$, while in the continued presence of 10 μM ryanodine (6 min at each $[Ca^{2+}]$), $[Ca^{2+}]_i$ increased to 20 ± 3, 28 ± 4, and 46 ± 4% above baseline, respectively. Subsequent caffeine challenge produced only an additional 7 ± 2% increase in $[Ca^{2+}]_i$ above that attained by ryanodine alone in 10 mM $Ca^{2+}$ PSS.

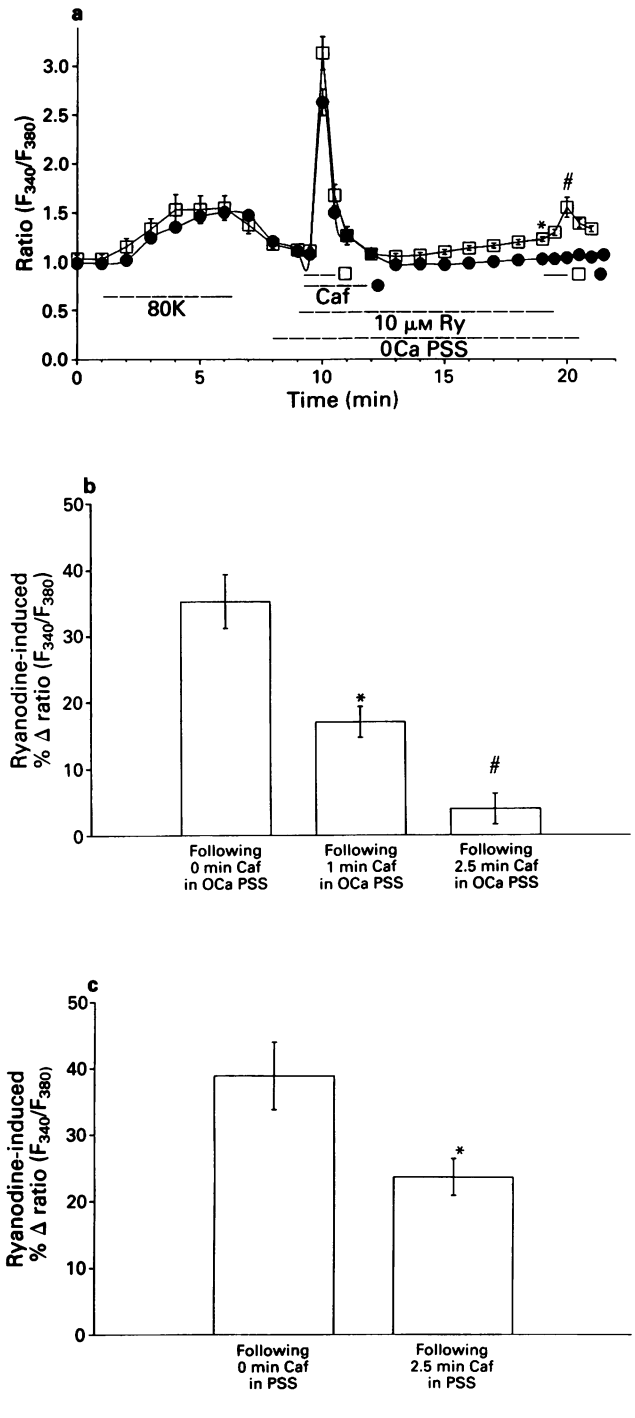



**Figure 6** Caffeine-induced $Ca^{2+}$ release from sarcoplasmic reticulum (SR) decreases the subsequent ryanodine-induced $[Ca^{2+}]_i$ increase in bovine coronary artery smooth muscle cells. (a) Time course of the experiments conducted in which 1 min (□) or 2.5 min (●) caffeine (Caf; 5 mM) plus ryanodine (Ry; 10 μM) is followed by 9 or 7.5 min, respectively, ryanodine exposure (10 min total exposure to ryanodine; * indicates + Ry fluorescence ratio is significantly greater than the − Ry fluorescence ratio at that data point; # indicates the fluorescence ratios are significantly different at that data point). As with previously described protocols, cells were first depolarized in 80 mM potassium (80 K) for 5 min. (b) Ryanodine-induced $[Ca^{2+}]_i$ changes in 0 Ca PSS (% Ry Δ ratio = [Ratio ($F_{340}/F_{380}$) at 19 min]/[Ratio ($F_{340}/F_{380}$) at 9.5 min] × 100−100) following each of the indicated caffeine exposures (0 min: $n = 9$; 1 min; $n = 9$, * indicates significantly different than the 0 min and 2.5 min caffeine-induced responses; 2.5 min: $n = 30$, # indicates significantly different than the 0 min and 1 min caffeine-induced responses). (c) Ryanodine-induced $[Ca^{2+}]_i$ changes in $Ca^{2+}$-containing PSS following each of the indicated caffeine exposures (0 min; $n = 13$; 2.5 min: $n = 10$, *significantly different from the 0 min caffeine-induced response; % Ry Δ ratio calculated as indicated in (b) above).

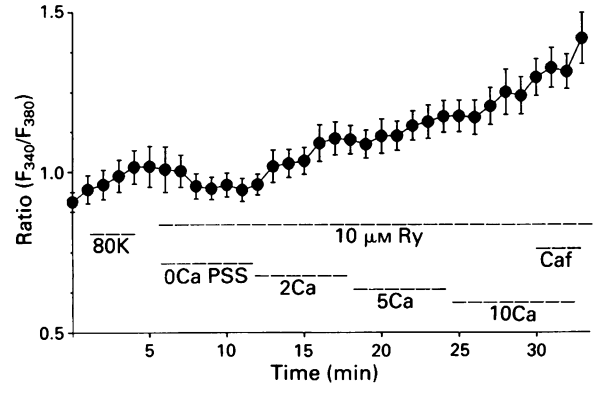



**Figure 7** Increases in superfusing $[Ca^{2+}]$ (0, 2, 5 and 10 mM) are associated with increases in ryanodine-induced ([ryanodine] = 10 μM) $[Ca^{2+}]_i$ changes in bovine cells ($n = 16$). It should be noted that while a return to precise baseline ratio values (fluorescence ratio at 0 min) was never achieved following the 80 mM potassium (80 K) exposure, the fluorescence ratio values during the 0 Ca PSS plus 10 μM ryanodine exposure were not significantly greater than baseline. Caffeine (Caf, 5 mM) was without effect.

When ryanodine exposure resulted in elevated $[Ca^{2+}]_i$ (Figures 3–7), the increase in $[Ca^{2+}]_i$ was sustained. This finding implied that $[Ca^{2+}]_i$ removal processes, presumably via the SR and sarcolemmal $Ca^{2+}$ pumps and $Na^+$-$Ca^{2+}$ exchange (Van Breemen *et al.*, 1986; Slaughter *et al.*, 1989), were not as effective as noted during large increases in $[Ca^{2+}]_i$ evident in response to caffeine without prolonged pretreatment with ryanodine (Figures 3a, 4a, 6a). To test whether these ryanodine-associated sustained elevations of $[Ca^{2+}]_i$ were the result of altered $Na^+$-$Ca^{2+}$ exchange, the caffeine-sensitive SR $Ca^{2+}$ store was depleted and then the cells were preloaded with barium by depolarization with 80 K (2 Ba, 80 K). The cells were then allowed to remove the intracellular free $Ba^{2+}$ ($[Ba^{2+}]_i$) in 0 Ca PSS in the presence or absence of ryanodine. In cells exposed to ryanodine following the 2 Ba, 80 K challenge, the decrease in $[Ba^{2+}]_i$ was significantly ($P < 0.05$) smaller than the decrease in $[Ba^{2+}]_i$ for cells not exposed to ryanodine (Figure 8).

## Discussion

The main findings from these studies on regulation of $[Ca^{2+}]_i$ by ryanodine in bovine and porcine smooth muscle cells are: (1) over the range of dosages examined (0.001 μM to 100 μM), ryanodine effects the release of $Ca^{2+}$ from the caffeine-sensitive internal store; (2) ryanodine (10 μM) also inhibits $Ca^{2+}$-efflux from these cells; and finally, (3) while the above two effects of ryanodine are detectable in both bovine and porcine smooth muscle cells, there are primary differences between the species.

In these studies 10 μM ryanodine induced an increase in $[Ca^{2+}]_i$, whether the cells were superfused with a $Ca^{2+}$-containing or $Ca^{2+}$-free medium. Further, the ryanodine-associated increase in $[Ca^{2+}]_i$ was not different in bovine cells whether the cells were superfused in PSS or 0 Ca PSS. These data support a ryanodine-induced release of $Ca^{2+}$ from an internal store, probably the sarcoplasmic reticulum (SR), over the upper end of the range of dosages studied. While this is in contrast to the findings of Nagasaki & Fleischer (1989), Chu *et al.* (1990), and McGrew *et al.* (1989), who have reported the release of $Ca^{2+}$ in SR vesicles from skeletal muscle at lower concentrations of ryanodine (<μM), and blockade of $Ca^{2+}$ release from the SR at higher concentrations (≥ 10 μM), in smooth muscle ≥ 10 μM concentrations of ryanodine have been shown to induce SR $Ca^{2+}$-release (Hwang & Van Breemen, 1987; Ashida *et al.*, 1988; Nish-

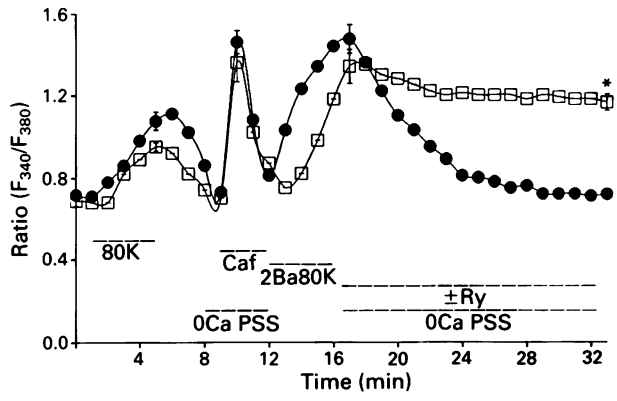



**Figure 8** Ryanodine decreases efflux of intracellular free barium ($[Ba^{2+}]_i$ in porcine cells. After depletion of the sarcoplasmic reticulum (SR) with caffeine, $Ba^{2+}$ was substituted for $Ca^{2+}$ (equimolar) during depolarization-induced influx (2 Ba, 80 K). − Ry = control (no ryanodine), $n = 11$, ●; + Ry = 10 μM ryanodine, $n = 14$, □ (s.e. are indicated only at min 4, 9, 16, and 32). Sustained $[Ba^{2+}]_i$ during exposure to 10 μM ryanodine suggests that ryanodine inhibits $Na^+$-$Ca^{2+}$ exchange. * indicates fluorescence ratios of the two curves at that time (33 min) are significantly different.

imura *et al.*, 1989; Hisayama *et al.*, 1990). The discrepancy between our findings regarding ryanodine-induced $Ca^{2+}$-release in $Ca^{2+}$-free solution and those of Kanmura *et al.* (1988), who demonstrated a $Ca^{2+}$-dependence of the actions of ryanodine might be reconciled by the different species (rabbit versus bovine/porcine), or different smooth muscle source (peripheral versus coronary vasculature). Furthermore, while it is clear that ⩾ 10 μM ryanodine may lock the SR $Ca^{2+}$ release channel in a low subconductance state (Nagasaki & Fleischer, 1989; McGrew *et al.*, 1989), the long duration (> 10 min) exposures to ryanodine in the present studies may result in substantial concentrations of $Ca^{2+}$ being released, thus accumulating as $[Ca^{2+}]_i$ in the intact cell. As indicated by Sutko & Kenyon (1990), the net effect of ryanodine on $[Ca^{2+}]_i$ depends on other $[Ca^{2+}]_i$ removal processes, including the sarcolemmal $Ca^{2+}$ pump and $Na^+$-$Ca^{2+}$ exchange. Direct bilayer (Ehrlich & Watras, 1988; Nagasaki & Fleischer, 1989; McGrew *et al.*, 1989) or *in situ* (Palade *et al.*, 1989) recordings of single $Ca^{2+}$ release channel activity will be necessary to resolve precise mechanisms of ryanodine action on channels in vascular smooth muscle. The only report of single $Ca^{2+}$ release channel activity in smooth muscle SR membranes did not assess the effects of ryanodine (Ehrlich & Watras, 1988).

Another finding was that both ryanodine and caffeine act on a common internal store of $Ca^{2+}$. The protocols included in this study were designed to determine both the effects of ryanodine on subsequent caffeine-induced $Ca^{2+}$ release and *vice versa*. The former set of experiments demonstrated that at the middle to upper end of the ryanodine concentrations studied (micromolar), pretreatment with ryanodine resulted in a measurable decrease in the caffeine-induced $Ca^{2+}$ transient. Furthermore, for [ryanodine] ⩾ 10 μM, there was also a sustained increase in $[Ca^{2+}]_i$. In cells pretreated with caffeine the anticipated ryanodine-induced increase in $[Ca^{2+}]_i$ was decreased (1 min caffeine) or abolished (2.5 min caffeine), consistent with a caffeine-induced depletion of the ryanodine-releasable $Ca^{2+}$ store. These data are consistent with other findings regarding the actions of ryanodine on the smooth muscle caffeine-sensitive $Ca^{2+}$ store (Hisayama & Takayanagi, 1988; Kanmura *et al.*, 1988; Ashida *et al.*, 1988).

Differences were detected between bovine and porcine tissue in the ryanodine-induced $[Ca^{2+}]_i$ changes (Figure 4). Compared with the $[Ca^{2+}]_i$ changes in bovine cells, the ryanodine-induced increases in $[Ca^{2+}]_i$ occurred more slowly in porcine cells. Furthermore, the maximum ryanodine-induced $[Ca^{2+}]_i$ increases were greater in bovine than porcine tissue in 0 Ca PSS. Finally, the results were not due to differences in handling of fura-2/AM by the cells as $[Ca^{2+}]$ sensitivity of myoplasmic fura-2 was not different between bovine and porcine cells (Figure 2). Thus, the data collected in this study support the conclusion that the caffeine-sensitive SR $Ca^{2+}$ store in bovine smooth muscle cells may be larger than that in porcine smooth muscle. This would imply a greater dependence on $Ca^{2+}$ influx to increase $[Ca^{2+}]_i$ in porcine cells.

Perhaps the most controversial, and certainly the most challenging, point is the ryanodine-associated sustained elevation in $[Ca^{2+}]_i$. Maintenance of elevated $[Ca^{2+}]_i$ might be explained by relatively enhanced influx over efflux of $Ca^{2+}$ across the sarcolemma or relatively decreased efflux over influx. We have been unable to identify any published data supporting or even suggesting that ryanodine decreases $Ca^{2+}$ influx in any detectable way. In contrast, ryanodine may increase $Ca^{2+}$ influx via decreased inactivation of voltage-gated $Ca^{2+}$ channels in ventricular myoctyes normally resulting from $Ca^{2+}$ release (Balke & Wier, 1991). A ryanodine-induced sustained increase in $[Ca^{2+}]_i$ has been reported in the literature (Hansford & Lakatta, 1987; Nishimura *et al.*, 1989). Results of studies utilizing $^{45}Ca$ to measure unidirectional $Ca^{2+}$ flux across the sarcolemma have not been consistent. While Kanmura *et al.* (1988) and Hwang & van Breemen (1987) have reported a ryanodine-enhanced $^{45}Ca$ efflux, Ito *et al.* (1986) found that ryanodine exposure resulted in a decreased agonist-induced $^{45}Ca$ efflux. These published studies plus the data from this study led to the hypothesis that ryanodine inhibits $Ca^{2+}$ efflux in vascular smooth muscle.

Maintenance of resting $[Ca^{2+}]_i$ in smooth muscle is achieved by the functional $Ca^{2+}$ buffering activity of the SR and $Ca^{2+}$ extrusion via sarcolemmal $Ca^{2+}$-ATPase activity, and $Na^+$-$Ca^{2+}$ exchange (Smith *et al.*, 1987; Matlib, 1988; Blaustein, 1988; Slaughter *et al.*, 1989). With elimination of the potential contribution of the SR to $Ca^{2+}$ buffering by caffeine treatment and elimination of extrusion by the sarcolemmal $Ca^{2+}$ pump by using barium ($Ba^{2+}$) in 0 Ca PSS (Schilling *et al.*, 1989), we were able to evaluate qualitatively the effect of ryanodine on $Na^+$-$Ca^{2+}$ exchange. Significantly decreased ($P < 0.05$) efflux of $[Ba^{2+}]_i$ from the smooth muscle cells challenged with 10 μM ryanodine versus cells not challenged with ryanodine provided evidence that ryanodine inhibits $Ca^{2+}$ efflux via $Na^+$-$Ca^{2+}$ exchange. This is of particular interest because 10 μM ryanodine has been used almost exclusively to modulate specifically SR function in intact smooth muscle cells (Hwang & Van Breemen, 1987; Kanmura *et al.*, 1988; Ashida *et al.*, 1988; Hisayama & Takayanagi, 1988; Hisayama *et al.*, 1990). Identification of multiple actions of ryanodine, therefore, is significant.

In conclusion, ryanodine (⩾ 10 μM) releases $Ca^{2+}$ from the SR and decreases $Ca^{2+}$ efflux in bovine and porcine vascular smooth muscle cells, thus causing a sustained increase in $[Ca^{2+}]_i$. Ryanodine is, nonetheless, a valuable tool to aid in the delineation of SR function, as shown by the identification of a greater dependence on $Ca^{2+}$ release from the SR to increase $[Ca^{2+}]_i$ in bovine as compared with porcine smooth muscle.

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# Dexamethasone-induced gastric mucosal damage in the rat: possible role of platelet-activating factor

[1*†]János G. Filep, [†]Ferenc Hermán, [*†]Éva Földes-Filep, [†]Ferenc Schneider & [**]Pierre Braquet

*Department of Pharmacology, Faculty of Medicine, University of Sherbrooke, Sherbrooke, P.Q., Canada J1H 5N4, †Department of Pathophysiology, Semmelweis University Medical School, Budapest, 1445 Hungary and **Institut Henri Beaufour, Le Plessis Robinson, 92350 France

**1** The aim of the present experiments was to study the possible role of platelet-activating factor (PAF) in mediating gastric mucosal damage induced by dexamethasone in the rat by measuring gastric tissue levels of PAF during dexamethasone-treatment and by investigating the effects of specific PAF receptor antagonists on dexamethasone-induced gastric lesions. PAF-like bioactivity extracted from the rat glandular stomach was determined by a platelet aggregation assay.

**2** Dexamethasone treatment (0.4– 4 mg $kg^{-1}$, daily for 1– 6 days) produced time- and dose-dependent damage to the glandular mucosa of the stomach as characterized by extensive, uniform hyperaemia with multiple, focal petechiae and erosions.

**3** These changes were accompanied by a time-, and dose-dependent increase in PAF content of the glandular stomach. Control rat stomach contained small amounts of PAF (0.14 ± 0.04 ng per g wet weight), which increased over 40 fold in response to dexamethasone treatment (4 mg $kg^{-1}$, daily for 6 consecutive days). The presence of PAF-like material in the stomach extract was ascertained by thin-layer chromatography, high performance liquid chromatography and by alkaline hydrolysis.

**4** Pretreatment of the animals with one or other of the structurally unrelated PAF receptor antagonists, BN 52021 (10 mg $kg^{-1}$, i.p.) or BN 50727 (1 mg $kg^{-1}$, i.p.) significantly reduced dexamethasone-induced gastric damage. In these animals neither petechiae nor erosions were observed.

**5** These observations suggest that PAF is a likely endogenous mediator of glucocorticoid-induced gastric mucosal damage in the rat.

**Keywords:** PAF; dexamethasone; glucocorticoid-induced gastric ulcer; PAF antagonists; BN 52021 (ginkgolide B); BN 50727

## Introduction

Although there is a long-standing debate over whether corticosteroid therapy leads to peptic ulcer disease in man (recently reviewed by Piper *et al.*, 1991), it is well established that administration of glucocorticoids to laboratory animals can result in an acute gastric erosion formation (Robert & Nezamis, 1958; Nobuhara *et al.*, 1985; Wallace, 1987). The mechanism of this damaging action of glucocorticoids is, however, far from being understood. Glucocorticoids have been shown to inhibit synthesis of gastric mucus (Menguy & Masters, 1963), to induce G cell and parietal cell hyperplasia (Delaney *et al.*, 1979) and to affect epithelial cell turnover (Eastwood, 1984). Dexamethasone and prednisolone have been shown to be ineffective in blocking prostaglandin (Whittle, 1978; Wallace, 1987), but not leukotriene $C_4$ (Wallace, 1987) synthesis in the rat stomach, suggesting that their ulcerogenic action may not be related to decreased prostaglandin formation.

Platelet-activating factor (PAF) is a phospholipid mediator of allergy and inflammation with a wide variety of biological activities including platelet aggregation, activation of neutrophil granulocytes, contraction of smooth muscle, increasing vascular permeability and inducing hypotension (for a recent review see Braquet *et al.*, 1987). Administration of picomol doses of PAF into the gastric artery induced substantial gastric mucosal hyperaemia, haemorrhage and erosions (Rosam *et al.*, 1986; Wallace & Whittle, 1986; Whittle *et al.*, 1986; Espluques & Whittle, 1988), changes similar to those observed following glucocorticoid administration (Robert & Nezamis, 1958; Nobuhara *et al.*, 1985; Wallace, 1987). These findings raised the possibility that release of endogenous PAF may underlie or contribute to certain forms of gastric ulceration.

The aim of the present experiments was to study the possible role of PAF in mediating gastric damage to dexamethasone by measuring the PAF content in the rat stomach during dexamethasone treatment and by investigating the effects of two structurally unrelated, specific PAF receptor antagonists, BN 52021 (ginkgolide B) (Braquet *et al.*, 1985; Földes-Filep *et al.*, 1987) and the benzodiazepine derivative, BN 50727 (Braquet & Esanu, 1991) on dexamethasone-induced gastric lesions.

## Methods

Male, Wistar rats weighing 160–205 g were housed in groups of 4 and were fed standard laboratory chow and tap water *ad libitum*.

### *Drug treatment*

Between 09 h 00 min and 10 h 00 min each day the animals were given an intraperitoneal injection of the PAF receptor antagonists, BN 52021 (10 mg $kg^{-1}$) or BN 50727 (1 mg $kg^{-1}$), or their vehicle, and 30 min later they received a subcutaneous injection of dexamethasone sodium phosphate or vehicle. The effectiveness of BN 52021 and BN 50727 treatments was tested in preliminary experiments in anaesthetized (Inactin, 100 mg $kg^{-1}$, i.p.) male rats by injecting 25 and 50 ng $kg^{-1}$ PAF into the right femoral vein 30 min before and 30 min after administration of either BN 52021, 10 mg $kg^{-1}$, i.p., or BN 50727, 1 mg $kg^{-1}$, i.p. Mean arterial

[1] Author for correspondence at: Department of Pharmacology, Faculty of Medicine, University of Sherbrooke, Sherbrooke, P.Q., Canada J1H 5N4.

blood pressure was monitored through a catheter inserted into the right femoral artery by an electromanometer (EM 61, Medicor, Budapest, Hungary) using a Statham P23 dB pressure transducer (Hato Ray, Puerto Rico). The following sets of experiments were performed:

*Series 1* The animals received 0.4, 1 or 4 mg $kg^{-1}$ of dexamethasone or its vehicle daily for 6 consecutive days.

*Series 2* The animals were given 4 mg $kg^{-1}$ of dexamethasone each day for 1, 2, 3, 4, 5 or 6 consecutive days.

*Series 3* The animals were divided into four groups: (a) control (receiving vehicle only), (b) BN 52021 (i.e. BN 52021, 10 mg $kg^{-1}$, i.p. plus vehicle s.c.), (c) dexamethasone (vehicle i.p. plus dexamethasone, 4 mg $kg^{-1}$ s.c. ) and (d) BN 52021, 10 mg $kg^{-1}$ plus dexamethasone, 4 mg $kg^{-1}$. The treatments were continued for 6 consecutive days.

*Series 4* In these series of experiments a protocol similar to that of Series 3 was followed except that another PAF receptor antagonist, BN 50727 (1 mg $kg^{-1}$, i.p.) was used instead of BN 52021.

Depending on the experimental protocol used, on days 1, 2, 3, 4, 5 or 6 the animals were anaesthetized with sodium pentobarbitone (40 mg $kg^{-1}$, i.p.) 5 h after the second injection. The animals were deprived of food, but not water, for 18–20 h before being anaesthetized. The stomach was immediately removed, opened along the greater curvature and examined. The extent of mucosal damage was scored by two observers in a randomized, blind manner using a 0 to 3 scoring system (Rosam *et al.*, 1986; Wallace, 1987). A score of 0 was assigned if the mucosa appeared normal. A stomach exhibiting hyperaemia or haemorrhage was assigned a score of 1 (limited to diffuse superficial hyperaemia), 2 (severe, extensive hyperaemia or focal haemorrhage) or 3 (multiple sites of bleeding or erosion). The correlation between the scores assigned by the two observers was greater than 0.860 ($n = 20$, $P < 0.001$) in any one series of experiments. The incidence and area of mucosal damage (petechiae and erosions) were quantified under an operation microscope (Wild, Heerbrugg, Germany; magnification: × 25) using a 1 × 1 mm eye grid. Then sections for microscopic examination were taken from the damaged areas, or if none was observed, randomly. Tissues were fixed in neutral buffered formalin, embedded into Durcupan ACM (Araldit). One μm thick sections were made and stained with toluidine blue. During histological evaluation special attention was given to the integrity of the surface epithelial cells and presence of erosions.

### *Extraction and purification of PAF*

Following scoring, the glandular stomach was weighed, minced and homogenized in 1 volume of 10 mM N-morpholinoethanesulphonic acid (MES) buffer containing 130 mM $Na^+$, 5.4 mM $K^+$, 1.8 mM $Ca^{2+}$, 112 mM $Cl^-$, 0.25% (w/v) fatty acid free bovine serum albumin, pH 5.2. [$^3$H]-PAF at 220,000 d.p.m. was added to monitor procedural losses and then lipids were extracted. Briefly, 3 volumes of ice-cold methanol acidified with acetic acid (49:1 vol/vol) was mixed with the sample. After 30 min at room temperature the mixture was centrifuged. The precipitated protein was again mixed with 5 ml of ice-cold methanol, stirred for 30 min at room temperature and centrifuged. Both methanol phases were pooled. Then chloroform and water were added to effect phase separation (chloroform:methanol:water 1:1:0.9 vol/vol/vol). The lower chloroform-rich phase contained all PAF activity. Chloroform was evaporated under a stream of nitrogen and PAF was extracted on silica cartridges (Sep Pak, Waters, Milford MA, U.S.A.) (López-Farré *et al.*, 1988). The fractions containing PAF were collected and dried under a stream of nitrogen.

### *Bioassay and characterization of PAF-like activity*

Bioassayable PAF activity was determined by a platelet aggregation assay (Filep *et al.*, 1989). Washed rabbit platelets were prepared as described by Ardlie *et al.* (1970). Platelet aggregation was determined at 37°C in a computerized four-channel aggregometer (CARAT, Budapest, Hungary) using 400 μl of platelet suspension ($2 \times 10^5$ cells per μl) containing 10 μM indomethacin and 0.4 u $ml^{-1}$ apyrase. Samples or standards were added in a volume of 10 μl. A standard curve of platelet aggregation by authentic PAF was obtained, yielding progressive platelet aggregation ranging from 300 pM to 100 nM. PAF bioactivity of experimental samples was determined in duplicate and compared against the standard curve. Values were corrected for recovery (ranging from 48 ± 81%) and were expressed as ng PAF generated per g wet tissue weight. In some experiments, BN 52021 (10 μM) was added to the platelets 2 min before adding PAF standards or the samples. In preliminary experiments, the PAF values obtained by either measuring aggregation or [$^3$H]-5-hydroxytryptramine release from preloaded rabbit platelets (Pinckard *et al.*, 1979) were consistent with each other ($r = 0.886$, $n = 6$, $P < 0.01$), thus only the aggregation assay was used in later experiments.

Characterization of PAF-like activity was performed with the following techniques (Filep *et al.*, 1989): (a) One dimensional thin-layer chromatography. After bioassay some samples were pooled, re-extracted, dissolved in chloroform and spotted on silicagel plates (Merck, Darmstadt, FRG) and developed with chloroform:methanol:water (65:35:6 vol/vol/vol). In separate lanes standard PAF was run. PAF was visualized by iodine vapor. (b) Analysis by alkaline hydrolysis. In some experiments, half of the sample (after purification over silica cartridge) was subjected to alkaline hydrolysis (15 min in 0.5 M KOH at 45°C) followed by re-extraction and bioassay. (c) High performance liquid chromatography (h.p.l.c.). Reverse-phase h.p.l.c. was performed using a Liquopump 312/1, a Liquochrom 2010 variable wavelength detector (Labor MIM, Budapest, Hungary) and an octadecylsilyl column (Chromsil, 6 μm particle size, 250 × 4 mm). The elution solvent was methanol:water:acetonitrile (85:10:5 vol/vol/vol) containing 20 mM choline chloride at a flow rate of 1 ml $min^{-1}$ (Pinckard *et al.*, 1984).

### *Effects of BN 52021 on gastric prostanoid formation*

The animals were treated with BN 52021, 10 mg $kg^{-1}$ or its vehicle daily for 6 consecutive days. On day 6, following scoring of the mucosa, samples of the fundic region of the stomach (200–300 mg wet weight) were excised for determination of tissue levels of thromboxane $A_2$ and prostacyclin. The tissue sample was homogenized for 15 s in 1.5 ml of Tris buffer (50 mM, pH 8.4), and then centrifuged for 1 min at 10,000 *g*. The supernatant was decanted into a tube containing 10 μM indomethacin, then [$^3$H]-prostaglandin $F_{1\alpha}$ ([$^3$H]-$PGF_{1\alpha}$, 4000 d.p.m.) was added to monitor procedural losses and prostanoids were extracted according to the method of Green *et al.* (1978). Concentrations of 6-keto $PGF_{1\alpha}$ and thromboxane $B_2$, the stable metabolites of prostacyclin and thromboxane $A_2$, respectively, were measured by radioimmunoassay kits (IZINTA, Budapest, Hungary and Amersham, UK, respectively). The 6-keto $PGF_{1\alpha}$ and thromboxane $B_2$ antisera had less than 0.9 and 1.2% cross reactivity with other prostanoids, respectively. Intra-assay coefficients of variation were 3.9% ($n = 6$) and 1.2% ($n = 7$), respectively. Values were corrected for individual recovery ranged between 52 and 97%.

### *Drugs and chemicals*

Dexamethasone sodium phosphate (Decadron, Merck, Sharp & Dohme-Chibret, Zürich, Switzerland) was dissolved to

10 mg ml$^{-1}$ in sterile 0.9% NaCl solution. BN 52021 (ginkgolide B, 9H-1, 7a-(epoxymethanol)-1H, 6aH, cyclopenta[c] [2-3-b]furo-[3,2':3,4] cyclopenta[1-2-d]-furan-5, 9, 12-[4H]- trione, 3-*tert*-butyl-hexahydro-4, 7b, 11 hydroxy-8 methyl) and BN 50727 (tetrahydro-4, 7, 8, 10 methyl-1 (chloro-2 phenyl) -6 (methoxy-4-phenyl-carbamoyl)-9 pyrido [4', 3'-4-5] thieno [3, 2-f] triazolo-1, 2, 4[4, 3-a]diazepine-1,4) (Institut Henri Beaufour, Le Plessis Robinson, France) were dissolved in dimethylsulphoxide at a concentration of 50 mg ml$^{-1}$ and were further diluted with 0.9% NaCl. Indomethacin, apyrase, bovine serum albumin, N-morpholinoethanesulphonic acid were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; Inactin was purchased from CEVA, Hamburg, Germany; PAF (1-0-hexadecyl-2-0-acetyl-sn-glycero-3-phosphorylcholine) was purchased from Bachem, Bubendorf, Switzerland. [$^3$H]-PAF (specific activity: 80 Ci mmol$^{-1}$, purity greater than 95%) was obtained from Amersham, U.K.

### *Statistical analysis*

Results are expressed as means ± s.e.mean. Statistical evaluation of the data were performed by Dunn's test for multiple comparison (i.e. control versus dexamethasone-treated, control versus BN 52021-treated, etc.) (Dunn, 1964) and by Student's *t* tests, as appropriate. Correlation coefficients were calculated by the Spearman correlation test using ranks. A $P<0.05$ level was considered significant for all tests.

## Results

### *Dexamethasone-induced gastric mucosal damage*

In order to study the time-, and dose-related effects of dexamethasone on gastric mucosa, a series of experiments were performed in which groups of rats were given various doses of dexamethasone daily for 6 days and groups of rats were treated with dexamethasone, 4 mg kg$^{-1}$ each day for 1 to 6 days. There was a dose-response relationship in terms of the activity of dexamethasone in evoking mucosal damage (Figure 1). The highest dose of dexamethasone tested (4 mg kg$^{-1}$ daily for 6 days) caused significant gastric damage (Figure 1). In rats killed after 1–3 days of dexamethasone administration (4 mg kg$^{-1}$ daily) gastric damage scores did not differ significantly from the control group (Figure 2). However, after 4 days of dexamethasone treatment, severe gastric mucosal damage was detected (Figure 2). This damage was characterized by extensive, uniform hyperaemia with multiple, focal regions of haemorrhage (petechiae) and in 10 out of 14 rats by mucosal erosions. Histological evaluation revealed exfoliation of the surface epithelium, necrotic lesions of focal character penetrating into one-fourth to one-third of the entire mucosal thickness (Figure 3). Administration of BN 52021 in itself did not affect the appearance of the gastric mucosa, while it was highly effective in inhibiting dexamethasone-induced alterations (Table 1). In BN 52021 plus dexamethasone-treated animals, neither erosions nor in 8 out of 9 rats petechiae were observed (Table 1). BN 52021 also reduced the severity of hyperaemia: in 4 out of 9 rats receiving BN 52021 plus dexamethasone, the gastric mucosa appeared to be normal both macroscopically and microscopically (Figure 3), whereas in the other 5 animals only a superficial hyperaemia was observed.

Like BN 52021, the other PAF receptor antagonist, BN 50727 by itself did not evoke any mucosal damage (Table 1). Pretreatment of the animals with BN 50727 (1 mg kg$^{-1}$, i.p.) resulted in similar inhibition of dexamethasone-induced gastric lesions as that seen in animals treated with BN 52021 (Table 1). Neither erosions nor petechiae were observed in these animals and even the gastric mucosa appeared to be normal in 2 out of 5 animals treated with this PAF receptor antagonist plus dexamethasone.

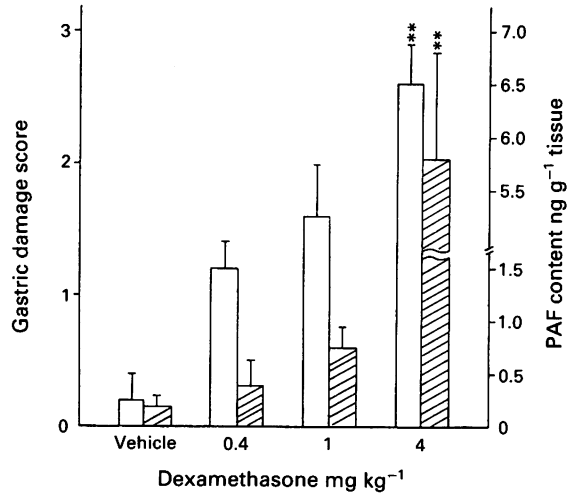


**Figure 1** Gastric damage scores (open columns) and tissue PAF contents (hatched columns) for rats treated with various doses of dexamethasone daily for 6 days. Values are means for 5 rats; s.e.mean shown by vertical bars. Multiple comparisons were performed by Dunn's multiple contrast hypothesis test using ranks. $^{**}P<0.01$ (compared to vehicle).

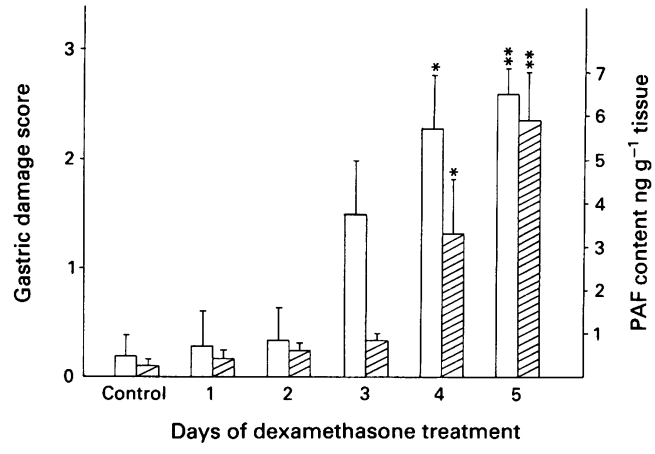


**Figure 2** Gastric damage scores (open columns) and gastric tissue PAF contents (hatched columns) following administration of dexamethasone (4 mg kg$^{-1}$ daily) to rats for up to 5 days. Control animals received 0.9% NaCl solution daily for 5 days. Values are means for 3–5 animals; s.e.mean shown by vertical lines. Multiple comparisons were performed by Dunn's multiple contrast hypothesis test using ranks. $^{*}P<0.05$; $^{**}P<0.01$ (compared to control).

### *Effectiveness of the PAF receptor antagonists*

Intraperitoneal injection of BN 52021 (10 mg kg$^{-1}$, 30 min prior to i.v. administration of PAF) almost completely abolished the hypotensive response to 25 ng kg$^{-1}$ (21.7 ± 2.7 mmHg versus 0.6 ± 1.0 mmHg decrease in mean arterial blood pressure before and after BN 52021, respectively; $n=3$, $P<0.05$) and to 50 ng kg$^{-1}$ of exogenous PAF (27.7 ± 2.7 mmHg versus 4.7 ± 1.9 mmHg decrease in mean arterial blood pressure before and after BN 52021, respectively; $n=3$, $P<0.05$). Similarly, pretreatment of the animals with BN 50727 (1 mg kg$^{-1}$, i.p., 30 min before i.v. injection of PAF) markedly attenuated the hypotensive effect of exogenous PAF. Mean arterial blood pressure decreased by 22.3 ± 3.1 mmHg versus 0.8 ± 1.2 mmHg, $n=4$, $P<0.05$, and by 29.4 ± 3.5 mmHg versus 4.2 ± 0.7 mmHg, $n=4$, $P<0.05$ in response to 25 and 50 ng kg$^{-1}$ PAF in the absence and presence of BN 50727, respectively.

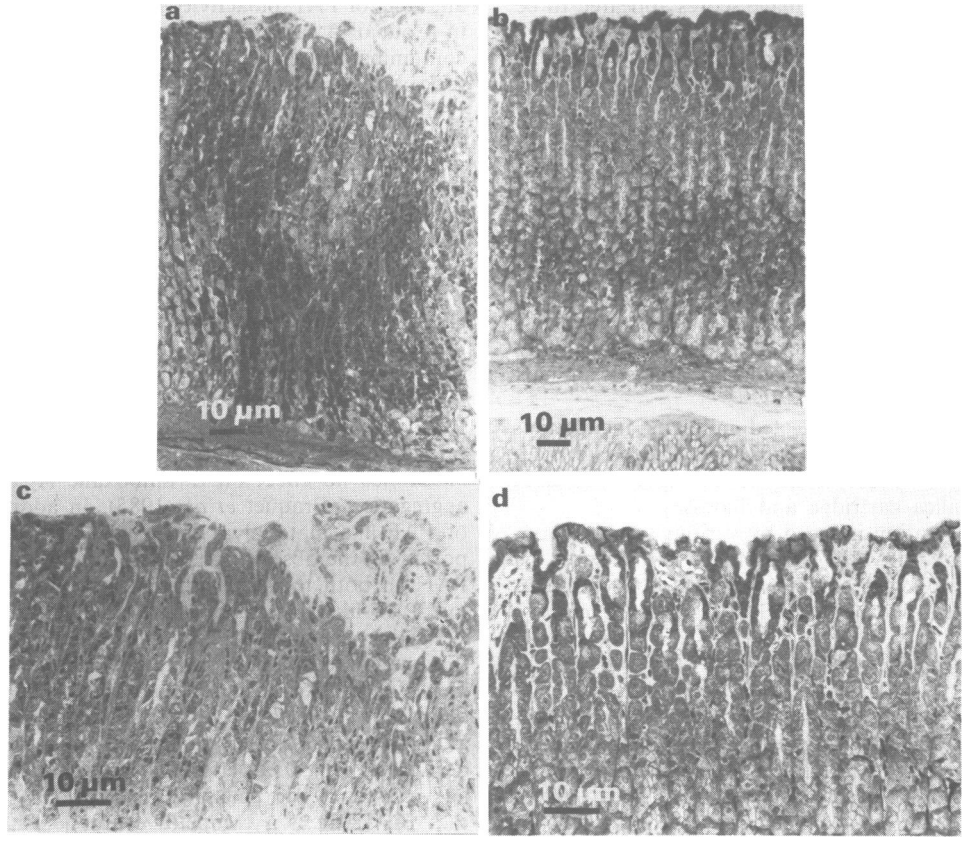


**Figure 3** Prevention by BN 52021 of dexamethasone-induced gastric mucosal damage. Dexamethasone (4 mg $kg^{-1}$), BN 52021 (10 mg $kg^{-1}$) or vehicle were administered daily to rats for 6 days. On day 6, sections of the stomach were prepared for histological examination as described under Methods. (a and c) Dexamethasone-induced haemorrhagic erosion. The necrotic region extends down to one-fourth of the mucosa and does not penetrate the muscularis mucosae (toluidine blue stain; a, magnification × 900). Necrotic tissues and extruded cells can be seen in the lumen (c, magnification: × 1400; upper right corner). (b and d) Gastric structure in rats treated with BN 52021 plus dexamethasone. Macroscopically, this region also appeared to be normal (b, magnification: × 900; d, magnification: × 1400).

**Table 1** Effects of dexamethasone treatment on gastric mucosa and PAF content in the rat glandular stomach and prevention by the PAF receptor antagonists, BN 52021 and BN 50727

| | *Damage scores* | | | *Area of petechiae and erosions* | | | *PAF content* (ng $g^{-1}$ tissue wet weight) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Treatment*[a] | | n | P[b] | $mm^2$ | n | P[b] | | (range) | n | P[b] |
| Vehicle | 0.4 ± 0.1 | 14 | — | 0 | 14 | — | 0.14 ± 0.04 | (0[c]–0.23) | 5 | — |
| BN 52021 | 0.4 ± 0.2 | 8 | NS | 0 | 8 | NS | 0.12 ± 0.05 | (0[c]–0.26) | 4 | NS |
| BN 50727 | 0.2 ± 0.2 | 5 | NS | 0 | 5 | NS | NM | | | |
| Dexamethasone | 2.6 ± 0.2 | 14 | <0.001 | 4.1 ± 0.7 | 14 | <0.001 | 5.88 ± 1.19 | (2.02–8.54) | 5 | <0.05 |
| BN 52021 plus dexamethasone | 0.7 ± 0.2†† | 9 | NS | 0.1 ± 0.1†† | 9 | NS | 1.60 ± 0.47†† | (0.24–2.79) | 5 | <0.05 |
| BN 50727 plus dexamethasone | 0.6 ± 0.2†† | 5 | NS | 0.3 ± 0.2† | 5 | NS | NM | | | |

[a]The animals were given BN 52021 (10 mg $kg^{-1}$), BN 50727 (1 mg $kg^{-1}$) or vehicle i.p. 30 min before subcutaneous injection of dexamethasone (4 mg $kg^{-1}$) or its vehicle, daily for 6 consecutive days. Values are means ± s.e.mean. *n*, number of the animals; NM, not measured.
[b]Compared to untreated rats; †$P<0.05$; ††$P<0.01$, compared to dexamethasone-treated animals. Statistical analysis was performed by Dunn's multiple contrast hypothesis test using ranks. NS, not significant.
[c]Below the detection limit (300 pM) of the platelet aggregation assay.

### *Determination of bioassayable PAF activity*

We used the rabbit platelet aggregation assay for measuring PAF bioactivity. After lipid extraction samples were further purified over silica cartridge before bioassay. Gastric tissue extracts from untreated and BN 52021-treated animals contained very low amounts of PAF-like activity; in 2 out of 9 samples measured the amount of PAF was below the detection limit of the assay. Samples prepared from rats treated with dexamethasone contained higher amounts of PAF-like activity than those prepared from control animals. This effect of dexamethasone appeared to be dose related (Figure 1). However, due to the close proximity of the dose, which caused severe mucosal damage (4 mg $kg^{-1}$) and the dose, which caused slight changes (i.e. superficial hyperaemia) (0.4 mg $kg^{-1}$), a definite dose-dependency could not be dem-

onstrated. Nevertheless, higher damage scores appeared to be associated with higher tissue PAF levels (Figure 1).

Treatment of the animals with dexamethasone (4 mg $kg^{-1}$) daily for 1–3 days resulted in an elevation in gastric PAF content, which was not statistically significant (Figure 2). Four days of dexamethasone treatment was required to elicit significant mucosal damage or increase in tissue level of PAF (Figure 2). Here again, higher damage scores were associated with higher tissue PAF contents (Figure 2). Samples prepared from rats treated with dexamethasone (4 mg $kg^{-1}$, daily for 6 days) contained about 40 fold higher amount of PAF-like activity than those prepared from control animals (Table 1). When BN 52021 and dexamethasone treatments were combined, PAF-like content of gastric extracts was significantly lower than that found in dexamethasone-treated animals (Table 1).

No PAF-like activity was detected in blank incubations (i.e. when dexamethasone or BN 52021 were added to incubation buffer without tissue) followed by lipid extraction, purification over silica cartridge and bioassay.

The platelet aggregation induced by various samples (containing 8–120 ng $ml^{-1}$ PAF-like bioactivity as measured by the aggregation assay) was completely blocked by pretreatment of the platelets with BN 52021, 10 $\mu$M, for 2 min.

### *Identification of PAF-like bioactivity*

The identity of the material recovered in the extraction procedure with ability to induce aggregation of washed rabbit platelets was deduced from the following results: (a) in thin layer chromatography it has an $R_F$ value of $0.23 \pm 0.02$ (3 independent determinations), which is identical to that of authentic PAF ($R_F$ value $0.25 \pm 0.02$, $n = 3$); (b) h.p.l.c. analysis of the pooled samples used in the platelet aggregation assay showed a single peak with a retention time of $7.5 \pm 0.2$ min ($n = 3$) similar to that of synthetic PAF ($7.4 \pm 0.2$ min, $n = 4$); (c) Pooled samples containing 8.6, 27.2, 41.8 and 104.3 ng PAF bioactivity per ml were subjected to alkaline hydrolysis. This resulted in loss of detectable PAF activity in the platelet aggregation assay.

### *Effects of BN 52021 on gastric prostanoid formation*

Tissue levels of 6-keto $PGF_{1\alpha}$ were $412 \pm 83$ and $487 \pm 135$ pg per mg tissue wet weight, ($n = 4$, $P > 0.05$) in animals given vehicle or BN 52021, 10 mg $kg^{-1}$ daily for 6 days, respectively. Similarly, no significant differences could be detected in tissue thromboxane $B_2$ levels ($94 \pm 12$ pg $mg^{-1}$ tissue in control and $113 \pm 20$ pg $mg^{-1}$ tissue in BN 52021-treated animals, $n = 4$).

## Discussion

Recent studies have shown that PAF is a potent ulcerogenic agent in the rat stomach (Rosam *et al.*, 1986; Whittle *et al.*, 1986; Espluques & Whittle, 1988). This ulcerogenic property of PAF is neither mediated via platelet activation or generation of cyclo-oxygenase products, nor via stimulation of histamine $H_1$, $H_2$ and $\alpha$-adrenoceptors (Rosam *et al.*, 1986). Microvascular changes (i.e. local vasoconstriction, exudation of plasma and aggregation of one or more components of blood) (Rosam *et al.*, 1986; Whittle *et al.*, 1986) observed after injection of exogenous PAF could lead to stasis and may therefore, contribute to the ulcerogenic action of PAF.

The present results suggest that PAF may be a mediator of dexamethasone-induced gastric lesions. Dexamethasone treatment resulted in a time-, and dose-related increase in gastric PAF levels. Stomachs from rats treated with dexamethasone, at a dose that caused haemorrhagic mucosal damage contained significantly greater amounts of PAF than controls. Furthermore, higher damage scores were associated with higher tissue PAF contents. We were able to detect very low amounts of PAF bioactivity in the stomach of untreated rats in 4 out of 5 cases. Although great care was taken to avoid contamination of the samples with PAF during the extraction procedure and aggregometry, it is still uncertain whether PAF is actually present *in situ*, or generated by mechanical or chemical stimuli during the analytical procedure. It is worth mentioning that similarly low amounts of PAF bioactivity were found in the rat forestomach, glandular stomach and duodenum (Sugatani *et al.*, 1989), kidney (Blank *et al.*, 1979), and uterus (Yasuda *et al.*, 1986). The biological role of PAF present in normal tissues is still unknown.

PAF was determined as a substance able to induce aggregation of washed rabbit platelets. Since this assay was performed in the presence of indomethacin, a cyclo-oxygenase blocker and apyrase, an ADP scavenger, it is highly unlikely that aggregation was induced by thromboxane $A_2$ or ADP. Furthermore, platelet aggregation evoked by extracts from rat stomach can be inhibited by BN 52021, whereas BN 52021 has no effect on thromboxane $A_2$ and ADP-induced aggregation (Braquet *et al.*, 1985). In addition, the presence of PAF-like material in the tissue extract was further supported by the observations that it co-migrated with authentic PAF on both thin-layer chromatography and h.p.l.c. and it lost PAF-like bioactivity following alkaline treatment.

Further evidence suggesting a role for PAF in dexamethasone-induced gastric damage is derived from the experiments with PAF receptor antagonists. Pretreatment of the animals with the structurally unrelated, specific PAF receptor antagonists, BN 52021 (ginkgolide B) or the benzodiazepine derivative, BN 50727 effectively inhibited the mucosal damage caused by dexamethasone. Previously we have shown that BN 52021 is a competitive antagonist of PAF with an approximately tenfold lower affinity for the PAF receptor of various cells than PAF itself (Braquet *et al.*, 1985; Földes-Filep *et al.*, 1987). BN 52021 and BN 50727 at the doses used markedly inhibited the hypotensive response to exogenous PAF. Furthermore, BN 52021 and BN 50727 at the dose of 10 and 1 mg $kg^{-1}$, respectively, significantly reduced gastric damage evoked by PAF injected i.v. (Braquet *et al.*, 1988; Braquet & Esanu, 1991). Both BN 52021 and benzodiazepine-derivative PAF receptor antagonists possess similar potency in inhibiting the effects of exogenous PAF on vascular permeability in the gastrointestinal tract (Filep *et al.*, 1991). These observations indicate that the effects of BN 52021 and BN 50727 were related to antagonism of PAF.

PAF content of gastric tissues from animals treated with BN 52021 plus dexamethasone was lower than those measured in rats receiving dexamethasone. The reasons for this effect are unclear. Since PAF, once released, can stimulate various cells to synthesize PAF (Doebber & Wu, 1987; Tessner *et al.*, 1989), an explanation might be that inhibition of PAF-induced PAF formation by BN 52021 led to reduced tissue PAF contents. Inhibition by BN 52021 of PAF synthesis through the remodelling pathway is unlikely, since BN 52021 did not affect phospholipase activity as shown by the unchanged gastric prostanoid formation.

The present experiments give little information on the mechanism(s) by which dexamethasone-treatment led to increased PAF formation. Since higher gastric damage scores were associated with higher gastric PAF levels, one might consider that either enhanced PAF formation induced mucosal damage or the increased tissue PAF levels may only reflect damage to the mucosa. This latter speculation should, however, be tempered by the fact that cold-water immersion stress-induced gastric mucosal lesions were associated with decreased tissue PAF levels in the rat (Sugatani *et al.*, 1989). In a wide range of cells, glucocorticoids have been demonstrated to inhibit phospholipase $A_2$ (Blackwell *et al.*, 1980; Hirata *et al.*, 1980) which is also involved in the release of precursors for PAF through the remodelling pathway (Snyder, 1987). Thus, one could anticipate that the reverse effect might be observed, i.e. reduced PAF levels following dexamethasone treatment. However, glucocorticoids do not in-

hibit prostanoid synthesis in the rat stomach, even at doses which caused haemorrhagic damage (Whittle, 1978; Wallace, 1987).

The cellular sources of PAF cannot be deduced from the present experiments. Mast cells (Wallace, 1989), gastric mucosal cells (Fernandez-Gallardo *et al.*, 1988), vascular endothelial cells (McIntyre *et al.*, 1985) as well as blood-borne cells are capable of synthesizing PAF (cf. Braquet *et al.*, 1987), and consequently one or more of them might be responsible for the enhanced tissue PAF levels observed in the present study.

In conclusion, the present results show that dexamethasone-induced gastric mucosal lesions are associated with a markedly elevated tissue PAF content in rat stomach and demonstrate the effectiveness of PAF receptor antagonists in preventing this damage. These data suggest that PAF is probably an endogenous mediator of gastric mucosal damage evoked by dexamethasone.

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# Bradykinin stimulation of phosphoinositide hydrolysis in guinea-pig ileum longitudinal muscle

[1]Richard W. Ransom, *Carl B. Goodman & Gretchen S. Young

Department of New Lead Pharmacology, Merck Sharp & Dohme Research Laboratories, West Point, PA 19486-0004 and *College of Pharmacy, Florida A&M University, Tallahassee, FL 32307, U.S.A.

1 Bradykinin (BK)-induced contraction of ileal smooth muscle is assumed to be due to phosphoinositide hydrolysis but this has never been reported. We have investigated whether BK receptors are linked to this transduction mechanism in guinea-pig ileum longitudinal muscle and determined whether these receptors are equivalent to those labelled in [$^3$H]-BK binding assays.

2 In membranes prepared from longitudinal muscle, [$^3$H]-BK bound to a single class of sites with high affinity. Characterization of the binding with BK analogues indicated that the radioligand selectivity labelled a $B_2$ type receptor.

3 BK significantly elevated tissue levels of [$^3$H]-inositol phosphates in longitudinal muscle slices preincubated with [$^3$H]-*myo*-inositol. The agonists potencies of BK, Lys-BK, Met-Lys-BK, Tyr$^5$-BK and Tyr$^8$-BK were in agreement with their relative potencies in the binding assay. The $B_1$ receptor agonist des-Arg$^9$-BK, did not stimulate inositol phosphate production. The response to BK was blocked by known $B_2$ receptor antagonists but not by the $B_1$ antagonist des-Arg$^9$, Leu$^8$-BK.

4 BK-induced phosphoinositide hydrolysis was unaffected by exposure of muscle slices to either atropine or indomethacin.

5 The results indicate that the $B_2$ receptors linked to phosphoinositide turnover in ileal longitudinal muscle exhibit properties similar to those involved in contractile responses. Also, the receptor mediating the phosphoinositide response is likely to be that labelled in the [$^3$H]-BK binding studies.

**Keywords:** Bradykinin; phosphoinositide hydrolysis; $B_2$ receptors

## Introduction

The nonapeptide bradykinin (BK) has been shown to exhibit an array of biological activities. These include contraction and/or relaxation of vascular smooth muscles, stimulation of epithelial ion transport, activation of nociceptive sensory nerves, and modulation of neurotransmitter release. The membrane receptors that mediate these diverse actions have been categorized as being either of the $B_1$ or $B_2$ type. These two classes are distinguished on the basis of their relative affinities for des-Arg$^9$ kinins which exhibit significant activity at $B_1$ receptors while being virtually inactive at $B_2$ receptors (Regoli & Barabe, 1980). Studies to date have shown that the predominant physiological and pathological (e.g., pain sensation, bronchoconstriction and inflammation) responses to BK involve $B_2$ receptors although it is becoming increasingly clear that $B_2$ receptor subtypes exist (Llona *et al.*, 1987; Farmer *et al.*, 1989). The two primary signal transduction mechanisms linked to $B_2$ receptors are stimulation of phosphoinositide hydrolysis and eicosanoid biosynthesis (Hong & Levine, 1976; Yano *et al.*, 1984; Miller, 1987). Elevation of cellular inositol phosphates by BK involves G-protein coupled activation of a phospholipase C and there is some evidence that arachidonic acid release may be due to stimulation of phospholipase $A_2$ through a G-protein-mediated mechanism (Burch & Axelrod, 1987).

The ileum of several species has been used to investigate the pharamcology and physiology of BK. Receptors in this tissue have been characterized as being of the $B_2$ type and mediate both smooth muscle contraction and the ion secretory activity of the mucosa (Manning *et al.*, 1982; Kachur *et al.*, 1987). Chloride ion transport across the muscosa is associated with prostaglandin biosynthesis as it is sensitive to cyclo-oxygenase inhibitors such as indomethacin (Cuthbert & Margolius, 1982). The second messenger system involved in the stimulation by BK of ileal longitudinal muscle has not been defined but this action does not appear to involve prostaglandins as the contraction is not appeciably attenuated by cyclo-oxygenase inhibitors (Walker & Wilson, 1989). In this paper we have examined kinin effects on phosphoinositide turnover in guinea-pig ileum longitudinal muscle. The results suggest it is this mechanism that underlies the contractile actions of BK in the tissue.

## Methods

### *Tissue preparation*

Male Hartley guinea-pigs (300–500 g) were anaesthetized with ether and decapitated. At a level 2 cm above the ileocecal junction, a section of ileum approximately 40 cm in length was removed, dissected free of fat and flushed with isotonic saline. The longitudinal muscle was obtained as described by Rang (1964). Briefly, 10 cm sections of tissues were stretched over a glass rod and a piece of surgical gauze was used to separate the longitudinal muscle from the underlying tissue by gently rubbing away from the mesenteric border. For membrane binding assays the tissue was homogenized with a Polytron in 20 vol of ice-cold 25 mM N-tris (hydroxymethyl)methyl-2-aminoethane sulphonic acid (TES, pH 6.8 at room temperature) buffer containing 1 mM 1,10-phenanthroline. The homogenate was centrifuged for 15 min at 48,000 *g* and the resulting pellet was rehomogenized in fresh buffer and then centrifuged once again. The final pellet was resuspended in assay buffer (above buffer with 0.1% protease-free bovine serum albumin, 5 μM MK-422 (enalaprilate; Gross *et al.*, 1981) and 140 μg ml$^{-1}$ bacitracin) by use of a motor-driven teflon-glass tissue grinder. In some assays a

[1] Author for correspondence.

physiological buffer (components listed below) was used in place of TES. Protein determinations were performed by the method of Bradford (1976) with bovine IgG used as the standard.

### *Binding assay*

Membrane binding assays followed the method of Manning *et al.* (1986). [$^3$H]-BK was incubated for 60 min with 0.5 mg tissue (wet weight) at 25°C in a final volume of 1 ml. The assay was terminated by filtration over 0.1% polyethyleneimine soaked (3 h) Whatman GF/B filters using a Brandel M-24 cell harvester. The tubes were rinsed two times with 4 ml ice-cold 10 mM TES and filter-bound radioactivity was quantitated by liquid scintillation spectrometry. Nonspecific binding was determined by performing incubations in the presence of 1 μM BK and represented less than 5% of the total binding at 50 pM [$^3$H]-BK. Competition and saturation experiments were analyzed with the EBDA programme of McPherson (1985).

### *Phosphatidylinositol hydrolysis*

The longitudinal muscle was obtained as described above except that it was maintained in an oxygenated (100% $O_2$) physiological buffer (concentrations in mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $KH_2PO_4$ 1.2, $NaHCO_3$ 5, HEPES 20 and glucose 11; pH 7.4 at 37°C). The tissue was sliced (350 μm) in two directors at 90° angles with a McIlwain Tissue Chopper and the slices were washed several times by adding fresh buffer, allowing the slices to settle and aspirating the media. Following a 75 min incubation at 37°C in the presence of 0.6 μM [$^3$H]-*myo*-inositol, the buffer was removed and the slices washed repeatedly over a 10 min period with buffer supplemented with 0.1% BSA, 100 μg ml$^{-1}$ bacitracin, 5 μM MK-422 and 10 mM LiCl (Berridge *et al.*, 1982). In the experiments performed in the absence of $Ca^{2+}$, $CaCl_2$ was omitted from the LiCl-supplemented buffer used for the washing of the slices and during the actual assay. EGTA (1 mM) was also present during the final wash and throughout the incubation with BK. Thirty microlitre aliquots of gravity packed tissue were allocated to each assay tube followed by 200 μl of LiCl buffer. The final volume was 250 μl after the addition of agonist and, when used, antagonist. Antagonists were present for 2 min before the addition of agonist. Tubes were incubated for 25 min at 37°C and the reaction was stopped by the addition of 1 ml chloroform-methanol-12 N HCl (1:2:0.01). After 45 min, 300 μl each of chloroform and water were added and the tubes were centrifuged to separate the phases. A 750 μl aliquot of the upper phase was diluted to 4 ml with $H_2O$ and analyzed for [$^3$H]-inositol phosphates as described by Bone *et al.* (1984). Columns (0.75 ml AG1-X8 resin; Bio-Rad, Richmond, CA, U.S.A.) were sequentially washed with 10 ml $H_2O$, 15 ml 5 mM sodium tetraborate/60 mM ammonium formate, 8 ml 5 mM sodium tetraborate/200 mM amonium formate, 8 ml 400 mM ammonium formate/0.1 M formic acid and 8 ml 1 M ammonium formate/0.1 M formic acid. The tritium content in 5 ml aliquots of the latter three eluates were determined to quantitate the levels of [$^3$H]-inositol mono-, di- and triphosphates, respectively.

### *Radioreceptor assay*

The stabilities of BK and Lys-BK were estimated by means of a radioreceptor binding assay (Enna & Snyder, 1976). Each peptide (100 nM) was incubated with longitudinal muscle membranes and slices under the conditions used for the binding and phosphoinositide assays, respectively. At the end of the incubation, the tissue was centrifuged at 48,000 *g* for 15 min and the supernatants were recovered. Aliquots of each sample were then examined for their ability to inhibit [$^3$H]-BK binding to longitudinal muscle membranes by the TES assay buffer. Standard curves were obtained for each peptide by conducting a competition experiment with that peptide on the day of assay. Each of the tubes used for the standard curve received the same volume of buffer used for determining the experimental peptide concentrations. Preliminary control experiments were performed with supernatants from membrane and tissue slice incubations from which peptides were omitted. In each case, there was no inhibition of binding when 100 μl of sample was added to the [$^3$H]-BK binding assay. The standard curves were linearized by the logit transformation and sample peptide concentrations were obtained with GRAPHAD (ISI Software, Philadelphia, PA, U.S.A.).

### *Materials*

[$^3$H]-BK (96-103 Ci mmol$^{-1}$) and [$^3$H]-*myo*-inositol (10–20 Ci mmol$^{-1}$) were obtained from New England Nuclear (Boston, MA, U.S.A.). BK and all other peptides were from Peninsula Laboratories (Belmont, CA, U.S.A.). MK-422 was obtained from Merck Sharp & Dohme Research Laboratories (Rahway, NJ, U.S.A.). All other reagents used for these studies were of the highest grade commercially available.

## Results

In TES buffer at 25°C [$^3$H]-BK binding reached equilibrium within 45 min and was stable for at least 2 h. Saturation experiments in the low ionic strength buffer showed that [$^3$H]-BK labelled a single class of sites with an equilibrium dissociation constant ($K_D$) of 16 ± 4 pM (mean ± s.e.mean, $n = 8$) and a maximal binding density of 244 ± 13 fmol mg$^{-1}$ protein (Figure 1). Saturation studies were performed in physiological buffer at pH 7.4 to provide a better estimation of the true biological affinity of BK for its receptors. In these experiments, [$^3$H]-BK was again found to label a homogeneous class of sites with a similar $B_{max}$ value (266 ± 18 fmol mg$^{-1}$ protein, $n = 3$) but with much lower affinity ($K_D$ = 289 ± 14 pM). The [$^3$H]-BK sites were further characterized by use of both agonist and antagonist BK analogues in competition studies under optimal binding conditions (low ionic strength, low pH) and in the near-physiological buffer. The results of Table 1 show that there was nearly a uniform 20 fold reduction in inhibitory potency for all of the peptides when assayed in the physiological medium. The Hill slopes of the inhibition curves for each peptide under the different conditions were consistently near one.

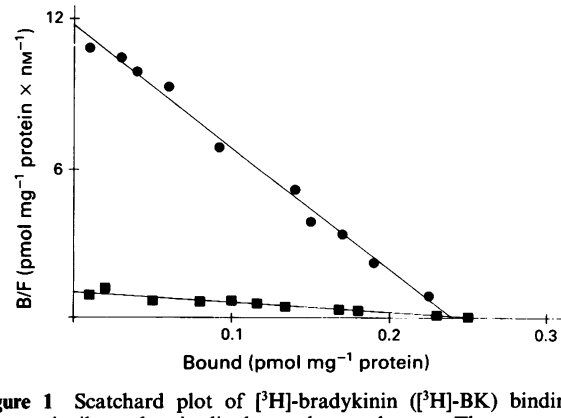


**Figure 1** Scatchard plot of [$^3$H]-bradykinin ([$^3$H]-BK) binding to guinea-pig ileum longitudinal muscle membranes. The assays were conducted in a low ionic strength buffer (●) or a physiological buffer (■). Each data point is mean of triplicate determinations that varied by < 10%. The experiment was repeated two additional times with similar results. Binding constants are given in the text.

**Table 1** Inhibitory potencies for bradykinin (BK) analogues in the [$^3$H]-BK binding assay

| | $K_i$ (nM) | |
|---|---|---|
| *Inhibitor* | *TES buffer* | *Kreb HEPES buffer* |
| BK | 0.021 ± 0.004 | 0.35 ± 0.05 |
| Lys-BK | 0.029 ± 0.006 | 0.60 ± 0.08 |
| Met-Lys-BK | 0.076 ± 0.005 | 1.81 ± 0.19 |
| Tyr$^8$-BK | 0.168 ± 0.009 | 3.69 ± 0.23 |
| Tyr$^5$-BK | 2.2 ± 0.9 | 51 ± 10 |
| Des-Arg$^9$-BK | 10200 ± 741 | $>10^5$ |
| D-Arg[Hyp$^3$,Thi$^{5,8}$,D-Phe$^7$]BK | 3.1 ± 0.4 | 49 ± 7 |
| D-Arg[Hyp$^{2,3}$,Thi$^{5,8}$,D-Phe$^7$]BK | 5.4 ± 0.9 | 97 ± 9 |
| Lys,Lys[Hyp$^3$,Thi$^{5,8}$,D-Phe$^7$]BK | 9.2 ± 1.8 | 166 ± 13 |
| [Thi$^{5,8}$,D-Phe$^7$]BK | 31 ± 7 | 496 ± 38 |
| Des-Arg$^9$,Leu$^8$-BK | $28 \times 10^3$ ± 1800 | $>10^5$ |

The radioligand concentration was 50 pM for assays performed in TES buffer at pH 6.8 and 150 pM for assays in the physiological buffer. The $K_i$ values were calculated by the equation of Cheng & Prusoff (1973) using the appropriate $K_D$ for BK under each assay condition (in text). The values are the mean ± s.e.mean from at least three experiments for each inhibitor.

In the phosphoinositide assay, preliminary experiments showed that an optimal incubation time for detecting BK-induced elevation of [$^3$H]-inositiol phosphates was 25 min. The relative contributions of [$^3$H]-inositol mono-, bis- and tris-phosphates to the signal are shown in Figure 2. Since the signal to noise ratio was found to be much greater for the [$^3$H]-IP$_1$ column eluate, only this fraction was monitored in subsequent experiments. The accumulation of [$^3$H]-IP$_1$ was linear during the 25 min incubation period. The concentration of BK required for half-maximal ($EC_{50}$) stimulation of [$^3$H]-IP$_1$ levels was 13 ± 4 nM ($n = 5$). BK maximally elevated tissue [$^3$H]-IP$_1$ by 342 ± 19% relative to basal values. The response was not dependent on extracellular $Ca^{2+}$ as BK elevated [$^3$H]-IP$_1$ levels by 315 ± 18% ($n = 3$) relative to basal levels when slices were stimulated in nominally $Ca^{2+}$-free media. For comparative purposes, the responses to substance P and carbachol were also examined. High concentrations of the tachykinin (3 μM) and the muscarinic agonist (300 μM) stimulated [$^3$H]-IP$_1$ production by 689 ± 51% ($n = 3$) and 1679 ± 204% ($n = 3$), respectively. Dose-response curves for other BK receptor agonists were also obtained (Figure 3). The $EC_{50}$ values for Lys-BK, Met-Lys-BK, Tyr$^8$-BK and Tyr$^5$-Bk were 18 ± 5 nM, 139 ± 15 nM, 268 ± 21 nM and 4.7 ± 1.1 μM, respectively. These potencies are in general agreement with the relative affinities of the peptides in the [$^3$H]-BK binding assay (Table 1). There was no response to 10 μM des-Arg$^9$-BK indicating that the kinin induced PI hydrolysis is mediated by a $B_2$ receptor. This was confirmed by the antagonism of the response by known $B_2$ receptor antagonists (Figure 4; Stewart & Vavrek, 1987). The $IC_{50}$ values obtained for D-Arg[Hyp$^3$,Thi$^{5,8}$,D-Phe$^7$]BK, Lys-Lys [Hyp$^3$,Thi$^{5,8}$,D-Phe$^7$]BK and [Thi$^{5,8}$,D-Phe$^7$]BK were 110 ± 14 nM, 503 ± 25 nM and 982 ± 104 nM, respectively. This order of potencies is also in agreement with the observed effects of the inhibitions in the binding assay. The $B_1$ receptor antagonist des-Arg$^9$,Leu$^8$-BK at 10 μM had no effect on BK stimulated [$^3$H]-IP$_1$ production. None of the peptides that antagonized the BK response were found to inhibit either substance P (100 nM) or carbachol (30 μM) stimulation of phosphoinositide turnover.

There remained a significant disparity between the absolute potencies of the kinin agonists in the binding assay using the physiological buffer and the phosphoinositide assay. A radioreceptor assay was therefore used to determine whether the difference is due to the loss of agonist during incubations with tissue slices. The results (Table 2) show that there was a time-dependent loss of BK and Lys-BK. This is in contrast to the stability of these two peptides in the binding assay. After a 60 min incubation at 25°C with longitudinal muscle membranes, the radioreceptor assay indicates that 92 ± 3% ($n = 3$) of BK and 89 ± 6% ($n = 3$) of Lys-BK was intact.

BK has been reported to release acetylcholine from guinea-pig ileum longitudinal muscle-myenteric strip preparations (Goldstein *et al.*, 1983). Also, one study has indicated that BK-induced release of acetylcholine is an indirect action that follows increased prostanoid biosynthesis (Yau *et al.*, 1986). As such, the possible contribution of these mechanisms to the BK phosphoinositide response were evaluated. In slices pre-exposed to 10 μM indomethacin (30 min, 37°C) BK (20 nM)-stimulated [$^3$H]-IP$_1$ accumulation was 97 ± 5% ($n = 3$) of the control value. In the presence of 10 μM atropine the response was 96 ± 4% ($n = 3$) of the corresponding control value.

## Discussion

These experiments have shown that BK significantly stimulates phosphoinositide hydrolysis in guinea-pig ileum longitudinal muscle. This effect may result in the elevation of

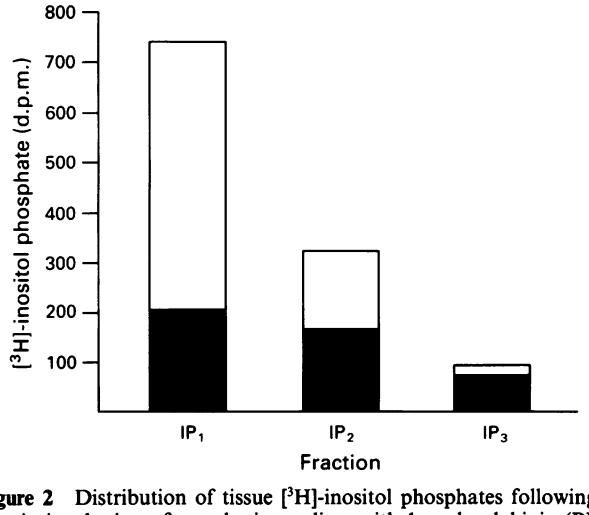


**Figure 2** Distribution of tissue [$^3$H]-inositol phosphates following a 25 min incubation of muscle tissue slices with 1 μM bradykinin (BK). The column elutions were performed according to Bone *et al.* (1984) as described in the Methods section. The solid portion of each column represents the tritium in each fraction in the absence of BK while the open area indicates the increase observed in the presence of the agonist. The same results were obtained in two other experiments.

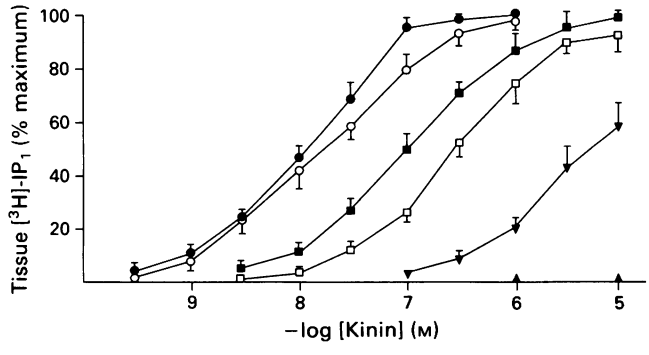


**Figure 3** Dose-response curves for the stimulation of [$^3$H]-inositol monophosphate accumulation by bradykinin (BK) and its analogues. The 100% value indicates the response due to 1 μM BK. The points are means for at least three experiments with each agonist; s.e.mean shown by verticle bars. BK (●); Lys-BK (○); Met-Lys-BK (■); Tyr$^8$-BK (□); Tyr$^5$-BK (▼); des-Arg$^9$-BK (▲).

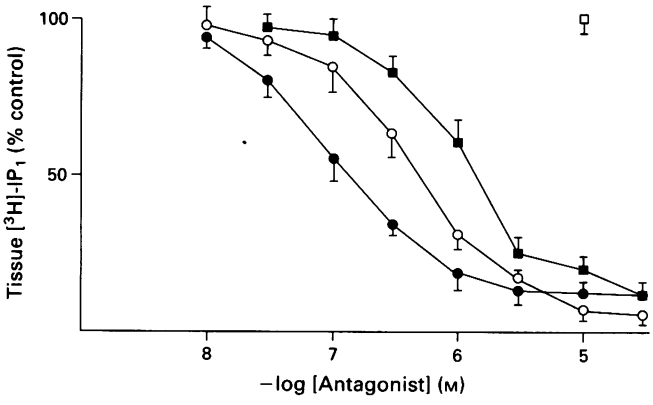


**Figure 4** Inhibition of bradykinin (BK) stimulated accumulation of [$^3$H]-inositol monophosphate by D-Arg[Hyp$^3$,Thi$^{5,8}$,D-Phe$^7$]BK (●), Lys,Lys[Hyp$^3$,Thi$^{5,8}$,D-Phe$^7$]BK (○) and [Thi$^{5,8}$,D-Phe$^7$]BK (■). The data points are mean from three experiments; vertical bars show s.e.mean. The 100% value of the ordinate is the response to 20 nM BK. The $IC_{50}$ values for the antagonists are presented in the text. There was no inhibition of the BK response by the $B_1$ receptor antagonist des-Arg$^9$,Leu$^8$-BK at 10 μM (□).

**Table 2** Estimation of bradykinin (BK) and Lys-BK stability by radioreceptor assay

| | *Peptide recovered (% control)* | |
|---|---|---|
| *Time (min)* | *BK* | *Lys-BK* |
| 5 | 89 ± 6 | 95 ± 4 |
| 10 | 78 ± 6 | 83 ± 7 |
| 15 | 59 ± 5 | 64 ± 6 |
| 20 | 43 ± 3 | 48 ± 5 |
| 25 | 29 ± 4 | 37 ± 5 |

Incubations were performed with 100 nM of each peptide under the conditions of the phosphoinositide assay. At the indicated times the tissue was centrifuged and aliquots were assayed in the [$^3$H]-BK binding assay described under Methods. The results are means ± s.e.mean from at least three separate determinations performed in duplicate. The values are expressed as a percentage of control recovery in which the peptides were incubated for the appropriate times in the absence of tissue.

cytosolic calcium ion levels and underlie the contractile actions of kinins in the tissue. However, it has been shown that influx of extracellular $Ca^{2+}$ by increasing buffer $K^+$ concentrations results in phosphoinositide hydrolysis in this tissue (Sasaguri & Watson, 1988). In order to examine the possibility that the kinin response is secondary to an increase in membrane $Ca^{2+}$ permeability, experiments were performed in the nominal absence of the divalent cation. The BK response was not appreciably decreased suggesting that kinin induced phosphoinositide hydrolysis results from a receptor-mediated activation of phospholipase C. The high potency of BK and the lack of agonist activity of des-Arg$^9$-BK indicate that the response is mediated through a $B_2$ receptor. This was also evident from the inhibition of the response by documented $B_2$ recetor antagonists while the $B_1$ antagonist des-Arg$^9$,Leu$^8$-BK (Regoli & Barabe, 1980) had no effect on BK stimulation of [$^3$H]-$IP_1$ production. These results are in agreement with previous findings that kinin contraction of ileal smooth muscle is due to $B_2$ receptor activation (Kachur *et al.*, 1987). In organ bath studies, BK contraction of guinea-pig ileum is not atropine-sensitive and therefore does not appear to involve activation of cholinergic neurones in the myenteric plexus (Ambache & Roche e Silva, 1951). Nevertheless, it has been reported that BK can release acetylcholine from longitudinal muscle-myenteric plexus preparations (Goldstein *et al.*, 1983). Also, Yau *et al.* (1986) presented results that BK-induced release of [$^3$H]-acetylcholine from the preparation is sensitive to indomethacin, implying that this kinin effect was dependent upon stimulation of prostaglandin biosynthesis. In as much as carbachol produced a very large increase in [$^3$H]-$IP_1$ levels in the slice preparation used in the present experiments, we examined the possibility that the BK response is linked to either prostaglandin biosynthesis or the release of endogenous acetylcholine which would then activate smooth muscle phosphoinositide-linked muscarinic receptors. It was found that neither exposure of the slices to indomethacin nor atropine inhibited BK stimulation of phosphoinositide hydrolysis. These results suggest that the BK response is due to a direct effect on smooth muscle cells and are consistent with the findings that contraction of longitudinal smooth muscle occurs independently of whatever effects BK may have on cholinergic neurones in the tissue.

[$^3$H]-BK binding to longitudinal muscle membranes were performed in order to provide a correlate for the activity of the kinins in the PI hydrolysis assay. Under conditions previously described by others (Manning *et al.*, 1986), [$^3$H]-BK labelled a single class of sites with very high affinity ($K_D$ = 16 pM). A site with similar affinity and binding density was observed by Manning *et al.* (1986) in membranes prepared from whole ileum. However, they also reported a second lower affinity ($K_D$ = 910 pM) site with a density almost twice that of high affinity site. Such a site was not observed in our experiments using longitudinal muscle membranes nor have we detected a lower affinity site in membranes prepared from whole ileum (data not included). The reason for this discrepancy is not clear since the two assays are identical except that Manning *et al.* (1986) used captopril rather than MK-422 to inhibit kininase II (angiotensin converting enzyme). Dithiothreitol was included to maintain the sulphhydryl of captopril in a reduced state but this agent has no effect on [$^3$H]-BK binding in our assay. Nevertheless, Manning *et al.* (1986) did conclude that the biological activities (ileum contraction and mucosal chloride ion secretion) of BK and its analogues correlated with their potencies in inhibiting [$^3$H]-BK binding to the high affinity site. The present study has shown that there is a reasonably good correlation between binding assay affinities of agonists and antagonists and their relative effects in the phosphoinositide assay. The correlation was maintained whether [$^3$H]-BK binding was performed in the low ionic strength-low pH buffer or in a near physiological buffer. Thus, the results show that for all of the kinin analogues examined there was between a 16 fold and 24 fold reduction in binding affinity when assayed in the physiological buffer. Although the relative potencies of the agonists in the more physiological binding assay and the phosphoinositide assay were in general agreement, there did remain a rather large difference in the absolute potencies. For example, the $K_i$ for BK was about 0.30 nM in the binding assay yet its $EC_{50}$ for stimulating phosphoinositide hydrolysis was over 10 nM. This discrepancy may be due to several factors. For instance, the phosphoinositide assay is performed with a high concentration of tissue in a small volume and secondary sites, even of low affinity, may reduce available agonist concentrations. It is also known that at 37°C, the temperature used for the phosphoinositide assay, BK is labile due to increased peptidase activity. A radioreceptor assay was used to investigate whether loss of agonist through either metabolism or nonspecific tissue absorption could account for the discrepancy. The data show that there is a time-dependent decrease in BK and Lys-BK during the incubation with the tissue slices though it does not appear to be sufficient to account for the almost 40 fold difference in the potencies of these agonists in the binding and phosphoinositide assays. It is presumed that the radioreceptor assay is measuring intact BK and Lys-BK as there are no hydrolysis products of the peptides that have appreciable affinity for $B_2$ receptors at the concentrations at which they would be present in the assay (Fredrick *et al.*, 1984). Thus, other factors must be responsi-

ble for the disparity between kinin agonist activity in the two assays. Certainly, the assays are quite different in that binding is performed under equilbrium conditions while such a stiuation is not possible to reproduce in an assay measuring receptor-mediated phospholipid hydrolysis in tissue slices. Yet, aside from this concern it should be noted that the absolute potencies of the kinin agonists in promoting phosphoinositide hydrolysis are quite similar to those reported for contracting longitudinal muscle in organ bath studies. For instance, the concentrations of BK and Lys-BK required to produce 50% of the maximal contraction of the ileum were found to be in the 10–20 nM range (Regoli *et al.*, 1990). Also, the agonists Met-Lys-BK, Tyr$^8$-BK and Tyr$^5$-BK are reported to have 30%, 25% and 1%, respectively, of the contractile potency of BK in the guinea-pig ileum (Stewart, 1979). Though not in absolute agreement with the relative activities of these peptides in stimulating phosphoinositide hydrolysis, there is a sufficient similarity to support the proposal that phosphoinositide hydrolysis in smooth muscle cells underlines the contractile activity of the kinins. The potencies of the antagonists required to block the BK response are in agreement with those needed to inhibit BK contractions of ileal longitudinal muscle as well (Steranka *et al.*, 1988; Tousignant *et al.*, 1991).

The receptor labelled in the binding studies appears to be similar to that which mediates phosphoinositide turnover and the contractile actions of kinins in longitudinal muscle. Yet the results of Yau *et al.* (1986) indicate that BK receptors are also coupled to prostaglandin biosynthesis in longitudinal muscle-myenteric plexus preparations. Inasmuch as only a single binding site was detected, either the receptors associated with the two transduction mechanisms are pharmacologically identical or one may not be detectable in the binding assay, possibly because of low density. The effects of kinins on eicosanoid production in guinea-pig ileum longitudinal muscle have not been adequately explored. Muscle contractions are not sensitive to indomethacin (Stewart, 1979) but that in itself does not refute the results of Yau *et al.* (1986). More work is needed before any conclusions can be drawn regarding the BK receptors linked to eicosanoid biosynthesis. The presence of two transduction systems would not necessarily imply the existence of separate receptors since it is possible that the same receptor may couple to more than a single type of G-protein. Alternatively, stimulation of phospholipase $A_2$ activity may be a consequence of elevated cytosolic diacylglycerol and $Ca^{2+}$ levels following receptor activation of phospholipase C (Burch & Axelrod, 1987).

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# Differences in neurokinin receptor pharmacology between rat and guinea-pig superior cervical ganglia

[1]G.R. Seabrook, M. Main, B. Bowery, N. Wood & R.G. Hill

Department of Pharmacology, Merck Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Road, Harlow, Essex, CM20 2QR

**1** The depolarizations elicited by seven neurokinin receptor agonists were examined in both rat and guinea-pig superior cervical ganglia by use of grease-gap methodology in the presence of tetrodotoxin (0.1 μM). Responses were normalised with respect to 1 μM eledoisin.

**2** The rank order of agonist potency in the rat ganglia was senktide > substance P > substance P methyl ester = eledoisin = Sar-Met-substance P > neurokinin B > neurokinin A, whereas in guinea-pig superior cervical ganglion (SCG) the rank order was senktide > Sar-Met-substance P > neurokinin B = eledoisin = substance P methyl ester. The concentration-effect curves for substance P and neurokinin A in guinea-pig ganglia were biphasic which precluded the determination of meaningful potency values.

**3** The maximal depolarization achieved by subtype selective ligands was different between these two species. On rat and guinea-pig SCG, the $NK_3$-selective ligand, senktide, produced a maximal depolarization of 27% and 274% respectively, whereas the $NK_1$-selective ligand, substance P methyl ester, produced depolarizations of 77% and 64% respectively.

**4** The depolarizations induced by substance P methyl ester and senktide in either species were unaffected by atropine (1 μM), suggesting a lack of involvement of presynaptic neurokinin receptors in the generation of the response.

**5** The potency of substance P methyl ester, senktide, and neurokinin A were unaffected by pretreating ganglia with the peptidase inhibitors bacitracin (40 μg $ml^{-1}$), leupeptin (4 μg $ml^{-1}$), and chymostatin (2 μg $ml^{-1}$). Similarly, these peptidase inhibitors had no effect on the maximal depolarizations achieved by any of these agonists.

**6** It is evident that rat and guinea-pig superior cervical ganglia possess both $NK_1$ and $NK_3$ receptors, but that their net contribution to depolarizations are different between the two species. The depolarizations in guinea-pig SCG are mediated predominantly by an $NK_3$ subtype and in rat SCG by an $NK_1$ receptor subtype.

**Keywords:** Substance P; senktide; sympathetic ganglia; tachykinin; neurokinin receptors

## Introduction

Substance P is an eleven amino acid peptide discovered by von Euler & Gaddum in 1931, and subsequently sequenced from bovine hypothalamus by Chang & Leeman in 1970. In mammals receptors to substance P have since been shown to be widely distributed, in the central nervous system, autonomic ganglia and peripheral musculature. Consequently activation of these receptors elicits a diversity of physiological effects. Such responses are principally excitatory, and include the increase in firing rate of monoamine neurones in the CNS (Guyenet & Aghajanian, 1977), the depolarization of autonomic ganglia (Dun & Karczmar, 1979; Konishi *et al.*, 1985), and contraction of smooth muscle (Lee *et al.*, 1982). The coupling of these receptors to physiological responses involves G proteins, usually linked to the inhibition of one or more potassium conductances (Nakajima *et al.*, 1988; Stanfield *et al.*, 1985; Nowak & Macdonald, 1982). In sympathetic ganglia substance P is localized in ganglionic cell bodies and fibres of intrinsic interneurones (Hokfelt *et al.*, 1977; Robinson *et al.*, 1980; Dalsgaard *et al.*, 1982). When released by either electrical or chemical stimulation this peptide elicits a postsynaptic depolarization caused partly by the inhibition of postsynaptic potassium conductances which includes, in some ganglia, the voltage-dependent M-current (Adams *et al.*, 1983; Konishi *et al.*, 1985).

At least three neurokinin receptor subtypes have been identified in the mammalian nervous system (reviewed in Iversen *et al.*, 1990). To date their characterization has been based principally on the potency of the following agonists, substance P, neurokinin A, and neurokinin B, which are the prototypic $NK_1$, $NK_2$ and $NK_3$ receptor selective ligands respectively. Recently, some important progress has been made in the development of specific $NK_1$ and $NK_2$ receptor antagonists (Williams *et al.*, 1988; McKnight *et al.*, 1988b; Ward *et al.*, 1990; Snider *et al.*, 1991). However, currently no selective $NK_3$ antagonist is available, and thus agonist pharmacology still remains an important criterion in receptor classification. More than one receptor subtype can presumably coexist within any one tissue and the majority of tachykinin agonists can act upon more than one subtype; thus definitive studies into neurokinin receptor subtypes required full evaluation of both the potency and efficacy of a range of neurokinin agonists.

It has been suggested that in a number of tissues the neurokinin agonist pharmacology does not conform to described receptor subtypes. These tissues include guinea-pig trachea where the existence of an $NK_4$ receptor subtype has been suggested (McKnight *et al.*, 1988a), although, with the use of $NK_1$- and $NK_2$-selective antagonists it is clear that more than one neurokinin receptor subtype may be present in this tissue (Ireland *et al.*, 1991). In rat superior cervical ganglia an anomalous agonist profile has also been reported (Brown *et al.*, 1983). In this study we present evidence for a heterogeneity of neurokinin receptors in rat and guinea-pig

[1] Author for correspondence.

superior cervical ganglia, demonstrating that their relative contribution to the maximal depolarization which can be elicited with tachykinins, exhibits differences between these two mammalian species.

## Methods

Superior cervical ganglia were excised from male Sprague-Dawley rats (150–250 g), and male Dunkin Hartley guinea-pigs (200–300 g), which had previously been concussed and then exsanguinated. Ganglia were desheathed and then placed in a grease-gap recording chamber (Brown *et al.*, 1980) through which warm physiological salt solution was continually perfused (25°C, 1–2 ml $min^{-1}$). The saline contained (in mM): NaCl 125, KCl 5, $KH_2PO_4$ 1, $CaCl_2$ 2.5, $MgSO_4$ 1, $NaHCO_3$ 25, glucose 10 and tetrodotoxin 0.1 μM and was gassed with 95% $O_2$: 5% $CO_2$.

The potential difference between the ganglion cell body (earthed) and the postganglionic trunk was monitored via Ag/AgCl electrodes connected via a d.c. amplifier to a chart recorder. Drugs were applied to the ganglion cell body for 1 min, at 40 min intervals to minimize receptor desensitization. Ganglia were allowed to stabilize until consecutive applications of either eledoisin (1 μM) or senktide (1 μM) produced depolarizations of equilavent amplitude and then the test ligand was applied in increasing concentration. The absolute potential changes were dependent upon the electrical seal and cell density of each preparation, and therefore data were normalized to a control application of eledoisin (1 μM) in each ganglion. This concentration produced submaximal depolarizations in ganglia of both species, and as such minimized errors associated with receptor desensitization. Eledoisin was used as the standard, in preference to substance P, because it produced large depolarizations in both rat and guinea-pig ganglia.

Concentration-effect curves were fitted by least-squares analysis of variance to the equation $Y = Ymax/1( + (EC_{50}/\text{agonist concentration})^{nH})$, where $EC_{50}$ is the half maximally effective concentration and nH is the Hill coefficient, using an iterative procedure on a VAX computer with Research System 1 software (BBN Software Products Corporation). Data are expressed as the mean ± standard error.

Drugs were obtained from the following companies: neurokinin A (NKA), neurokinin B (NKB), senktide (SE), and Sar-Met-substance P (Sar-Met-SP) from Cambridge Research Biochemicals; eledoisin (E), substance P (SP), substance P-methyl ester (SPOMe), tetrodotoxin (TTX), atropine sulphate, and bacitracin from Sigma; chymostatin and leupeptin from Bachem. Drugs were dissolved in distilled water, except for senktide which was dissolved in dimethylsulphoxide. Peptide stock solutions were stored in aliquots at −70°C.

## Results

Both rat and guinea-pig superior cervical ganglia were depolarized by the application of a range of neurokinin agonists. However, the relative potency and maximal ability of these ligands to depolarize ganglia was observed to vary between these two species.

### *Agonist pharmacology of rat ganglia*

In rat superior cervical ganglia, eledoisin was observed to be a highly efficacious neurokinin agonist (Figure 1a), with an $EC_{50}$ of 37 nM. The $NK_3$-selective ligand, senktide (Wormser *et al.*, 1986; Guard *et al.*, 1990) proved to be the most potent ligand examined with an $EC_{50}$ of 4 nM, although the maximum depolarization produced in rat ganglia was only 27% that of eledoisin and smaller than that of alternative agonists (Figure 1a and b). Similarly the $NK_1$-selective ligands, SP, Sar-Met-SP and SPOMe, all exhibited maximal depolarizations of only 72–80% that of eledoisin (Table 1). The potency of substance P was unaffected during continuous perfusion of senktide (1 μM; $n = 4$) which desensitized $NK_3$ receptors. Pretreatment of rat ganglia with the peptidase inhibitors bacitracin (40 μg $ml^{-1}$), chymostatin (2 μg $ml^{-1}$) and leupeptin (4 μg $ml^{-1}$) had no effect upon the potency or efficacy of SPOMe (36 ± 13 nM; 117 ± 7%, $n = 7$) or neurokinin A (288 ± 95 nM, 119 ± 7%, $n = 6$). Both neurokinin A and neurokinin B, peptides which are preferentially, although not exclusively, selective for $NK_2$ and $NK_3$ receptors respectively, both exhibited weak potencies and had Hill slopes significantly less than one. The maximal responses obtained with NKA and NKB at high concentrations were greater than those obtained with eledoisin (Table 1).

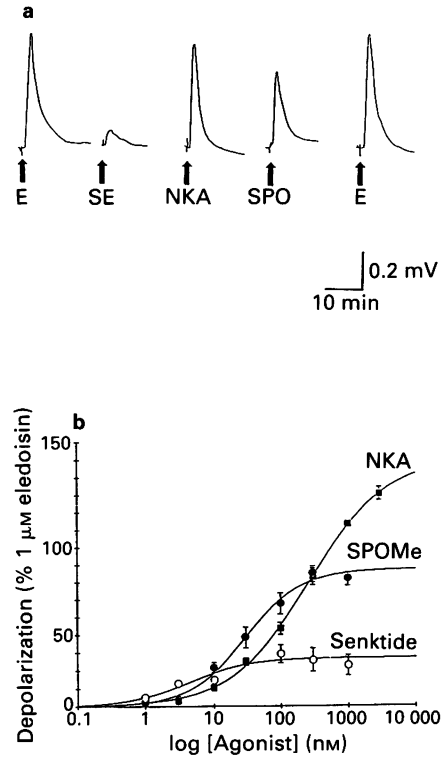


**Figure 1** The depolarization of rat superior cervical ganglia by subtype selective neurokinin agonists. (a) At the time points indicated by the arrows the following neurokinin agonists were applied (3 μM) for a 1 min period in the presence of atropine (1 μM) and tetrodotoxin (0.1 μM): eledoisin (E), senktide (SE), neurokinin A (NKA), and substance P methyl ester (SPO). (b) The resultant depolarizations in the same ganglia were normalized to the control response with eledoisin. These normalized depolarizations, from a number of ganglia, were used to calculate concentration-effect curves for each agonist (see Table 1). Eledoisin proved to be the most efficacious compound at a concentration of 1 μM, with SPOMe and senktide producing maximal depolarizations of 72% and 27% respectively.

### *Agonist pharmacology of guinea-pig ganglia*

In guinea-pig superior cervical ganglia, in contrast to the rat, senktide proved to be the most active compound in terms of both efficacy and potency (Figure 2a and b). The maximal depolarization achieved by the $NK_1$ selective ligand SPOMe was only 23% that of senktide, and 64% that of 1 μM eledoisin (Table 2). With NKA and SP, the concentration-effect curves were clearly biphasic, suggestive of an interaction at more than one receptor type. However, the lack of a distinct maximal depolarization with these weaker agonists prevented a reasonable fit to either a single- or two-site logistic model of ligand-receptor interaction. The depolariza-

**Table 1** Neurokinin receptor agonist potency and ability to depolarize rat superior cervical ganglia

| Ligand | $EC_{50}$ (nM) | Max (% 1 μM eledoisin) | Hill slope | n |
|---|---|---|---|---|
| Senktide | 4 ± 2 | 27 ± 3 | 0.99 ± 0.45 | 7 |
| Substance P | 12 ± 3 | 72 ± 4 | 0.94 ± 0.16 | 8 |
| SPOMe | 30 ± 6 | 77 ± 5 | 1.04 ± 0.17 | 8 |
| Eledoisin | 33 ± 9 | 103 ± 8 | 1.06 ± 0.21 | 9 |
| Sar-Met-SP | 39 ± 4 | 80 ± 3 | 1.52 ± 0.22 | 7 |
| NKB | 117 ± 65 | 132 ± 18 | 0.67 ± 0.15 | 7 |
| NKA | 268 ± 35 | 140 ± 5 | 0.76 ± 0.04 | 7 |

SPOMe = substance P methyl ester; NKA, NKB = neurokinins A and B; Sar-Met-SP = Sar-Met-subtance P.

**Table 2** Neurokinin receptor agonist potency and ability to depolarize guinea-pig superior cervical ganglia

| Ligand | $EC_{50}$ (nM) | Max (% 1 μM eledoisin) | Hill slope | n |
|---|---|---|---|---|
| Senktide | 37 ± 14 | 274 ± 24 | 0.74 ± 0.12 | 8 |
| Sar-Met-SP | 90 ± 26 | 102 ± 8 | 0.89 ± 0.23 | 4 |
| NKB | 1473 ± 545 | 259 ± 20 | 0.53 ± 0.06 | 6 |
| Eledoisin | 1918 ± 97 | 273 ± 5 | 0.85 ± 0.02 | 9 |
| SPOMe | 2052 ± 4552 | 64 ± 41 | 0.67 ± 0.47 | 9 |
| NKA | Indeterminate | 26% at 1 μM | Biphasic | 8 |
| Substance P | Indeterminate | 25% at 1 μM | Biphasic | 3 |

Sar-Met-SP = Sar-Met-substance P; SPOMe = substance P methyl ester; NKA = neurokinin A.

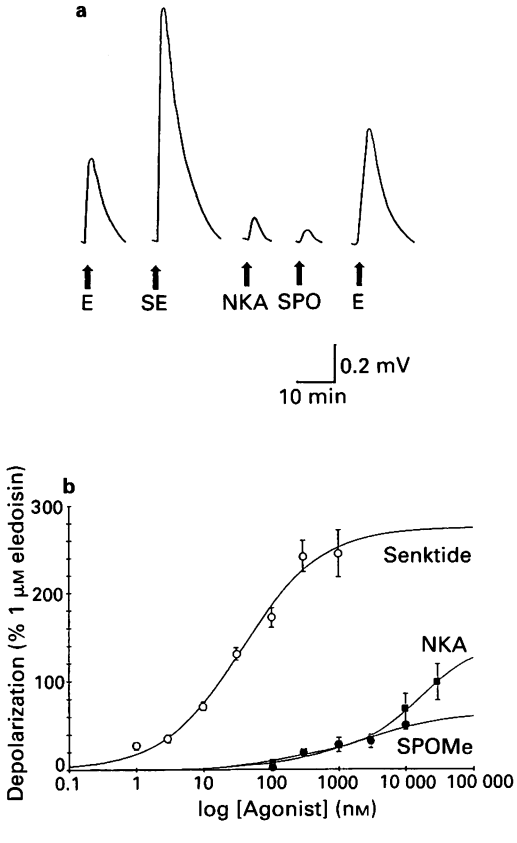


**Figure 2** The depolarization of guinea-pig superior cervical ganglia by subtype selective neurokinin agonists. (a) At the time points indicated by the arrows the following neurokinin agonists were applied (3 μM) for a 1 min period in the presence of atropine (1 μM) and tetrodotoxin (0.1 μM): eledoisin (E), senktide (SE), neurokinin A (NKA), and substance P methyl ester (SPO). The slower rate of decay of these traces relative to Figure 1a reflect differences in flow rate of the drug solutions through the bath. (b) The normalized depolarizations in a number of ganglia were used to calculate concentration-effect curves for each agonist (see Table 2). Senktide proved to be the most efficacious agonist, the maximal depolarization achieved by SPOMe was 23% that of senktide. The concentration-effect relationship with neurokinin A was biphasic, and the depolarization was not maximal at 30 μM.

tions elicited by senktide or Sar-Met-SP were not blocked by application of atropine (Figure 2a), and similarly the potency of senktide was unaffected by continuous perfusion of substance P (1 μM; $n = 4$) which desensitizes $NK_1$ receptors. As was seen in rat tissue, pretreatment of guinea-pig ganglia with the peptidase inhibitors bacitracin (40 μg ml$^{-1}$), chymostatin (2 μg ml$^{-1}$) and leupeptin (4 μg ml$^{-1}$) had no significant effect upon the potency or efficacy of SPOMe (2.9 ± 2.1 μM, 44 ± 18%, $n = 4$), senktide (23 ± 4 nM, 200 ± 9%, $n = 4$), or neurokinin A (biphasic, 15% at 1 μM).

## Discussion

This study demonstrates that neurokinin receptor pharmacology differs in rat and guinea-pig superior cervical ganglia. These differences exist not only in terms of agonist potency, but also in terms of relative agonist efficacy.

It has previously been reported that substance P depolarizes rat superior cervical ganglia (Hawcock *et al.*, 1982), but that it is a relatively weak agonist with an $EC_{50}$ of 297 nM (Brown *et al.*, 1983). In the present study, substance P was found to be 20 times more potent. The rank order of agonist potency in this tissue is therefore consistent with an $NK_1$ receptor as the predominant receptor population. The lack of involvement of $NK_2$ receptors was indicated by the absence of a response to low concentrations of NKA, and the insensitivity of the SPOMe-mediated depolarizations to the $NK_2$ selective antagonist L-659,877 (data not shown).

Senktide binds selectively to both central and peripheral $NK_3$ receptors with high affinity (Guard *et al.*, 1990). The high potency and relatively weak efficacy of senktide in rat ganglia thus provides evidence for the presence of an $NK_3$ receptor subtype. Similarly the greater efficacy of eledoisin relative to $NK_1$ selective ligands suggests that the response to this ligand is a consequence of activation of both $NK_1$ and $NK_3$ receptors. The depolarization caused by the $NK_1$ receptor agonist SPOMe (72%) and the $NK_3$ receptor agonist senktide (27%) were equieffective to the maximal depolarization achieveable with the non-selective agonist, eledoisin (see Figures 1b, 2b). In guinea-pig ileum, both $NK_1$ and $NK_3$ receptors contribute to the contraction of smooth muscle (Lee *et al.*, 1982). These $NK_3$ receptors are located on the myenteric plexus and mediate their effects by the release of acetylcholine. The $NK_3$-mediated contraction can be blocked by incubation of the tissue in atropine to block the postjunctional muscarinic receptors. However, unlike guinea-pig ileum, application of atropine did not antagonize the neurokinin-mediated depolarizations in rat ganglia (Figure 1a), which suggests the absence of presynaptic neurokinin receptors in this response.

The variation in efficacy of neurokinin agonists in this tissue can be explained by receptor heterogeneity, and/or by partial agonism. The possibility of heterogeneity of receptor subtypes is difficult to test due to the lack of a selective $NK_3$ receptor antagonist. However, as previously discussed there is some evidence for such heterogeneity in rat ganglia based upon the relative potency of senktide. Also consistent with this interpretation is the observation that desensitization of presumptive $NK_3$ receptors with prolonged application of senktide did not reduce the maximal depolarization obtain-

able with substance P. During this experiment the potency of substance P remained unaffected, therefore it is unlikely that senktide acted as partial agonist at an identical receptor population. It is not clear as to whether both $NK_1$ and $NK_3$ receptors are located on common neuronal cell bodies, or whether their distribution is more discrete, in that the receptor distribution reflects separate neurokinin pathways through the ganglia.

It is interesting to note that the pharmacology of superior cervical ganglia may be different between species for 5-hydroxytryptamine (Newberry *et al.*, 1991). Indeed in the present study the response to neurokinin receptor agonists was also found to differ between species. In guinea-pig superior cervical ganglia, in contrast to the rat, the majority of ligands tested were only weak agonists with $EC_{50}$s typically greater than 1 μM (Table 2). The most potent ligand senktide, 37 nM, also exhibited the most efficacious response, whereas substance P was only weakly active in this species. It is unlikely that $NK_3$ receptors are the exclusive neurokinin receptor in guinea-pig superior cervical ganglia as biphasic concentration-effect curves were observed with NKA and substance P. The binding of senktide to neurokinin receptors in cerebral cortex is similar for both rats and guinea-pigs (Renzetti *et al.*, 1991), whereas NKB binding was shown to be 27 fold weaker in guinea-pig tissue; this may indicate subtle differences in the agonist recognition site between the two species.

In conclusion we have shown that neurokinin receptor agonists depolarize both rat and guinea-pig superior cervical ganglia, but that the potency and efficacy of these ligands differ between these two species. Rat ganglia appear to express multiple neurokinin receptor subtypes, the predominant receptor population being of an $NK_1$ subtype, whereas in guinea-pig ganglia $NK_3$ receptors appear to be the principal subtype involved. The development of a selective $NK_3$ receptor antagonist will help classify the receptor subtypes present in this tissue.

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# Phosphoramidon potentiates the contractile response to endothelin-3, but not endothelin-1 in isolated airway tissue

[1]K.O. McKay, J.L. Black & *C.L. Armour

Departments of Pharmacology and *Pharmacy, The University of Sydney, Australia

**1.** Phosphoramidon (10 μM) markedly increased the contractile response to endothelin-3 in human and rabbit bronchus *in vitro*. In human tissue the contractile response to 0.3 μM endothelin-3 was significantly increased from 54 ± 12% to 137 ± 34% (of the response to 1 mM acetylcholine) in the presence of phosphoramidon. Similarly, in rabbit isolated bronchus, the endothelin-3-induced response was increased from 34 ± 5% to 61 ± 7%.

**2.** In addition, the potency (as measured by $EC_{30}$ values) of this peptide in human and rabbit airways was significantly augmented in the presence of the enzyme inhibitor. The geometric mean $EC_{30}$ value was decreased from 53 nM (95% CI:15, 190) to 8 nM (95% CI:3, 23) in human bronchus and from 150 nM (95% CI:89, 250) to 23 nM (95% CI:11, 50) in rabbit tissue.

**3** Neither the potency nor the response (at 0.3 μM) to endothelin-3 in canine bronchial rings was altered after incubation of the tissue in phosphoramidon.

**4** A previous study carried out in human airways has implied that the difference in potency between endothelin-1 and endothelin-3 may be attributed to a heterogeneous endothelin receptor population. The results of our study, while also demonstrating this difference in potency, have shown that this marked difference, as well as that obvious in rabbit airway tissue can be abolished in the presence of phosphoramidon.

**5** Phosphoramidon produced no change in the cumulative concentration-response curve for endothelin-1 in airway tissue from the three species studied.

**6** These results suggest that a phosphoramidon-sensitive enzyme (probably neutral endopeptidase) found in lung, may be responsible for local degradation of endothelin-3, but not endothelin-1 in human and rabbit isolated bronchus.

**Keywords:** Endothelin-1; endothelin-3; human bronchus; rabbit bronchus; canine bronchus; phosphoramidon; neutral endopeptidase

## Introduction

The endothelins are a group of 21 amino acid peptides. Currently, three isoforms of endothelin have been isolated and characterized. Endothelin-1 induces potent and prolonged contraction of airways both *in vivo* and *in vitro*. Removal of epithelium and inhibition of neutral endopeptidase increases the contractile effects of endothelin in guinea-pig isolated airways (Maggi *et al.*, 1989; Hay, 1989). In addition, pretreatment of guinea-pigs with phosphoramidon significantly augments the bronchopulmonary response to aerosolized endothelin-1 (Boichot *et al.*, 1991). Phosphoramidon increases the contractile response to various neuropeptides including neurokinin A in human and rabbit isolated airways (Black *et al.*, 1988; 1990). Neutral endopeptidase (EC 3.4.24.11) is a phosphoramidon-sensitive enzyme located in the pulmonary epithelium (Johnson *et al.*, 1985; Sekizawa *et al.*, 1987). It is believed that inhibition of this enzyme by phosphoramidon is responsible for the potentiation we and others have previously observed in airways from a variety of species including man. In this study we investigated the possibility that a phosphoramidon-sensitive enzyme may be responsible for the local metabolism of endothelin in human, rabbit and canine airway tissue. Various isoforms of endothelin have significantly different potencies in human bronchus (Advenier *et al.*, 1990; McKay *et al.*, 1991a) and there appear to be marked species differences in the action of endothelin in airway tissue (McKay *et al.*, 1991b). We have therefore examined the effect of phosphoramidon on the cumulative concentration-response curves to both endothelin-1 and endothelin-3 in human, rabbit and canine bronchus.

[1]Author for correspondence at Department of Pharmacology, Bosch Building [D05], The University of Sydney, N.S.W. 2006, Australia.

## Methods

Samples of human lung were obtained from tissue surgically removed from 14 patients with pulmonary carcinoma. Lungs were also removed from New Zealand white rabbits and adult mongrel dogs immediately after they had been killed (cervical disslocation or pentobarbitone sodium overdosage) by methods approved by the Animal Ethical Review Committee of The University of Sydney. The tissue was placed in oxygenated ice-cold Krebs-Henseleit solution (composition in mM: NaCl 118.4, KCl 4.7, $CaCl_2.2H_2O$ 2.5, $MgSO_4.7H_2O$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25.0 and D-glucose 11.1). Bronchi were dissected free from surrounding parenchyma and cut into rings measuring 3–6 mm in internal diameter and 4–5 mm in length. Paired rings of bronchus were mounted and equilibrated against a 1–2 g load in 5 ml water jacketed organ baths containing Krebs-Henseleit solution maintained at 37°C and aerated with 5% $CO_2$ in $O_2$ as previously described (Black *et al.*, 1990). When possible the tissue pairs were studied in duplicate. When stable tone had been established, a supramaximal dose of acetylcholine (1 mM) was added to the baths and the contractile response (reference response) allowed to plateau (< 5 min) before the acetylcholine was removed from the baths by repeated washing of the tissues. When washout was complete and stable resting tone regained, one ring from each pair of bronchi was incubated in 10 μM phosphoramidon for 30 min. Cumulative concentration-response curves (CCRCs) for endothelin-1 or endothelin-3 over the range 1 pM–0.3 μM were then elicited in

each tissue. In a separate series of experiments, rings of canine bronchus from 3 dogs were studied in the presence and absence of 10 μM phosphoramidon, and increasing doses of substance P or neurokinin A over the concentration range 0.1 pM–3 μM were added to the baths.

Changes in the force of contraction were measured isometrically using Grass FT03 transducers and recorded on Grass polygraphs. The response at each concentration of endothelin was expressed as a percentage of the reference response to 1 mM acetylcholine obtained as described above. A graph was then constructed relating cumulative concentration of endothelin to response. From this an $EC_{30}$ value (the concentration of endothelin at which a contractile response equal to 30% of the response to 1 mM acetylcholine was attained) was derived. When duplicate preparations were studied, a mean response curve was constructed for each experiment and a geometric mean $EC_{30}$ value calculated. An overall mean curve was then constructed for each series of experiments for tissues in the presence and absence of phosphoramidon and a geometric mean $EC_{30}$ value with 95% confidence limits obtained. Two-tailed unpaired Student's $t$ tests were used to compare the mean results for treated and control tissues and differences were considered as having statistical significance if $P \leqslant 0.05$. Unpaired two-tailed $t$ tests were also used to compare differences in the $EC_{30}$ values and the responses to 0.3 μM to endothelin-1 and endothelin-3 between the three species, again differences were reported as being significant if $P \leqslant 0.05$.

Substance P, neurokinin A and both forms of endothelin were obtained from Auspep (Australia) and were dissolved in 0.1 M acetic acid to form stock solutions. Phosphoramidon (N-(a-rhamnopyranosyloxyphosphinyl)-L-leucyl-L-tryptophan) and acetylcholine were purchased from Sigma (U.S.A.) and dissolved in distilled water. Aliquots of substance P, neurokinin A and endothelin were stored at −70°C until required and serial dilutions made with Krebs-Henseleit solution on the day of experimentation.

## Results

Both endothelin-1 and endothelin-3 produced sustained contractions of human, rabbit and canine isolated bronchus. Due to limited availability of the peptides, the maximal bath concentration achieved in all experiments was 0.3 μM and at this concentration responses did not appear to be maximal. The mean values for the tension (expressed as a percentage of the reference contraction to acetylcholine) induced by endothelin-1 and endothelin-3 at a concentration of 0.3 μM in rabbit and canine airways were not significantly different from one another (Figure 1, Tables 1 and 2). However, the mean tension generated by 0.3 μM endothelin-1 was significantly greater than that by 0.3 μM endothelin-3 in human isolated bronchus ($P = 0.05$, see Tables 1 and 2 for values). When unpaired Student's $t$ tests were used to analyse differences in potency between endothelin-1 and endothelin-3, the geometric mean $EC_{30}$ value for endothelin-1 was found to be significantly lower than the value for endothelin-3 in tissue from all species studied.

Phosphoramidon significantly increased the response to 0.03, 0.1 and 0.3 μM endothelin-3 in the human and rabbit preparations, but had no effect upon the response elicited by this peptide in canine airways (Table 2, Figure 2). The potency of endothelin-3 in human and rabbit bronchial rings, but not in canine bronchus, was increased by phosphoramidon, in that the geometric mean $EC_{30}$ value in human bronchus was decreased from 53 nM to 8 nM ($n = 7$, $P = 0.04$) and in rabbit airways from 150 nM to 23 nM ($n = 7$, $P = 0.0004$) (Table 2). In contrast, neither the potency nor the magnitude of the response to any concentration of endothelin-1 (Table 1) in airway tissue from the three species was affected by phosphoramidon.

In view of the lack of effect of phosphoramidon on the

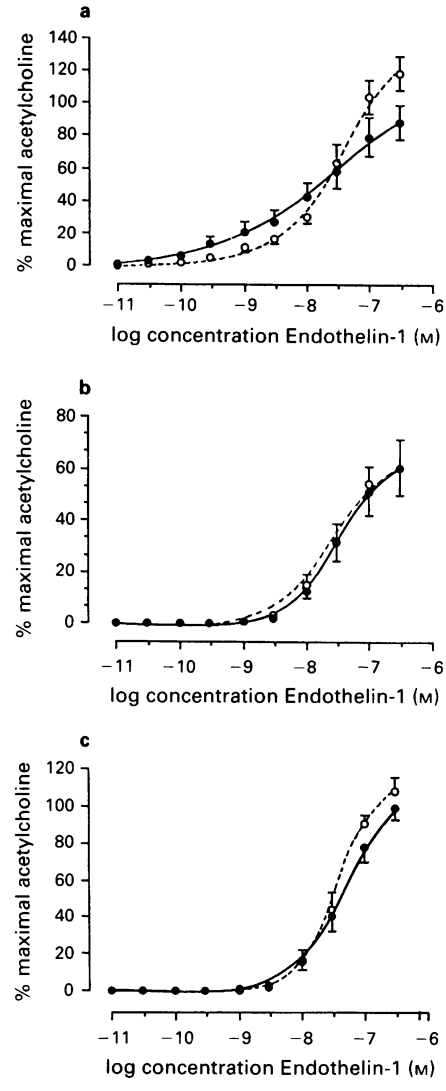


**Figure 1** Mean cumulative concentration-response curves for endothelin-1 in the presence (○---○) and absence (●——●) of phosphoramidon in human (a), rabbit (b) and dog (c) bronchus. Mean responses for 7 experiments are expressed as a percentage of the reference response obtained to 1 mM acetylcholine; s.e. values are shown as vertical bars; $n = 7$ in (a), (b) and (c).

**Table 1** The effect of phosphoramidon on the magnitude of the contractile response* and potency ($EC_{30}$)† of endothelin-1 in human, rabbit and canine bronchus

| | *Contractile response* (s.e.mean) | | *$EC_{30}$* (95% CI) | |
|---|---|---|---|---|
| | *Control* | *Phosph* | *Control* | *Phosph* |
| Human | 89.2 | 118.8 | 4.5 nM | 9.0 nM |
| ($n = 7$) | (10.5) | (10.5) | (0.6, 35.1) | (4.9, 17.0) |
| Rabbit | 60.7 | 60.6 | 42.2 nM | 32.7 nM |
| ($n = 7$) | (10.9) | (5.5) | (20.6, 86.3) | (17.0, 63.2) |
| Dog | 98.5 | 107.9 | 24.3 nM | 22.2 nM |
| ($n = 7$) | (6.1) | (7.5) | (11.8, 50.0) | (13.2, 37.5) |

Mean values are shown together with s.e. mean or 95% confidence intervals (CI).
*Response to 0.3 μM endothelin-1 (the highest bath concentration achieved) expressed as a percentage of the reference contraction to acetylcholine (see Methods).
†The concentration of endothelin-1 at which a contractile response equal to 30% of that induced by 1 mM acetylcholine is attained (see Methods).

**Table 2** The effect of phosphoramidon on the magnitude of the contractile response† and potency ($EC_{30}$)‡ of endothelin-3 in human, rabbit and canine bronchus

| | *Contractile response* (s.e.mean) | | *$EC_{30}$* (95% CI) | |
|---|---|---|---|---|
| | *Control* | *Phosph* | *Control* | *Phosph* |
| Human | 54.2 | 136.5* | 53.0 nM | 8.0 nM* |
| ($n = 7$) | (12.0) | (33.7) | (15,1, 185.8) | (2.8, 22.6) |
| Rabbit | 33.8 | 61.4** | 150.3 nM | 23.3 nM*** |
| ($n = 7$) | (5.0) | (7.0) | (89.1, 253.5) | (10.9, 50.1) |
| Dog | 79.9 | 96.5 | 87.7 nM | 49.6 nM |
| ($n = 7$) | (11.6) | (8.9) | (47.8, 161.1) | (28.7, 85.3) |

Mean values are shown together with s.e.mean or 95% confidence intervals (CI).
* Indicates significantly different from control value ($P \leq 0.05$).
**Indicates significantly different from control value ($P \leq 0.01$).
*** Indicates significantly different from control value ($P \leq 0.001$).
†Response to 0.3μM endothelin-3 (the highest bath concentration achieved) expressed as a percentage of the reference contraction to acetylcholine (see Methods).
‡The concentration of endothelin-3 at which a contractile response equal to 30% of that induced by 1 mM acetylcholine is attained (see Methods).

responses to endothelin in canine airways we decided to assess the effect of this enzyme inhibitor on the contractions to the neuropeptides substance P and neurokinin A. There was however, no contractile response to either neurokinin A or substance P in the presence or the absence of phosphoramidon ($n = 3$ dogs) although the mean tension generated in response to 1 mM acetylcholine was 5.8 ± 0.7 g.

## Discussion

This study has shown that the presence of phosphoramidon at a concentration of 10 μM greatly enhanced the contractile activity of endothelin-3 but not endothelin-1 in human and rabbit bronchus. Moreover, phosphoramidon abolished the differences in potency between endothelin-1 and endothelin-3 in these preparations. There was however, no change in the potency nor the magnitude of the contractile response to any concentration (up to 0.3 μM) of either isoform of endothelin in canine airways when the tissue had been incubated in phosphoramidon.

Previous studies have also shown increased effects of endothelin in the presence of an inhibitor of neutral endopeptidase. Maggi *et al.* (1989) reported that, in guinea-pig airways, thiorphan, another inhibitor of neutral endopeptidase, caused a greater enhancement of the action of endothelin-3 than endothelin-1 although the responses to both forms of endothelin were augmented. In another study also carried out in guinea-pig airways, Hay (1989) demonstrated that removal of the epithelium and incubation in phosphoramidon resulted in increased responsiveness to endothelin. We however, found no significant potentiation of the action of endothelin-1 by phosphoramidon. This discrepancy in the effect on endothelin-1 between our findings and those previously published could be a reflection of the species difference or variation in experimental protocol. Marked differences between species in the mechanism of action of endothelin have been reported. For example, cyclo-oxygenase product generation in response to endothelin, while involved in the contractile response in guinea-pig airways (Sarria *et al.*, 1990), is not responsible for the endothelin-induced contraction of human bronchus (Advenier *et al.*, 1990; McKay *et al.*, 1991a). Maggi *et al.* (1989) used captopril, bestatin and indomethacin simultaneously with thiorphan in the study in guinea-pig airways and found significant potentiation but it is possible that one of these other enzyme inhibitors produced the enhancement to endothelin-1.

A previous study carried out in human airways has implied that the difference in potency between endothelin-1 and endothelin-3 may be attributed to a heterogeneous endothelin receptor population in this tissue (Advenier *et al.*, 1990). The results of our study, while also demonstrating this difference in potency, have shown that this marked difference, as well as that obvious in rabbit airway tissue, can be abolished in the presence of phosphoramidon. It is possible that neutral endopeptidase has differential affinity for isoforms of the endothelin family and is responsible for the local degradation of endothelin-3 but not endothelin-1. Recently, all three isoforms of endothelin have been shown to be substrates for neutral endopeptidase (Vijayaraghavan *et al.*, 1990). Hydrolysis of the endothelins differs according to the amino acid sequence and conformation of the peptide. As one of the major sites for cleavage of endothelin was shown to be at position 6 of the peptide, and as endothelin-1 and endothelin-3 differ in the amino acid at this position, it is possible

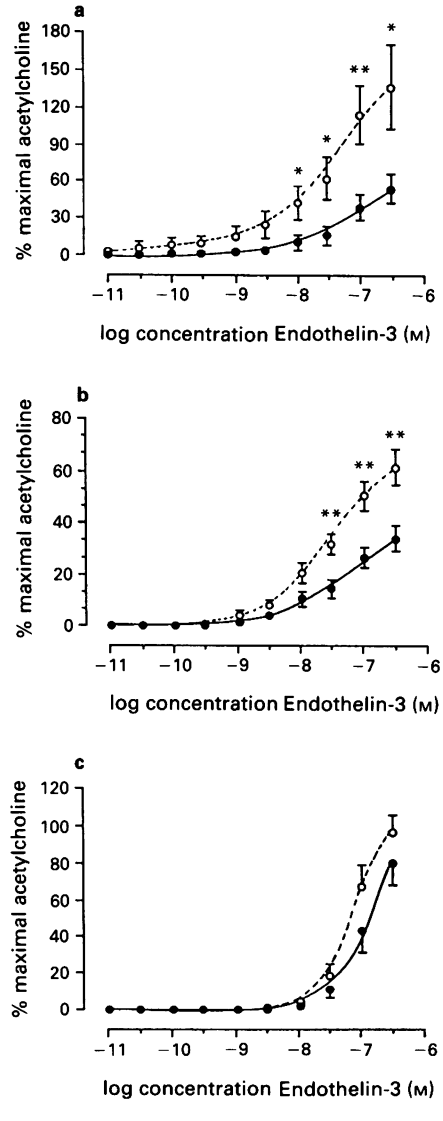


**Figure 2** Mean cumulative concentration-response curves for endothelin-3 in the presence (○---○) and absence (●——●) of phosphoramidon in human (a), rabbit (b) and dog (c) bronchus. Mean responses for 7 experiments are expressed as a percentage of the reference response obtained to 1 mM acetylcholine; s.e. values are shown as vertical bars; $n = 7$ in (a), (b) and (c). Significant differences between control and treated tissues are indicated (*$P \leq 0.05$; **$P \leq 0.01$ paired two-tailed Student's $t$ test).

that neutral endopeptidase located within the airways may differentially hydrolyse the isoforms of endothelin. Thus, endothelin-1 and endothelin-3 may act at the same receptor, the difference in potency perhaps being due to the available local concentration of endothelin-3.

Unlike human and rabbit bronchus, neither the potency nor the magnitude of the contractile response to endothelin-3 in canine bronchial rings was altered by prior incubation of the tissue in phosphoramidon. It is therefore possible that endothelin-3 is not a substrate for canine airway neutral endopeptidase, and hence this highlights an additional species difference in the mechanism of action of endothelin in airway tissue. It is also possible that canine airways have little or no neutral endopeptidase, or that this enzyme is not inhibited by phosphoramidon in this species. As we are unaware of any previous reports of *in vitro* studies with canine airways and phosphoramidon, we attempted to clarify which of the above possibilities may explain the lack of effect of phosphoramidon on the cumulative concentration-response curve for endothelin-3. We investigated the effect of phosphoramidon on the contractile effects of substance P and neurokinin A in canine bronchus, but in tissue taken from three dogs there was no response to either peptide at a concentration of 3 $\mu$M in the presence or absence of phosphoramidon. It thus appears that dog airways lack appreciable numbers of tachykinin $NK_1$ and $NK_2$ receptors mediating contraction and perhaps therefore neutral endopeptidase as well. Hence, we are unable to elucidate the reasons for the lack of effect of phosphoramidon on the response to endothelin-3 in canine bronchus.

We have therefore demonstrated that the contractile activity of endothelin-3 but not endothelin-1 is markedly potentiated in the presence of phosphoramidon in human and rabbit bronchus *in vitro*. In contrast to this finding, phosphoramidon was without effect on the response to either endothelin-1 or endothelin-3 in canine bronchus. As neutral endopeptidase is a phosphoramidon-sensitive enzyme found in lung, it is possible that endothelin-3 is a substrate for this enzyme in human and rabbit tissue. The source of this phosphoramidon-sensitive enzyme and the involvement of other peptidases in the metabolism of endothelin warrant further investigation.

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# Effects of BRL 38227 on neurally-mediated responses in the guinea-pig isolated bronchus

[1]D.M. Good, J.C. Clapham & T.C. Hamilton

SmithKline Beecham Pharmaceuticals, Coldharbour Road, The Pinnacles, Harlow, Essex CM19 5AD

**1** In guinea-pig isolated bronchus treated with indomethacin (2.8 μM), electrical field stimulation (EFS; 10 Hz, 0.5 ms, 60–70 V, for 10 s) evoked a tetrodotoxin (3 μM)-sensitive, biphasic contraction comprising a rapid, atropine (1 μM)-sensitive cholinergic response succeeded by a slowly developing, capsaicin (10 μM)-sensitive, non-adrenergic, non-cholinergic excitatory (NANCe) response.

**2** BRL 38227 (0.3–3 μM), salmeterol (0.003–3 μM) and ketotifen (1.0–300 μM) each produced concentration-dependent inhibition of both NANCe and cholinergic responses to EFS in guinea-pig isolated bronchus.

**3** Substance P (SP; 1 μM) and neurokinin A (NKA; 0.07 μM) produced contractions equivalent in magnitude to the NANCe response to EFS, which were inhibited by salmeterol (1 μM), but not by BRL 38227 (3 μM) or ketotifen (100 μM).

**4** Acetylcholine (ACh; 6 μM) was equi-effective with the electrical activation of cholinergic neurones. BRL 38227 (3 μM) slightly inhibited responses to ACh (6 μM). Salmeterol (1 μM) and ketotifen (100 μM) markedly inhibited responses to ACh (6 μM).

**5** In bronchial rings pre-contracted with ACh (100 μM), BRL 38227 (0.1– 30 μM), salmeterol (0.001–3 μM) and ketotifen (0.1–100 μM) each produced concentration-dependent relaxation. Unlike ketotifen, BRL 38227 and salmeterol only partially (18.8 ± 2.1% and 51.8 ± 3.9% respectively) reversed the ACh-induced contraction.

**6** The (+)-analogue of BRL 38227, BRL 38226 (0.3–100 μM), was without effect on responses to EFS and had no effect on the inhibition caused by BRL 38227. The $K^+$-channel activators pinacidil (3.0–30 μM) and RP 52891 (3.0–30 μM) exerted similar inhibitory actions on responses to EFS as BRL 38227, but were less potent. Glibenclamide (0.1–1.0 μM) and phentolamine (3 μM) antagonized the inhibitory effects of BRL 38227 on responses to EFS.

**7** It is concluded that BRL 38227 and ketotifen can inhibit NANCe neuroeffector transmission at concentrations exerting little or no inhibitory effects on responses to exogenously applied tachykinins. By contrast, in addition to suppressing NANCe responses to EFS, salmeterol also markedly inhibits responses to SP and NKA. At concentrations markedly suppressing cholinergic neuroeffector transmission, BRL 38227 has only minor effects on responses to exogenously-applied ACh. Salmeterol and ketotifen both depress responses to ACh within the concentration-range over which they inhibit cholinergic responses to EFS. The inhibitory effects of BRL 38227 on responses to EFS exhibit stereo-specificity and may involve the opening of a neuronal $K^+$-channel. This $K^+$-channel is glibenclamide- and phentolamine-sensitive and appears similar to the smooth muscle $K^+$-channel which is modulated by BRL 38227.

**Keywords:** BRL 38227; guinea-pig bronchus; cholinergic nerves; NANCe nerves; glibenclamide; phentolamine

## Introduction

It is becoming increasingly recognised that, in addition to bronchospastic episodes, chronic inflammatory changes in the airways are likely to be of importance in the pathogenesis of asthma. It has been suggested that the activation of a subset of sensory nerve endings, via an axon reflex, may contribute to the inflammatory reaction in asthmatic airways. In particular, the release of tachykinins such as substance P (SP) and neurokinin A (NKA) from non-adrenergic, non-cholinergic excitatory (NANCe) nerves has been implicated (Barnes *et al.*, 1990). Agents interfering with NANCe nerve activity might, therefore, exert an anti-inflammatory effect in the airways.

The ability of $K^+$-channel activators such as cromakalim to relax airways smooth muscle contracted by a variety of mediators is well established (Allen *et al.*, 1986; Arch *et al.*, 1988). More recently, work in guinea-pig airways has suggested that cromakalim might also exert an inhibitory effect on the activity of cholinergic and NANCe nerves (Hall & Maclagan, 1988; McCaig & de Jonckheere, 1989; Ichinose & Barnes, 1990; Lewis & Raeburn, 1990; Burka *et al.*, 1991). In these studies, cromakalim was more effective in suppressing responses evoked by the electrical activation of neuronal elements, than contractions produced by exogenously applied acetylcholine (ACh) or SP. It was therefore suggested that cromakalim was acting at a prejunctional site, to inhibit neurotransmitter release.

The present study was undertaken to assess the effects of BRL 38227, the active (−)-enantiomer of cromakalim (Buckingham *et al.*, 1986), on NANCe and cholinergic nerve-mediated responses to electrical field stimulation (EFS) in the guinea-pig isolated bronchus. The anti-asthma drugs ketotifen and the long-acting $\beta_2$-adrenoceptor agonist salmeterol were also included in this study in view of their suggested anti-inflammatory effects in the airways (Kamikawa, 1989; Grant *et al.*, 1990; Twentyman *et al.*, 1990; Whelan & Johnson, 1990). We have sought to establish whether BRL 38227, salmeterol and ketotifen inhibit excitatory neuroeffector transmission in the guinea-pig bronchus, and if so, whether this is mediated at a pre- or post-junctional site. In addition, we have attempted to characterize the mechanism by which BRL 38227 is producing its effects. A preliminary account of

[1] Author for correspondence.

this work has previously been communicated to the British Pharmacological Society (Good & Hamilton, 1991).

## Methods

Male, Dunkin-Hartley guinea-pigs (300–500 g) were killed by stunning and exsanguination. Ring segments 2–3 mm in length were prepared from the main bronchi. Each segment was suspended for the recording of isometric tension changes, under a resting tension of 0.5–1.0 g. The Krebs solution bathing the tissues contained indomethacin (2.8 μM). The tissues were allowed a half hour equilibration period.

### *Field stimulation experiments*

Bronchial rings were mounted between pairs of platinum electrodes, 1 cm apart. NANCe and cholinergic nerve-mediated responses were evoked by the application of 10 s trains of 60–70 V, biphasic, 0.5 ms pulses at a frequency of 10 Hz. A minimum of 15 min was allowed between successive stimuli to allow baseline levels of tension to be regained. Before the start of an experiment, 2–3 responses to electrical field stimulation (EFS) were obtained to establish their reproducibility.

Cumulative concentration-effect curves for one of BRL 38227 (0.3–1.0 μM), BRL 38226 (0.3–100 μM), pinacidil (0.3–10 μM) RP 52891 (0.3–10 μM), ketotifen (1.0–300 μM), salmeterol (0.03–3.0 μM), or the appropriate vehicle were constructed. Each concentration of drug was allowed a 10 min equilibration period before the tissues were subjected to EFS. An exception was salmeterol, where a 20 min pre-incubation was used. Response were expressed as a % of the response to EFS immediately prior to drug exposure.

The ability of glibenclamide (0.1–1.0 μM), phentolamine (3.0–30 μM) or BRL 38226 (0.5, 1.5, 5.0 and 30 μM) to antagonize the actions of BRL 38227 was investigated. Tissues were pre-incubated with a single concentration of glibenclamide, phentolamine or BRL 38226, or the relevant vehicle, for 30 min. EFS was then applied. Cumulative concentration-effect curves for BRL 38227 were then constructed as described above. Responses were expressed as a % of the response to EFS obtained following exposure to antagonist, but before exposure to BRL 38227.

### *Exogenously-applied spasmogens*

The effects of pretreatment with BRL 38227 (3 μM), salmeterol (1 μM) or ketotifen (100 μM) on contractions evoked by ACh, SP and NKA were examined. Preliminary experiments established that ACh (6 μM) evoked contractions equivalent in magnitude to the cholinergic response following EFS (~30% histamine (1 mM) maximum). Similarly SP (1 μM) and NKA (0.07 μM) were equi-effective with the activation of NANCe neurones by EFS (~20% histamine (1 mM) maximum).

Bronchial rings were initially challenged with one of SP (1 μM), NKA (0.07 μM) or ACh (6 μM). Once the evoked spasm had developed, the tissues were washed at 15 min intervals until baseline levels of tension were regained. Following incubation with BRL 38227 (3 μM for 10 min), ketotifen (100 μM for 10 min) or salmeterol (1 μM for 20 min) or their relevant vehicles, the tissues were re-challenged with SP, NKA or ACh, as appropriate. Responses are expressed as a % of the response to spasmogen before exposure to BRL 38227, salbutamol or ketotifen.

In a further set of experiments, the spasmolytic properties of BRL 38227, salmeterol and ketotifen in the guinea-pig bronchus were examined. Tissues were precontracted with ACh (100 μM; $EC_{70}$). Cumulative concentration-effect curves for BRL 38227 (0.1–30 μM), ketotifen (0.1–100 μM) and salmeterol (0.001–3 μM) were then constructed. Tissue contact times were 10 min for BRL 38227 and ketotifen and 20 min for salmeterol. Time-matched control tissues received the appropriate concentration of vehicle. Since BRL 38227 and salmeterol failed to produce full reversal of the tension increment induced by ACh, responses were expressed as % relaxation of the initial spasmogen-induced tone.

### *Drugs and solutions*

The physiological salt solution used in the present experiments was a Krebs solution of the following composition (mM): $Na^+$ 143, $K^+$ 5.9, $Mg^{2+}$ 1.2, $Ca^{2+}$ 2.5, $Cl^-$ 123, $SO_4^{2-}$ 1.2, $H_2PO_2^{4-}$ 1.2, $HCO_3^-$ 25, and glucose 10.1. Drugs used, together with their sources are listed below. Acetylcholine chloride (Sigma), aminophylline (Sigma), atropine hydrobromide (Sigma), capsaicin (Sigma), glibenclamide (Roussel), indomethacin (Sigma), ketotifen (Sandoz), neurokinin A (Sigma), phentolamine mesylate (Ciba), RP 52891 (Rhône-Poulenc Rorer), substance P (Sigma), tetrodotoxin (Sigma), BRL 38227, BRL 38226, pinacidil and salmeterol were synthesized by the laboratories of SmithKline Beecham Pharmaceuticals.

Stock solutions of acetylcholine, indomethacin and capsaicin were prepared in absolute ethanol. Glibenclamide was dissolved in dimethylsulphoxide (DMSO). Stock solutions of salmeterol and pinacidil were prepared in N/10 HCl. BRL 38227, BRL 38226, RP 52891 and ketotifen were each dissolved in polyethylene glycol (PEG) and diluted as appropriate with twice distilled water. All other stock solutions were prepared in twice distilled water.

### *Analysis of results*

Data are presented as mean values ± s.e.mean. Potency estimates represent the concentration of drug required to produced a 50% (NANCe) or 30% (cholinergic) decrease in the response to EFS. These were calculated as geometric means ± s.e.mean of values derived from individual log concentration-effect curves. The significance of differences between mean values was assessed by a two-way, unpaired Student's $t$ test, Duncan's multiple range test, or Dunnett's test. Differences were considered significant when calculated probability ($P$) values were less than 0.05.

Time- and vehicle-matched control experiments suggested that there was a progressive reduction in the size of the response of bronchial rings to repeated field stimulation, or successive challenges with spasmogenic agents. In order to prevent these changes influencing the inhibitory effects of any drugs tested, all responses in these experiments were corrected for changes in their individual time- and vehicle-matched controls. This procedure involved calculating the fractional change in the response of the individual time- and vehicle-matched control tissues and using this value to adjust the corresponding response of the test tissues.

## Results

### *General features of the response to EFS*

Field stimulation of guinea-pig isolated bronchial rings treated with indomethacin (2.8 μM) evoked a biphasic, contractile response (Figure 1a,b). This was abolished by tetrodotoxin (3 μM), suggesting it was neurogenic in origin (data not shown). The initial (cholinergic) component developed and decayed rapidly and was abolished by atropine (1 μM; Figure 1a). This was succeeded by a secondary component which developed over a period of 1–2.5 min and thereafter declined to baseline levels within 10–15 min (Figure 1a(i) and b(i)). This latter response persisted in the presence of atropine (Figure 1a(ii)), but was abolished by prior exposure to capsaicin (10 μM; Figure 1b(ii)), suggesting the involvement of NANCe nerves (Andersson & Grundstrom, 1983; Lundberg et al., 1983). The magnitude of the cholinergic response was

reduced following exposure to capsaicin (10 μM; Figure 1b(ii)).

### *Effects of potassium channel activators on responses to EFS*

BRL 38227 (0.1–3.0 μM), pinacidil (3.0–30 μM) and RP 52891 (3.0–30 μM) each produced a concentration-dependent suppression of EFS-evoked responses in guinea-pig isolated bronchus treated with indomethacin (2.8 μM) (Figure 2a,c,d). The rank order of potency for the inhibition of both the NANCe and the cholinergic components of the response was BRL 38227 > pinacidil ≥ RP 52891 (Table 1). None of the $K^+$-channel activators tested completely suppressed contractions of the bronchial smooth muscle evoked by EFS (Figure 2a,c,d). However, each drug was more effective as an inhibitor of NANCe, than of cholinergic responses (Table 1, Figure 2a,c,d). Over the range 0.3–100 μM, BRL 38226, the (+)-isomer of BRL 38227, failed to produce significant suppression of either NANCe or cholinergic responses to EFS (Figure 2b).

### *Effects of salmeterol and ketotifen on responses to EFS*

Salmeterol (0.03–3 μM) produced a concentration-dependent suppression of responses to EFS (Figure 3a). It inhibited NANCe responses more potently than cholinergic responses (Figure 3a, Table 1). Salmeterol was the most potent inhibitor of electrically-evoked responses tested (Table 1) and, in a concentration of 1 μM, virtually abolished the response to EFS (Figure 3a).

Over the range 30–300 μM, ketotifen produced a concentration-dependent suppression of responses to EFS (Figure 3b), but was the least potent agent tested (Table 1). In 5 out of the 6 tissues, the highest concentration of ketotifen tested (300 μM), evoked a marked contraction of the bronchial smooth muscle. Like salmeterol, ketotifen, in adequate concentrations, abolished completely EFS-evoked contractions (Figure 3b). However, unlike salmeterol and the $K^+$-channel activating agents, it inhibited cholinergic responses more potently than NANCe responses (Table 1).

**Table 1** Effects of potassium channel activators, salmeterol and ketotifen on responses to electrical field stimulation (EFS) in guinea-pig isolated bronchus

| *Drug* | | *NANCe response* | *Cholinergic response* |
|---|---|---|---|
| BRL 38227 | [a]potency | 6.09 ± 0.09 | 6.12 ± 0.16 |
| ($n$ = 6) | [b]maximum | 80.9 ± 3.8% | 57.1 ± 6.0% |
| Pinacidil | [a]potency | 5.45 ± 0.15* | 5.50 ± 0.02* |
| ($n$ = 5) | [b]maximum | 85.6 ± 4.8% | 53.5 ± 5.9% |
| RP 52891 | [a]potency | 5.38 ± 0.11* | 5.39 ± 0.13* |
| ($n$ = 6) | [b]maximum | 73.4 ± 11.2% | 48.0 ± 6.9% |
| Salmeterol | [a]potency | 7.22 ± 0.22* | 6.82 ± 0.10* |
| ($n$ = 6) | [b]maximum | 97.5 ± 2.5%* | 81.2 ± 3.6%* |
| Ketotifen | [a]potency | 3.84 ± 0.16* | 4.62 ± 0.11* |
| ($n$ = 6) | [b]maximum | 98.1 ± 1.9%* | 98.6 ± 1.4%* |

Values are means ± s.e.mean. [a]Potency estimates represent -log $IC_{50}$ values for NANCe responses and -log $IC_{30}$ values for cholinergic responses. [b]% reduction of initial response to EFS.
Significant difference from the response to BRL 38227: *$P < 0.05$.

Over the range 30–300 μM, ketotifen produced a concentration-dependent suppression of responses to EFS (Figure 3b), but was the least potent agent tested (Table 1). In 5 out of the 6 tissues, the highest concentration of ketotifen tested (300 μM), evoked a marked contraction of the bronchial smooth muscle. Like salmeterol, ketotifen, in adequate concentrations, abolished completely EFS-evoked contractions (Figure 3b). However, unlike salmeterol and the $K^+$-channel activating agents, it inhibited cholinergic responses more potently than NANCe responses (Table 1).

### *Effects of BRL 38227, salmeterol and ketotifen on responses to exogenously-applied spasmogens*

BRL 38227 (3 μM), a concentration which was maximally effective in causing suppression of NANCe responses to EFS (Figure 2a), had no significant effect on responses to exogenously-applied SP (1 μM) or NKA (0.07 μM) (Table 2). BRL 38227 (3 μM) produced a small, but nevertheless significant inhibition of responses to ACh (6 μM). However, this was less than its inhibitory effect on cholinergic responses to EFS (Table 2).

Ketotifen (100 μM) suppressed NANCe responses to EFS, but had no significant effect on contractions evoked by SP (1 μM) or NKA (0.07 μM) (Table 2). By contrast, responses to exogenously-applied ACh (6 μM) were almost completely abolished by ketotifen (100 μM). Indeed, ketotifen (100 μM) inhibited responses to ACh (6 μM) to a greater extent than the cholinergic component of the response to EFS (Table 2). Salmeterol (1 μM) markedly reduced responses to each of SP (1 μM), NKA (0.07 μM) and ACh (6 μM) (Table 2).

### *Spasmolytic effects of BRL 38227, salmeterol and ketotifen in ACh-precontracted bronchial rings*

In bronchial rings precontracted with ACh (100 μM), BRL 38227, salmeterol and ketotifen each evoked concentration-dependent relaxation (Figure 4). However, there were marked differences in the maximal effects of these three agonists, with only ketotifen causing a full suppression of ACh-induced tension (Figure 4). Salmeterol exhibited a greater potency than BRL 38227 and ketotifen. The latter two agents produced relaxation over a simlar concentration range (Figure 4).

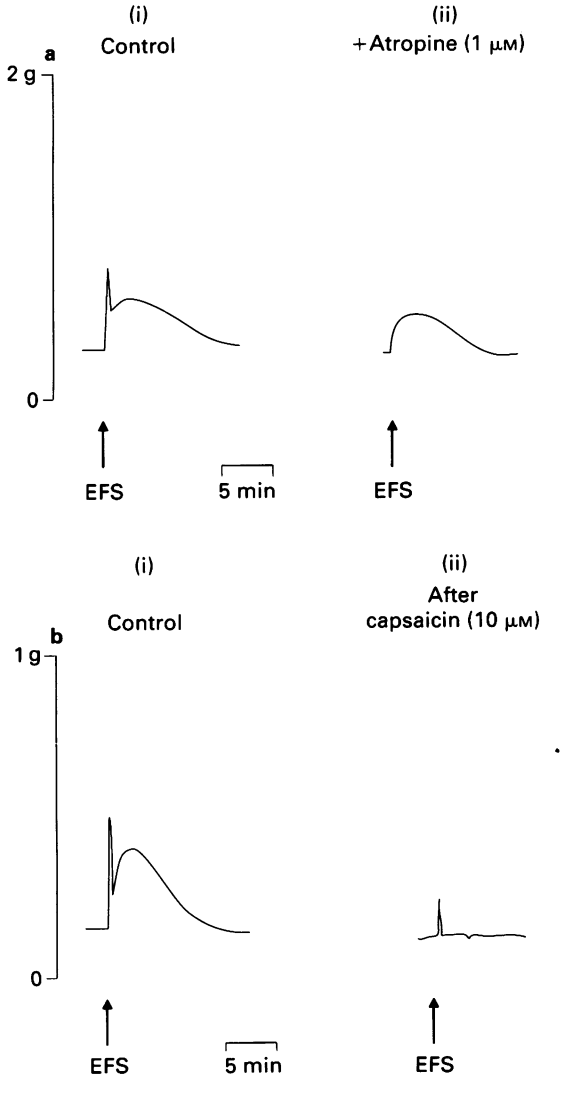


**Figure 1** Responses to electrical field stimulation (EFS) in guinea-pig isolated bronchus, before (i) and after (ii) exposure to (a) atropine (1 μM) or (b) capsaicin (10 μM). In both (a) and (b), panels (i) and (ii) show responses of the same bronchial ring.

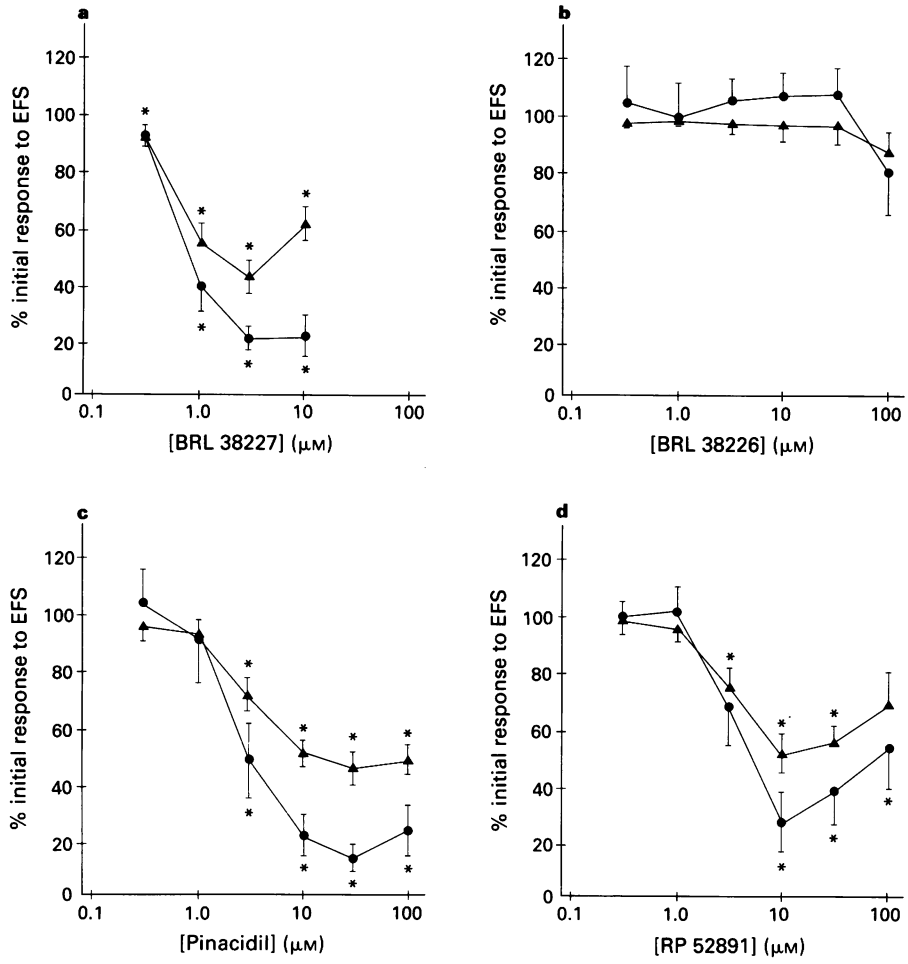


**Figure 2** Effects of (a) BRL 38227, (b) BRL 38226, (c) pinacidil and (d) RP 52891 on non-adrenergic, non-cholinergic excitatory (●) and cholinergic (▲) responses to electrical field stimulation (EFS) in guinea-pig isolated bronchus. Data are means of values from 6 tissues, s.e.means are shown by the vertical bars. *Significant suppression of the response to EFS ($P<0.05$).

### *Effects of BRL 38226, glibenclamide and phentolamine on BRL 38227-induced suppression of responses to EFS*

By itself, glibenclamide (0.1–1 μM) had no significant effect on responses to EFS (Table 3), but caused a concentration-dependent rightward shift of the log concentration-effect curve for BRL 38227 in field stimulated, guinea-pig isolated bronchus (Figure 5a,b). In that the shifts of the curves appeared parallel, and the maximum response to BRL 38227 was not significantly reduced, the antagonistic effect of glibenclamide appeared to be competitive.

Phentolamine (3 μM) had no effect on the response of guinea-pig isolated bronchus to EFS (Table 3), but reduced the suppressant effects of BRL 38227 (Figure 6a,b). Higher concentrations of phentolamine (10–30 μM) caused no further reduction in responses to BRL 38227, but there was a tendency for the log concentration-effect curve to become flattened and to shift back towards the control curve (Figure 6a,b). However, at concentrations greater than 3 μM, phentolamine itself caused a concentration-dependent suppression of responses to EFS (Table 3).

BRL 38226 (0.5, 1.5, 5 and 30 μM) itself had no effect on responses to EFS (Table 3) and, furthermore, did not modify the inhibition of responses to EFS produced by BRL 38227 (Figure 7a,b).

**Table 2** Comparative effects of BRL 38227, salmeterol and ketotifen on responses to electrical field stimulation, substance P (SP), neurokinin A (NKA) and acetylcholine (ACh)

| | *% initial response to spasmogenic stimulus* | | | | |
|---|---|---|---|---|---|
| | *NANC* | *SP* (1 μM) | *NKA* (0.07 μM) | *Cholinergic* | *ACh* (6 μM) |
| BRL 38227 (3 μM) | 13.0 ± 2.5* | 64.7 ± 10.0 | 64.3 ± 6.0 | 36.5 ± 3.6* | 76.4 ± 6.5* |
| Vehicle | 69.8 ± 2.2 | 80.8 ± 7.1 | 81.2 ± 7.6 | 87.9 ± 4.2 | 95.2 ± 5.0 |
| Salmeterol (1 μM) | 7.3 ± 3.9* | 45.8 ± 3.6* | 36.6 ± 8.5* | 28.4 ± 5.5* | 47.7 ± 3.3* |
| Vehicle | 86.5 ± 4.5 | 99.2 ± 8.0 | 101.7 ± 4.2 | 97.8 ± 2.8 | 96.8 ± 5.4 |
| Ketotifen (100 μM) | 54.0 ± 4.9* | 79.3 ± 4.4 | 92.9 ± 4.9 | 41.3 ± 8.9* | 2.3 ± 1.1* |
| Vehicle | 84.7 ± 3.7 | 75.2 ± 3.3 | 105.5 ± 9.8 | 97.1 ± 2.9 | 96.8 ± 5.4 |

Values are means ± s.e.mean, $n = 6–12$.
*Significant difference from vehicle control ($P<0.05$).

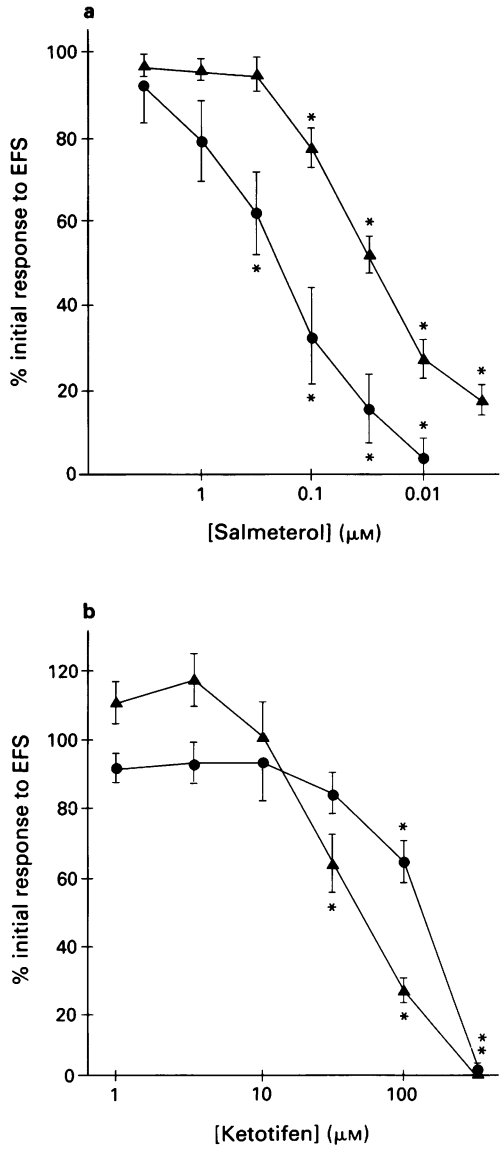


**Figure 3** Effects of (a) salmeterol and (b) ketotifen on non-adrenergic, non-cholinergic excitatory (●) and cholinergic (▲) responses to electrical field stimulation (EFS) in guinea-pig isolated bronchus. Data are means of values from 6 tissues, s.e.means are shown by the vertical bars. *Significant suppression of the response to EFS ($P<0.05$).

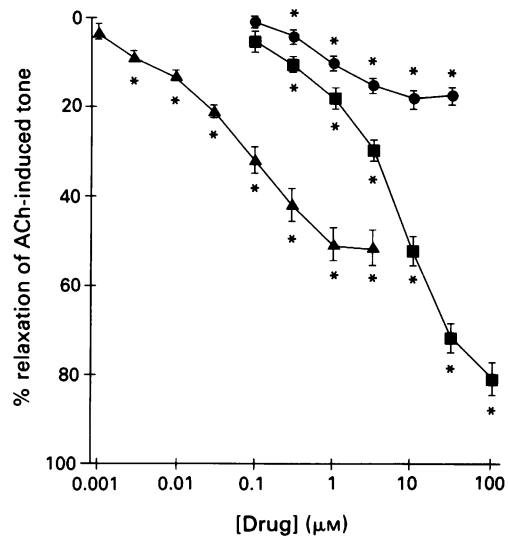


**Figure 4** Spasmolytic effects of BRL 38227 (●), salmeterol (▲) and ketotifen (■) in acetylcholine (ACh, 100 μM)-precontracted guinea-pig isolated bronchus. Data are means of values from 6 tissues, s.e.means are shown by the vertical bars. *Significant suppression of ACh-induced tone ($P<0.05$).

## Discussion

### *NANCe responses to EFS: pre- or post-junctional site of action?*

NANCe responses of the guinea-pig isolated bronchus to EFS are believed to reflect the antidromic activation of a subset of intramural sensory neurones (Andersson & Grundstrom, 1987). The ability of capsaicin to abolish NANCe responses (Andersson & Grundstrom, 1983; Lundberg *et al.*, 1983; Present Study, Figure 1b), suggests that the transmitter(s) in this pathway is a sensory neuropeptide (Buck & Burks, 1986), although its identity remains to be established. SP has previously been considered a strong candidate (Lundberg *et al.*, 1983). However, it has recently been suggested that NKA may in fact mediate NANCe nerve-mediated contractions. Thus, dactinomycin inhibited contractions of guinea-pig bronchus evoked by NANCe nerve stimulation and NKA, but had no effect on responses to SP (Lungberg & Lou, 1991).

BRL 38227 (3 μM) and ketotifen (100 μM) each attenuated NANCe responses to EFS, but had no significant effect on the spasm evoked by an equi-effective challenge with SP (1 μM) or NKA (0.07 μM). This shows good agreement with previous reports, where cromakalim was tested in guinea-pig isolated trachea (Burka *et al.*, 1991) and ketotifen in guinea-pig bronchus (Kamikawa, 1989). These findings may suggest that the inhibitory effects of BRL 38227 (3 μM) and ketotifen (100 μM) on NANCe responses to EFS are unlikely to result from inhibition of the response of the bronchial smooth muscle to the NANCe transmitter, but, instead, may reflect a pre-junctional effect on NANCe nerve neurotransmission. One possibility is that BRL 38227 and ketotifen may reduce the amount of transmitter released in response to EFS (Kamikawa, 1989; Burka *et al.*, 1991). However, this requires confirmation, for instance by direct measurement of tachykinin release from guinea-pig bronchus in response to EFS.

The ability of salmeterol (1 μM) to attenuate markedly responses to SP and NKA suggests that, by contrast, a large component of its inhibitory effects on NANCe responses to EFS can be attributed to depression of bronchial smooth muscle responsiveness. Similar conclusions were reached when another β-adrenoceptor agonist, isoprenaline, was tested in the guinea-pig bronchus (Kamikawa & Shimo, 1990).

### *Cholinergic responses to EFS: pre- or postjunctional site of action?*

That the initial, rapid response to EFS was mediated by cholinergic neurones is suggested by its sensitivity to atropine (1 μM) (Figure 1a). The present findings that capsaicin reduces the magnitude of cholinergic-nerve mediated responses agrees well with previous work by Stretton *et al.* (1989), and may suggest that NANCe nerves exert a facilitory effect on cholinergic neurotransmission in the guinea-pig bronchus.

The cholinergic component of the response to EFS was inhibited by BRL 38227, albeit less potently and effectively than were NANCe responses (Figure 2a). By contrast with its effect on nerve-mediated responses, BRL 38227 was relatively ineffective as a relaxant of ACh-induced tone in the guinea-pig bronchus (Figure 4), and caused only a small reduction in the response to a challenge with ACh (6 μM) (Table 2). This suggests that BRL 38227 may also modulate cholinergic

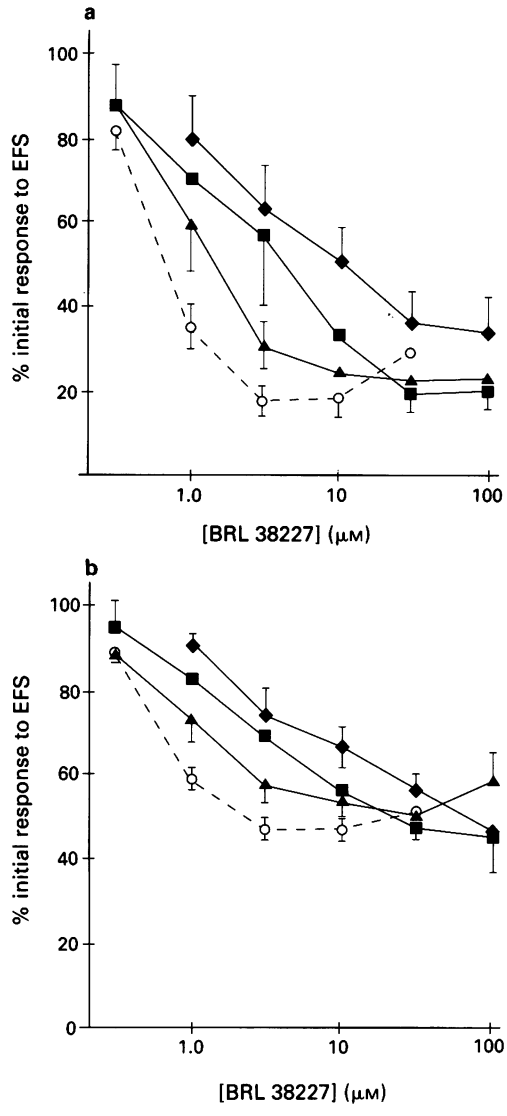


**Figure 5** Effects of glibenclamide 0.1 μM (▲), 0.3 μM (■), 1.0 μM (◆) or dimethylsulphoxide (--○--) on responses to BRL 38227 in field stimulated, guinea-pig isolated bronchus. (a) Non-adrenergic, non-cholinergic excitatory responses to electrical field stimulation (EFS), (b) cholinergic responses to EFS. Data are means of values from 5–6 tissues, s.e.means are shown by the vertical bars.

**Table 3** Effects of glibenclamide, phentolamine and BRL 38226 on responses to electrical field stimulation (EFS) in guinea-pig isolated bronchus

| | | *% Initial response to EFS* | |
|---|---|---|---|
| | | *NANC responses* | *Cholinergic responses* |
| Vehicle | | 87.8 ± 4.1 | 100.7 ± 2.3 |
| Glibenclamide | 0.1 μM | 101.4 ± 6.9 | 104.2 ± 2.7 |
| | 0.3 μM | 93.9 ± 7.1 | 107.9 ± 3.7 |
| | 1.0 μM | 95.9 ± 5.6 | 109.1 ± 3.3 |
| Vehicle | | 100.5 ± 4.1 | 102.5 ± 2.9 |
| Phentolamine | 3 μM | 99.3 ± 2.5 | 99.9 ± 2.9 |
| | 10 μM | 76.6 ± 10.4* | 88.8 ± 5.8* |
| | 30 μM | 43.8 ± 7.6* | 54.6 ± 4.1* |
| | 100 μM | 13.6 ± 7.2* | 22.7 ± 6.9* |
| Vehicle | | 95.6 ± 4.3 | 99.6 ± 3.7 |
| BRL 38226 | 0.5 μM | 88.6 ± 5.4 | 98.9 ± 3.8 |
| | 1.5 μM | 80.2 ± 9.2 | 91.5 ± 6.2 |
| | 5.0 μM | 94.4 ± 6.3 | 102.9 ± 4.2 |
| | 30 μM | 95.9 ± 4.9 | 100.3 ± 4.2 |

Values are means ± s.e.mean, $n = 4-6$.
*Significant difference from vehicle-treated tissues ($P < 0.05$).

nerve activity in the guinea-pig bronchus, by an action at a prejunctional site. These findings show good agreement with previous studies where BRL 38227 was tested in the electrically stimulated, guinea-pig isolated trachea (Cooper *et al.*, 1991) and cromakalim was tested against cholinergic nerve- and ACh-induced bronchoconstriction in the anaesthetized guinea-pig (Ichinose & Barnes, 1990; Lewis & Raeburn, 1990). However, the present study contrasts with the work of McCaig & de Jonckheere (1989), who reported that cromakalim failed to affect responses to EFS in the guinea-pig trachea, although responses to selective, preganglionic, stimulation of vagal nerves were inhibited.

Again, quantification of the effects of BRL 38227 on the amount of transmitter released by bronchial cholinergic nerves in response to EFS is required to establish its site of action. It is noteworthy that investigations of the effects of a $K^+$-channel activator on [$^3$H]-ACh release caused by field stimulation of cholinergic nerves in the guinea-pig trachea have failed to demonstrate any inhibition (Wessler *et al.*, 1991).

By contrast with BRL 38227, ketotifen (100 μM) and salmeterol (1 μM) each markedly inhibited responses to

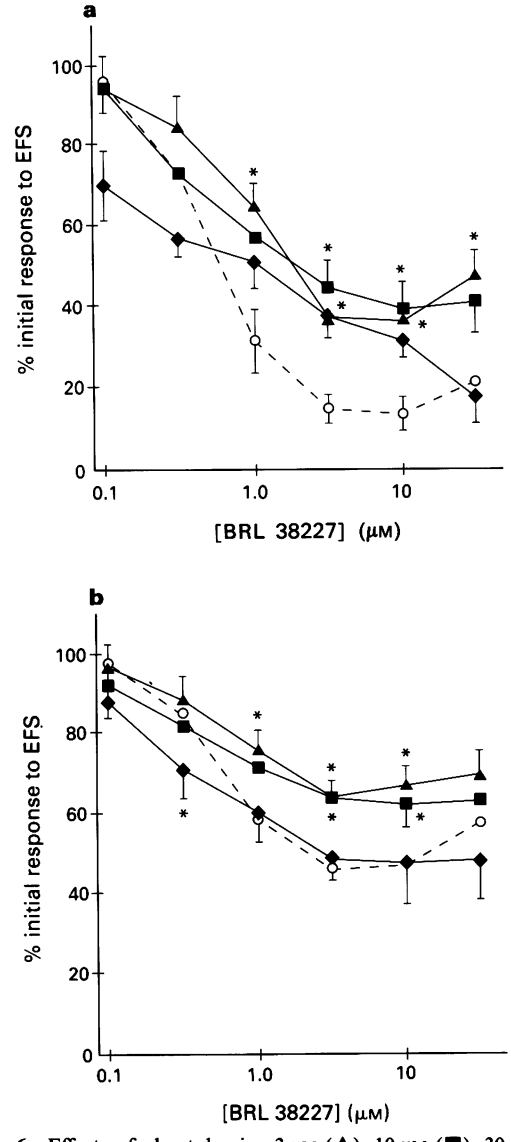


**Figure 6** Effects of phentolamine 3 μM (▲), 10 μM (■), 30 μM (◆), or twice distilled water (--○--) on responses to BRL 38227 in field stimulated guinea-pig isolated bronchus. (a) Non-adrenergic, non-cholinergic excitatory responses to electrical field stimulation (EFS), (b) cholinergic responses to EFS. Data are means of values from 5–6 tissues, s.e.means are shown by the vertical bars. *Significant difference from tissues treated with twice distilled water ($P < 0.05$).

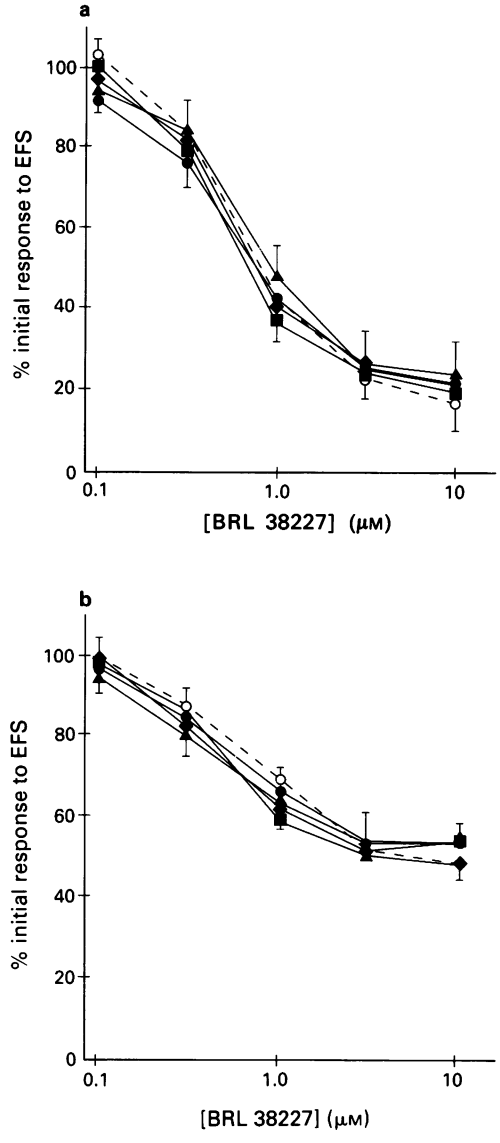


**Figure 7** Effects of BRL 38226 0.5 μM (▲), 1.5 μM (■), 5 μM (◆), 30 μM (●) or polyethylene glycol (--○--) on responses to BRL 38227 in field stimulated, guinea-pig isolated bronchus. (a) Non-adrenergic, non-cholinergic excitatory responses to electrical field stimulation (EFS), (b) cholinergic responses to EFS. Data are means of values from 5–6 tissues, s.e.means are shown by the vertical bars.

exogenously applied ACh (6 μM; Table 2). Moreover, these agonists were more effective than BRL 38227 at producing relaxation of ACh-induced spasm of the bronchial smooth muscle (Figure 4). These findings show good agreement with previous work using ketotifen and isoprenaline (Kamikawa, 1989; Kamikawa & Shimo, 1990), and suggest that the inhibitory effects of ketotifen and salmeterol on cholinergic responses to EFS can largely be attributed to a postjunctional action at the level of the bronchial smooth muscle.

### *Mechanism of action of BRL 38227 on responses to EFS*

BRL 38227, pinacidil and RP 52891, the active (−)-enantiomer of RP 49356 (Mondot *et al.*, 1990), are each thought to cause smooth muscle relaxation by opening $K^+$-channels in the plasmalemma of smooth muscle cells (Bray *et al.*, 1987; Arch *et al.*, 1988; Mondot *et al.*, 1988). The very similar profiles of action of BRL 38227, pinacidil and RP 52891 on responses to EFS may therefore suggest that $K^+$-channel activation is a common underlying mechanism for their suppressant effects on nerve-mediated contractions.

The sulphonylurea glibenclamide is considered to be a relatively specific blocker of the ATP-dependent $K^+$-channel in the pancreas (Ashcroft, 1988). At higher concentrations, it has been shown to block an ATP-dependent $K^+$-channel opened by cromakalim in rabbit mesenteric arterial smooth muscle cells (Standen *et al.*, 1989). Over the range 0.1–1 μM, glibenclamide selectively antagonized the tracheal smooth muscle relaxant actions of cromakalim in an apparently competitive manner (Murray *et al.*, 1989) and prevented the hyperpolarization of guinea-pig trachealis cells caused by cromakalim (Murray *et al.*, 1989).

In the present study, glibenclamide (0.1–1 μM) also antagonized the actions of BRL 38227 on responses to EFS in guinea-pig bronchus in a competitive manner. Similarly, previous studies have reported that glibenclamide antagonized the inhibition of NANCe and cholinergic nerve-mediated responses produced by cromakalim in the guinea-pig isolated trachea (Burka *et al.*, 1991; Cooper & Maclagan, 1990) and anaesthetized guinea-pig (Ichinose & Barnes, 1990). These findings lend support to the proposition that the effects of BRL 38227 on responses to EFS are mediated by $K^+$-channel activation, and may further suggest the involvement of an ATP-dependent $K^+$-channel (Quast & Cook, 1989). Nevertheless, the relatively high concentrations of glibenclamide required to inhibit responses to BRL 38227 suggest that the channel involved is unlikely to be identical with that found in pancreatic β cells (Schmid-Antomarchi *et al.*, 1987).

It has recently been suggested that phentolamine is also a blocker of ATP-dependent $K^+$-channels. Thus, in mouse pancreatic β cells and RINm5F insulin- secreting cells, phentolamine (5–100 μM) reduced diazoxide-induced $^{86}Rb^+$ efflux (Plant & Henquin, 1990) and inhibited ATP-sensitive $K^+$-currents (Plant & Henquin, 1990; Dunne, 1991). In vascular and tracheal smooth muscle, phentolamine (1.0–100 μM) specifically inhibited the relaxant effects of cromakalim, by a mechanism unrelated to blockade of α-adrenoceptors (McPherson & Angus, 1989; Murray *et al.*, 1989). Furthermore, phentolamine (100 μM) prevented the hyperpolarization of rat mesenteric arterial smooth muscle and of guinea-pig trachealis caused by cromakalim (Murray *et al.*, 1989; McPherson & Angus, 1991).

In the present study, the use of phentolamine as an analytical tool was weakened by the finding that, at concentrations greater than 3 μM, phentolamine itself caused inhibition of responses to EFS. Nevertheless, at a concentration exerting no inhibitory effects on responses to EFS (3 μM), phentolamine antagonized the actions of BRL 38227 on nerve-mediated responses in the guinea-pig bronchus (Figure 5a,b). In agreement with Murray *et al.* (1989), we found that the inhibition of responses to BRL 38227 by phentolamine (3 μM) was non-competitive in nature (Figure 5a,b). However, the present findings contrast with the report of Ichinose & Barnes (1990) that, although phentolamine antagonized the blood pressure lowering effects of cromakalim in the anaesthetised guinea-pig, it had no effect on the ability of cromakalim to cause suppression of NANCe nerve-mediated bronchoconstriction.

The inhibition of nerve-mediated responses in guinea-pig bronchus by BRL 38227 is observed over a concentration-range similar to that at which it causes relaxation of spontaneous tone in tracheal smooth muscle (0.3–3 μM; Arch *et al.*, 1988). Furthermore these actions exhibit stereo-specificity (Figures 1a,b), and can be blocked by known antagonists of the actions of $K^+$-channel activators on airways smooth muscle (Figures 5 and 6). If BRL 38227 is inhibiting NANCe and cholinergic nerve activity in the guinea-pig bronchus by a mechanism involving $K^+$-channel activation, then these findings suggest that the channel involved has characteristics similar to that mediating airways smooth muscle relaxation.

The (+)-isomer of BRL 38227, BRL 38226 is generally considered to be essentially devoid of activity (Buckingham *et al.*, 1986; Arch *et al.*, 1988). The possibility of an interaction between the two enantiomers was investigated in view of a recent report that, in guinea-pig tracheal cholinergic

nerves, BRL 38226 itself caused a slight suppression of responses to electrical stimulation and reduced the inhibitory effects of BRL 38227 on these nerves (Cooper *et al.*, 1991). However, we obtained no evidence for an interaction between BRL 38226 and BRL 38227 in guinea-pig bronchial cholinergic or NANCe nerves (Figure 7a,b). Our findings do not support the suggestion of Cooper *et al.* (1991) that BRL 38226 may be a partial agonist for the neuronal $K^+$-channel mediating the effects of BRL 38227.

It has been suggested that neural mechanisms are important in the pathogenesis of astham (Barnes, 1986). In particular, it is thought that peptidergic NANCe neurones may be involved in the generation and propagation of inflammatory changes in the airways of asthmatic patients (Barnes *et al.*, 1990). Accordingly, the present findings that BRL 38227 interferes with NANCe neuroeffector transmission may suggest that, in addition to providing direct bronchodilatation, BRL 38227 may also exert an anti-inflammatory effect in the airways.

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# Effects of monoamine uptake inhibitors on extracellular and platelet 5-hydroxytryptamine in rat blood: different effects of clomipramine and fluoxetine

Jordi Ortiz & [1]Francesc Artigas

Department of Neurochemistry, Consejo Superior de Investigaciones Científicas, Jordi Girona 18–26, 08034 Barcelona, Spain

**1** The concentration of 5-hydroxytryptamine (5-HT) in rat platelet-free plasma increased significantly 30 min after a single i.p. injection (10 mg $kg^{-1}$) of each of six inhibitors of the high-affinity 5-HT uptake (fluvoxamine, fluoxetine, alaproclate, paroxetine, sertraline and clomipramine). The increases ranged from 226% to 776% of control values. In contrast, imipramine, desipramine and femoxetine had no significant effect. The increase elicited by paroxetine was dependent on the dose (1, 5 and 10 mg $kg^{-1}$) and returned to control values after 4 h. That observed after clomipramine was also transient and paralleled the plasma concentration of the drug (Spearman-rank correlation $r = 0.43$).

**2** *In vivo*, the rat pulmonary vascular endothelium removed trace amounts (8.8 nmol in a bolus) of intravenously injected [$^{14}$C]-5-HT. Paroxetine pretreatment (10 mg $kg^{-1}$, 30 min before-hand) reduced this uptake by 73%.

**3** Repeated fluoxetine treatments reduced rat whole blood 5-HT concentration (ca. – 60% after daily 2 × 5 mg $kg^{-1}$, i.p. during 14 days). However, plasma (extracellular) 5-HT was not increased.

**4** Various repeated treatments with clomipramine (i.p. injections or osmotic minipumps, up to 30 mg $kg^{-1}$ $day^{-1}$), failed to decrease rat whole blood 5-HT concentrations. Platelet-free plasma 5-HT was also unchanged, even after treatments yielding plasma clomipramine levels 2.7 times higher than those that increased it acutely.

**5** These results indicate that the extracellular pool of 5-HT in rat blood (measured in the platelet-free plasma) is physiologically under the control of high-affinity 5-HT uptake systems. The sustained 5-HT uptake inhibition does not result in an increase of 5-HT in platelet-free plasma, suggesting that adaptative mechanisms are triggered. The distinct long-term effects of the two antidepressants clomipramine and fluoxetine on rat whole blood 5-HT suggest a differential *in vivo* action on the rat 5-HT uptake.

**Keywords:** 5-Hydroxytryptamine uptake; antidepressant drugs; uptake inhibitors; endothelial uptake; clomipramine; fluoxetine; imipramine; paroxetine; platelets; plasma

## Introduction

Central nervous system (CNS) 5-hydroxytryptaminergic neurotransmission is of key importance for the clinical effects of antidepressant drugs (Shopsin *et al.*, 1975; 1976; Delgado *et al.*, 1990). However, the study of the actions of antidepressants in human CNS is complicated by the fact that only indirect measures of neurotransmitter function can be used, which are scarce and difficult to interpret (see Murphy *et al.*, 1990 for review). Platelets have been used extensively because of their similarities with 5-hydroxytryptamine (5-HT) nerve terminals, particularly for the presence of amine storage granules and a high-affinity transporter for 5-HT (Stahl, 1985). However, platelets lack noticeable synthesis and metabolism of 5-HT, so that they provide only a restrictive view of the 5-HT system. In recent years, several laboratories have studied the effects of certain pathologies and pharmacological treatments on the extracellular 5-HT in the blood of psychiatric patients (Sarrias *et al.*, 1987; Cook *et al.*, 1988; Artigas *et al.*, 1989; Rupprecht *et al.*, 1989; Celada *et al.*, 1990). This variable is sensitive to rapid and sustained changes of 5-HT synthesis and metabolism (Ortiz *et al.*, 1988; Celada *et al.*, 1990) and therefore may overcome some of the limitations of the measure of 5-HT in platelets. Extracellular 5-HT concentrations in venous human blood (measured in platelet-free plasma) account for less than 1% of total blood 5-HT, the rest being stored in platelets (Ortiz *et al.*, 1988). The low concentration (5–15 nM) of extracellular 5-HT is a consequence of potent mechanisms removing 5-HT from the bloodstream (high-affinity uptake into platelets and endothelial cells and monoamine oxidase (MAO) deamination) after its release from enterochromaffin cells (Verbeuren, 1989). In different areas of rat brain, extracellular and tissue 5-HT maintain a similar proportion due to 5-HT reuptake (Adell *et al.*, 1991). Extracellular 5-HT in rat brain increases several fold after treatment with different uptake inhibitors (Kalén *et al.*, 1988; Auerbach *et al.*, 1989; Carboni & Di Chiara, 1989; Adell & Artigas, 1991) confirming *in vivo* that uptake is a basic mechanism of the control of extracellular- and therefore, receptor-available-5-HT. Since the 5-HT uptake systems also control the equilibrium between extra- and intracellular 5-HT in blood, the effects of 5-HT uptake inhibitors on extracellular 5-HT can also be studied *in vivo* outside the CNS. The aim of the present work was to study the effects of several high-affinity 5-HT uptake inhibitors, the working hypothesis being that they should increase the extracellular concentration of 5-HT in rat blood (measured in platelet-free plasma). Also, platelet 5-HT concentration should decrease only after sustained uptake inhibition. The hypothesis was confirmed after acute treatment, repeated treatment yielding unexpected results that are discussed.

## Methods

### *Animal treatment*

Male Wistar rats of 250–300 g (Iffa-Credo, Lyon, France) were kept in a controlled environment (22°C) with a light/

[1] Author for correspondence.

dark periodicity of 12 h, at least for one week before the experiments. They were housed four per cage with water and food *ad libitum*.

Acute administration of uptake inhibitors was carried out i.p. to groups of 7–12 animals randomly chosen. In each experiment, a control group was injected with saline and processed along with the treated groups. Usually the acute effects of 2 or 3 different treatments were examined during the same experiment.

For the repeated administration of drugs, Alzet mini-osmotic pumps (Model 2002, Palo Alto, U.S.A.) were implanted subcutaneously under light ether anaesthesia. Clomipramine was dissolved in 2% Tween and fluoxetine in 50% dimethyl sulphoxide (DMSO) due to the low volume of mini-pumps. Control rats were implanted with pumps filled with the corresponding vehicles. Interindividual variance of weights was corrected by the method of Greenshaw (1986). Since the animals increased their weights during the treatment (two weeks), the drug doses reported correspond to those expected on the 7th day. Animals with implanted minipumps were housed individually to avoid reciprocal gnawing of sutures observed after some treatments.

### *Blood sampling*

Blood sampling was routinely carried out between 16 h 00 min and 19 h 00 min. Under pentobarbitone anaesthesia (70 mg $kg^{-1}$, i.p. Sagatal, RMB, U.K.), the blood was drawn from the carotid artery through a polythene cannula (Portex, U.K.) cut bevelled (0.58 mm i.d., 4–15 cm long) into three Eppendorf tubes. The cannula and the sampling tubes contained $K_2$-EDTA calculated to give a final concentration of 0.5–1% (w/v) EDTA in blood. The use of this method of blood sampling is due to the necessity of avoiding 5-HT release from platelets that would occur after more traumatic methods. The blood of the first tube (0.8 ml) was used for the determination of whole blood 5-HT content. The second and third tubes (1.5 ml of blood each) were centrifuged (12,000 *g*, 5 min, 22°C). The resulting supernatant (platelet-free plasma) was carefully removed and frozen at − 80°C until analysed. Extracellular 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) concentrations were determined in platelet-free plasma of the second tube. With this method, mean values of 5-HT in the platelet-free plasma of rats are usually in the low nM range (Ortiz *et al.*, 1991a,b).

### *High performance liquid chromatography analysis*

The 5-HT concentration in platelet-free plasma was determined by high performance liquid chromatography (h.p.l.c.) coupled to electrochemical detection after a butanol/heptane/HCl extraction procedure (Artigas *et al.*, 1985). Whole blood 5-HT analysis was carried out as described in Ortiz *et al.* (1988). In both cases, bufotenine (N,N-dimethylserotonin) was used as internal standard. Plasma 5-HIAA determination was carried out as described for human blood (Martínez *et al.*, 1983). Minor modifications of these methods were introduced in order to use smaller volumes of sample. Plasma total tryptophan (TRP) was also analysed by h.p.l.c. and fluorescence detection (280/340 nm). Plasma clomipramine (CIM) levels were monitored by the method of Langerström *et al.* (1983), that involves a diethyl ether/HCl extraction from plasma at pH 3.5 and h.p.l.c. – u.v. determination with desipramine as internal standard.

### *Effect of paroxetine on the endothelial [$^{14}$C]-5-HT uptake*

The pulmonary removal of [$^{14}$C]-5-HT from blood was measured *in vivo* by a modification of the method described by Catravas & Gillis (1980). Rats (350–450 g) were pretreated with saline or paroxetine (10 mg $kg^{-1}$, i.p., 30 min before, $n = 4$ rats/group) and anaesthetized with pentobarbitone (70 mg $kg^{-1}$, i.p.). Then, a bolus of 500 nCi of [$^{14}$C]-5-HT (8.8 nmol) (Amersham) was injected into the jugular vein. Blood was being collected simultaneously at 1-s intervals through a cannula placed in the carotid artery into tubes containing $K_2$-EDTA (120–150 µl of blood/tube). Blood (50 µl) from each tube was processed as for whole blood 5-HT determination and then subjected to liquid scintillation counting instead of h.p.l.c. $^{14}$C d.p.m. $ml^{-1}$ of blood was calculated in each 1 s sample, by use of a [$^{14}$C]-5-HT standard addition curve that was processed in parallel. To another set of rats ($n = 3$), 1 mg indocyanine green (cardiogreen) was injected as reference for the calculation of [$^{14}$C]-5-HT uptake. This compound does not leave the cirulation in a single passage through the lungs. Its concentration was measured by optic densitometry at 675 nm in plasma, in parallel to a standard addition curve of colorant. The area under the curve (d.p.m. or optic density of colorant) was used to calculate [$^{14}$C]-5-HT uptake with the formula:

$$\%\ 5\text{-HT uptake} = \left[1 - \frac{(\text{d.p.m. ml}^{-1})/\text{d.p.m. injected}}{(\text{mg ml}^{-1})/\text{mg injected}}\right] \times 100$$

$^{14}$C uptake represents the percentage of the total amine injected that was removed by endothelial 5-HT uptake during a single passage through the lungs (Catravas & Gillis, 1980).

### *Drugs*

The following drugs were kindly provided: clomipramine, imipramine and desipramine (Ciba-Geigy), paroxetine (Beecham), fluoxetine (Eli, Lilly & Co.), fluvoxamine (Duphar), sertraline (Pfizer), alaproclate (Astra) and femoxetine (Ferrosan). The doses used correspond to the free compound. H.p.l.c. standards were from Sigma and the rest of the products were of the highest purity commercially available.

### *Statistics*

The statistical significance of the changes induced by uptake inhibitors was assessed by one-way ANOVA followed by Student-Neuman-Keuls comparisons between the drug and saline groups. The effects of treatments on plasma 5-HT were evaluated by use of non-parametric statistics (Kruskal-Wallis test followed by Mann-Whitney U-test) because of the skewed statistical distribution of the variable in control rats (Ortiz *et al.*, 1991a). Accordingly, plasma 5-HT is represented in the figures by its median. Statistical significance was set at $P < 0.05$.

## Results

### *Acute effects*

*Increase of plasma 5-HT by selective high-affinity 5-HT uptake inhibitors* A single dose of the following drugs (10 mg $kg^{-1}$, i.p., 30 min before blood sampling) increased rat plasma 5-HT ($P < 0.05$) vs saline-treated rats: fluvoxamine, fluoxetine, alaproclate, paroxetine, sertraline and clomipramine (Table 1). In contrast, the increases induced by imipramine, desipramine and femoxetine did not reach statistical significance. Whole blood 5-HT (>99% in platelets; Ortiz *et al.*, 1988), was not modified in a consistent manner although slight decreases were observed after fluvoxamine, fluoxetine and imipramine.

Plasma 5-HIAA increased significantly after the acute administration of paroxetine (+110%), fluoxetine (+90%) and sertraline (+49%) ($P < 0.05$). More moderate changes were elicited by alaproclate (+32%) and imipramine (−16%). Plasma tryptophan was unchanged by these treatments.

**Table 1** Increase of plasma 5-hydroxytryptamine (5-HT) produced by acute 5-HT uptake inhibitors

| Drug | *Controls* median interquartile range (n) | *Treated* median interquartile range (n) | *% of controls* |
|---|---|---|---|
| Fluvoxamine | 7.6 3.9–13.2 (10) | 59 19.4–104 (11) | 776%* |
| Fluoxetine | 7.6 3.9–13.2 (10) | 39.6 22–63 (11) | 520%* |
| Alaproclate | 8.1 5.4–28.8 (10) | 37.5 18.8–61.9 (10) | 465%* |
| Paroxetine | 14.1 7.7–24.8 (10) | 50.6 38.6–77.2 (11) | 358%* |
| Sertraline | 8.1 5.4–28.8 (10) | 27 15.6–86 (11) | 335%* |
| Desipramine | 8.1 5.4–28.8 (10) | 20.7 7.1–139 (12) | 257% |
| Clomipramine | 6.8 3.7–11.8 (9) | 15.5 9.5–43 (8) | 226%* |
| Femoxetine | 8.1 5.4–28.8 (10) | 15.1 10.5–131 (11) | 187% |
| Imipramine | 7.6 3.9–13.2 (10) | 12.4 7.6–116 (11) | 185% |

Plasma 5-HT is given in nmol $l^{-1}$. *$P<0.05$ versus respective saline-treated animals, Kruskal-Wallis and Mann-Whitney tests. The data shown correspond to four different experiments in which median control values were not different (Kruskal-Wallis test).

*Drug-effect relationships* Different doses of paroxetine were administered to randomized groups of animals (0, 1, 5 or 10 mg $kg^{-1}$, i.p., 30 min before), eliciting dose-dependent increases in plasma 5-HT and 5-HIAA as shown in Figure 1. Whole blood 5-HT was not modified by such treatments.

The kinetics of the increase of extracellular 5-HT were studied after the administration of clomipramine and paroxetine. Groups of animals treated with a single dose of either drug (10 mg $kg^{-1}$, i.p.) were killed at different times post-administration: 15 min (only for paroxetine), 30 min, 1 h, 2 h and 4 h. With both drugs, plasma 5-HT rose maximaly at 30 min, returning to control values after 4 h. The increase of plasma 5-HT elicited by clomipramine (CIM) clearly paralleled the plasma concentration of the drug (Figure 2). In addition, the individual values of 5-HT and CIM in plasma correlated significantly (Spearman rank correlation $r = 0.43$, $n = 41$, $P<0.05$).

*Effects of paroxetine pretreatment on the endothelial [$^{14}$C]-5-HT uptake* The pretreatment with paroxetine (10 mg $kg^{-1}$, i.p., 30 min before) elevated the amount of [$^{14}$C]-5-HT left in blood after a single passage of the lungs. This is shown by the difference in the area under the curve represented in Figure 3. Saline-pretreated rats removed 47 ± 22% of the [$^{14}$C]-5-HT bolus, while the paroxetine group removed 13 ± 27% (means ± s.d. of $n = 4$ rats per group, $P<0.05$ Mann-Whitney test). Thus, paroxetine elicited a 73% reduction in the endothelial 5-HT removal.

## *Effects of sustained uptake inhibition*

*Effects of repeated clomipramine* In a first experiment, repeated intraperitoneal administrations of CIM (15 mg $kg^{-1}$ daily for 2, 4, 7, 14 or 21 days, killing 24 h after the last injection) did not modify the concentrations of total tryptophan, 5-HT and 5-HIAA in plasma nor 5-HT in whole blood (data not shown). The lack of effect on whole blood 5-HT was surprising, since lower doses decrease by more than 90% platelet 5-HT in depressive patients (Sarrias *et al.*, 1987). Given the short half-life of CIM in rat plasma after repeated administration (Friedman & Cooper, 1983), it is

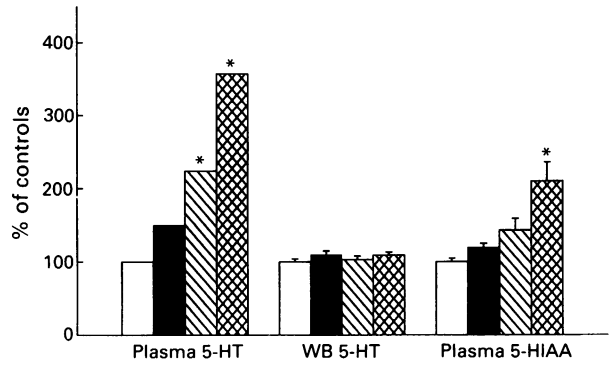


**Figure 1** Dose-related increase of extracellular 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in plasma elicited by the selective high-affinity 5-HT uptake inhibitor paroxetine. Saline (open column) or paroxetine 1 mg $kg^{-1}$ (solid column), 5 mg $kg^{-1}$ (hatched column) or 10 mg $kg^{-1}$ (cross-hatched column) were administered i.p. 30 min before blood sampling. Values are represented as medians (plasma 5-HT) or means ± s.e.mean (vertical lines) of 10 to 12 rats per group. Control values were: plasma 5-HT, 14.1 nM; whole blood (WB) 5-HT, 8.23 μM; plasma 5-HIAA, 81 nM. $P<0.05$ versus saline-treated animals, Kruskal-Wallis and Mann-Whitney tests (plasma 5-HT) or one-way ANOVA and Student-Newman-Keuls (plasma 5-HIAA).

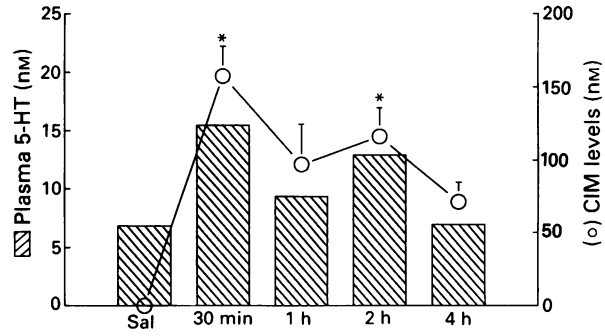


**Figure 2** Time- and drug level-dependent increase of extracellular 5-hydroxytryptamine (5-HT) in plasma elicited by i.p. administration of 10 mg $kg^{-1}$ of the high-affinity 5-HT uptake inhibitor clomipramine (CIM). Columns represent plasma 5-HT medians and (○) are means ± s.e.mean (vertical lines) of plasma CIM (7–10 rats per group). $P<0.05$ versus saline-treated animals, Kruskal-Wallis and Mann-Whitney tests. Plasma 5-HT and CIM levels correlated significantly (Spearman-rank correlation $r = 0.43$, $n = 41$, $P<0.05$).

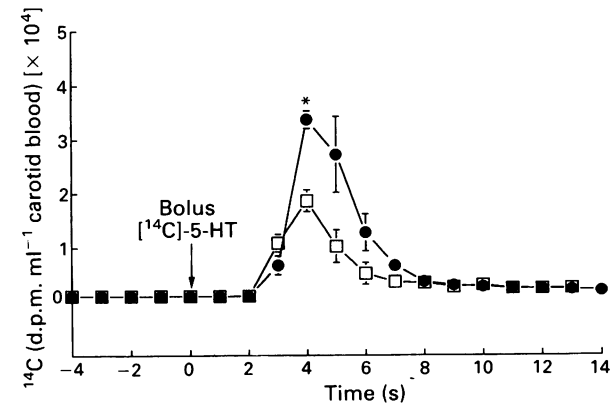


**Figure 3** Effect of paroxetine pretreatment on the *in vivo* pulmonary vascular endothelial removal of [$^{14}$C]-5-hydroxytryptamine ([$^{14}$C]-5-HT), 30 min before the rats were pretreated with saline (□) or paroxetine 10 mg $kg^{-1}$ (●). At time zero, 8.8 nmol of radiolabelled 5-HT was injected into the jugular vein while blood was being sampled each second through the carotid artery. The ordinate scale (d.p.m.) represents the radiolabelled 5-HT that escaped the endothelial removal of the amine. The data are means of four different experiments per group; s.e.mean shown by vertical lines. ($P<0.05$ two-tailed Student's $t$ test.

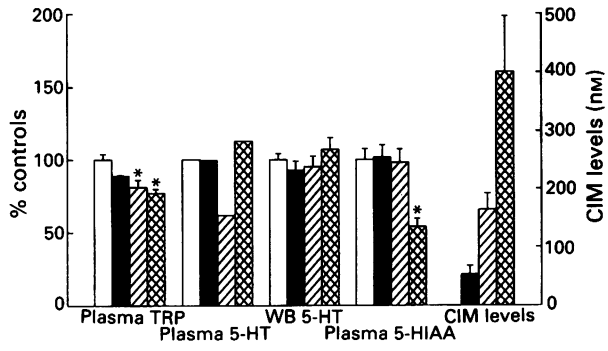


**Figure 4** Dose-related effects of chronic clomipramine administration by osmotic mini-pumps. Treatments used were vehicle (open column), clomipramine 15 mg kg$^{-1}$ day$^{-1}$ for 7 days (solid column), 15 mg kg$^{-1}$ day$^{-1}$ for 14 days (hatched column) or 30 mg kg$^{-1}$ day$^{-1}$ for 14 days (cross-hatched column). No change was observed in plasma and whole blood 5-hydroxytryptamine (5-HT) even at the highest drug levels. Values are expressed as medians (plasma 5-HT) or means ± s.e.mean (vertical lines) of 7 to 19 rats per group. $P<0.05$ versus controls, one-way ANOVA and Student-Newman-Keuls comparisons. Control group pools data from two experiments with slightly different values: plasma tryptophan (TRP), 82 μM and 74 μM; plasma 5-HT, 13.5 nM and 12.2 nM; whole blood (WB) 5-HT, 7.2 μM and 8.6 μM ($P<0.05$ two-tailed Student's $t$ test) and plasma 5-hydroxyindoleacetic acid (5-HIAA), 164 nM and 104 nM ($P<0.05$ two-tailed Student's $t$ test). The statistical significances did not vary when each control group was considered alone.

likely that steady-state levels were not achieved. Therefore, we used a more sustained administration, by means of subcutaneously implanted mini-osmotic pumps. The treatments used were: 15 mg kg$^{-1}$ daily for 7 or 14 days, and 30 mg kg$^{-1}$ daily for 14 days, with no wash-out period at the end of the administration. Although the drug plasma levels obtained were within the therapeutic concentrations found in depressed patients (mean values: 54, 164 and 401 nM CIM in plasma, respectively), plasma and whole blood 5-HT were not modified. Plasma total tryptophan decreased moderately both in the 15 mg kg$^{-1}$ day$^{-1}$, 14 days (−19%) and in the 30 mg kg$^{-1}$ day$^{-1}$, groups (−23%, $P<0.05$). Plasma 5-HIAA concentration decreased only in the 30 mg kg$^{-1}$ day$^{-1}$ group (−46%, $P<0.05$) (Figure 4). Regression analysis showed a positive correlation between plasma TRP and 5-HIAA (Pearson's correlation $r=0.52$, $P<0.05$, $n=34$), and a dependence of both variables on CIM levels ($r=-0.47$ and $-0.42$ respectively, $P<0.05$). Multiple regression analysis showed that the correlation between 5-HIAA and CIM was due to that between tryptophan and CIM. Body weight gain during the treatments was lower in the clomipramine groups than in controls (data not shown).

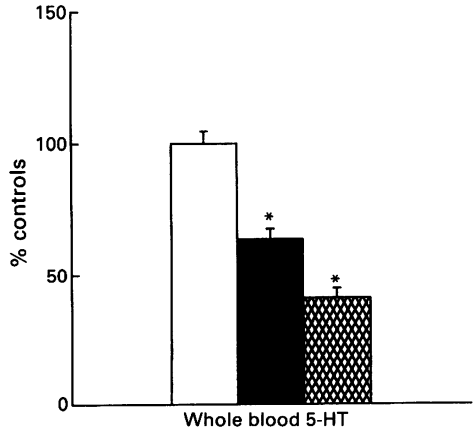


**Figure 5** Dose-related effects of chronic fluoxetine administration. Treatments used were vehicle (open column), fluoxetine 5 mg kg$^{-1}$ day$^{-1}$ for 14 days using mini-osmotic pumps, no washout (solid column) or 2 × 5 mg kg$^{-1}$ day$^{-1}$ i.p. for 14 days, 48 h wash-out (cross-hatched column). *$P<0.05$ versus controls, one-way ANOVA and Student-Newman-Keuls. Respective mean control values were 8.6 μM and 9.4 μM (not significantly different, two-tailed Student's $t$ test).

*Effects of repeated fluoxetine* Repeated administration of fluoxetine was carried out in two experiments, using either mini-osmotic pumps or i.p. injections. Subcutaneously implanted pumps (5 mg fluoxetine kg$^{-1}$ day$^{-1}$ for 14 days, no wash-out) decreased whole blood 5-HT (−36% vs. vehicle-treated rats, $P<0.05$ two-tailed Student's $t$ test) (Figure 5). Plasma 5-HT was unaltered (median control values: 12.2 nM; fluoxetine: 11.6 nM). Neither plasma 5-HIAA nor plasma total tryptophan concentration were significantly changed. Intraperitoneal injections (5 mg kg$^{-1}$, twice daily for 14 days, followed by 48 h wash-out) decreased whole blood 5-HT (−59% vs. saline-treated controls, $P<0.05$ Student's $t$ test).

Plasma 5-HIAA was not significantly changed by fluoxetine. Control values were significantly different between both experiments: Lower values were observed in the rats injected i.p. daily (80.4 ± 16.8 nM) than in those with an implanted pump (104.1 ± 13.7 nM; means ± s.d., $P<0.05$ two-tailed Student's $t$ test). This difference may result from the stress of handling non-habituated animals (pump group) at the time of the experiment. This can moderately increase plasma 5-HIAA probably through activation of 5-hydroxytryptaminergic function (unpublished observations).

## Discussion

In recent years it has been shown that an extracellular pool of 5-HT occurs in low nM concentrations in human blood, in addition to the well-known platelet pool (Artigas *et al.*, 1985; Anderson *et al.*, 1987; Ortiz *et al.*, 1988). When appropriate experimental conditions are used, the 5-HT found in platelet-free plasma is a good estimate of the extracellular pool. Independent changes of the extracellular and platelet pools in man have been reported in pathological conditions and after some pharmacological treatments known to affect the 5-HT system (Sarrias *et al.*, 1987; Cook *et al.*, 1989; Artigas *et al.*, 1989). In rats, little is known about this extracellular pool of 5-HT. Results from this laboratory show that its concentration is similar to that in man, in spite of the fact that rats contain about ten times more 5-HT than man in the platelet pool per unit of blood volume (Ortiz *et al.*, 1988; 1991a,b).

The present results indicate that in the rat, the plasma pool of 5-HT (but not the platelet one) is markedly increased after short-term uptake inhibition. This is consistent with the occurrence of high-affinity 5-HT uptake present in the platelets and vascular endothelial cells and perhaps in enterochromaffin cells and their physiological role in removing extracellular 5-HT (Verbeuren, 1989). The increase of plasma 5-HT was dependent on the dose of the uptake inhibitor (e.g. paroxetine). Also, it followed the evolution of drug plasma levels (clomipramine), which suggests that the turnover of extracellular 5-HT in blood is very rapid.

More marked plasma 5-HT increases were obtained with the new antidepressants which are selective 5-HT uptake inhibitors rather than with the tricyclic drugs. Also, the three drugs having the greatest effect (fluvoxamine, fluoxetine and alaproclate) displayed the same order of potencies as in the synaptosomal [$^3$H]-5-HT uptake inhibition (Wolf & Kuhn, 1991). This indicates that the increase of plasma 5-HT is representative of the actual effects of these drugs on the 5-HT transporter *in vivo*. In addition, unlike *in vitro* procedures (sometimes performed far from physiological conditions, such as the binding of [$^3$H]-imipramine to platelets at 0°C), the increase of plasma 5-HT takes into account other factors that may influence the interaction of the drug with its phar-

macological target (bioavailablity, metabolism and excretion, interaction with other mechanisms of control, etc).

Besides platelets, endothelial cells have a 5-HT transporter sensitive to imipramine (Catravas & Gillis, 1980; Bosin & Lahr, 1981; Robinson-White *et al.*, 1981; Lee & Fanburg, 1986). To examine the contribution of endothelial uptake to the increases of extracellular 5-HT in rats, we tested *in vivo* the effects of a single dose of paroxetine. Saline-treated animals removed almost 50% of the injected [$^{14}$C]-5-HT (8.8 nmol) in a single passage through the lungs. Paroxetine elicited an important reduction (− 73%) of the removal of this physiological amount of 5-HT (as [$^{14}$C]-5-HT) by the lungs, in parallel with a marked increase of plasma 5-HT (Table 1). These figures support the view that the endothelial uptake is a very important factor for the control of extracellular 5-HT in blood *in vivo*, confirming earlier data obtained with rat isolated lungs (Steinberg & Das, 1980). Also, the present results prove that the rat endothelial removal of 5-HT is sensitive to specific 5-HT uptake inhibitors, such as paroxetine.

Some of the specific 5-HT uptake inhibitors acutely increased plasma 5-HIAA concentration. This is formed by MAO-A in the liver and, after high-affinity 5-HT uptake, in the vascular endothelium (Verbeuren, 1989). The effects on the plasma 5-HIAA concentration probably derive from a major availability of extracellular 5-HT to the liver, since 5-HT can enter the hepatocytes from portal blood without specific uptake (Wiersma & Roth, 1980). However, the lack of a significant effect of fluvoxamine, which increased plasma 5-HT maximally, suggests that other factors may be differentially affected by the several drugs used (e.g. effects on the release of 5-HIAA by enterochromaffin cells, renal clearance, etc).

The next step was to examine the effects of repeated administration of 5-HT uptake inhibitors. Clomipramine and fluoxetine were chosen for their wide use in psychiatric practice for the treatment of depression, obsessive-compulsive and panic disorders. Two different repeated treatments with fluoxetine decreased whole blood 5-HT, indicating that the platelet high-affinity 5-HT uptake mechanism was effectively inhibited. Despite the 48 h wash-out used in the i.p. treatment, platelet 5-HT was markedly reduced. The slow metabolism of fluoxetine (Caccia *et al.*, 1990) probably accounts for this. However, plasma 5-HT was not increased as in the acute effects. This indicates the presence of adaptive mechanisms leading to a decrease of the circulating extracellular 5-HT. Repeated fluoxetine treatment does not increase extracellular 5-HT in the frontal cortex of rats (Sarkissian *et al.*, 1990), perhaps due to a reduction of brain 5-HT turnover (Fuller *et al.*, 1974). In the periphery, a decreased synthesis by or release from enterochromaffin cells could explain the reduction of 5-HT in the bloodstream after repeated fluoxetine treatment.

Long-term treatment with clomipramine failed to decrease rat whole blood 5-HT, despite achieving a stable plasma CIM concentration (up to 400 nM). In depressive patients, a two-week treatment with CIM (mean plasma levels: 205 nM) elicited a 90% reduction of platelet 5-HT (Sarrias *et al.*, 1987). Clomipramine, unlike other tricyclic antidepressants, has very potent and long-lasting effects on the [$^{3}$H]-imipramine binding and [$^{3}$H]-5-HT uptake in human platelets (Mellerup & Plenge, 1986; Poirier *et al.*, 1984; 1987). However, such long-lasting effects may not occur in rats. Acute CIM increased plasma 5-HT, but this change paralleled closely CIM levels in plasma. Therefore, interspecies differences in the interaction of CIM with the platelet 5-HT uptake transporter *in vivo* seem likely. Errebo *et al.* (1991) have studied in detail the drug-carrier interactions in synaptosomal preparations *in vitro*, revealing drug and species differences of the dissociation times of [$^{3}$H]-imipramine, [$^{3}$H]-paroxetine and [$^{3}$H]-citalopram from the 5-HT transporter. However, the relationship of these differences to the present results is uncertain. In addition, the CIM plasma levels obtained after long-term treatment were higher (up to 2.7 times) than those increasing plasma 5-HT acutely, but no change of this pool was observed. Thus, the triggering of adaptive mechanisms of peripheral 5-HT physiology is likely, as for fluoxetine.

In summary, the present work shows that plasma 5-HT is highly dependent on transient, but not sustained, inhibition of the high-affinity 5-HT uptake. Also, the marked differences in the effects elicited by fluoxetine and clomipramine suggest that their *in vivo* interaction with the rat platelet 5-HT transporter may be clearly distinct. Therefore, caution should be taken in extrapolating to man the effects of certain uptake inhibitors on the rat high-affinity 5-HT uptake.

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# α-Adrenoceptor modulation of the efferent function of capsaicin-sensitive sensory neurones in guinea-pig isolated atria

Sandra Amerini, Annalisa Rubino, Laura Mantelli & Fabrizio Ledda

Department of Pharmacology, University of Florence, Viale G.B. Morgagni 65, 50134 Florence, Italy

**1** Transmural nerve stimulation of guinea-pig atria, obtained from animals pretreated with reserpine (5 mg $kg^{-1}$, i.p.), in the presence of atropine 1 μM and of the β-adrenoceptor blocker CGP 20712A 1 μM, induced a positive inotropic effect which was reduced by the calcitonin gene-related peptide (CGRP) antagonist hCGRP-(8-37) and abolished by pretreatment with capsaicin 1 μM.

**2** Noradrenaline concentration-dependently (0.01 – 10 μM) reduced the increase in cardiac contractility induced by transmural nerve stimulation. The inhibitory effect of noradrenaline was antagonized by yohimbine (0.5 – 1 μM), in a dose-dependent manner. Prazosin (0.5–1 μM) antagonized the effect of noradrenaline and this effect was independent of concentration.

**3** In the presence of yohimbine, the lower part of the inhibitory-response curve for noradrenaline was slightly but significantly shifted by prazosin. A similar degree of antagonism was observed in the presence of 1 μM phenoxybenzamine.

**4** The selective $\alpha_2$ agonists BHT 920 and clonidine reduced, in the same concentration-range (0.01 – 1 μM), the cardiac response to transmural nerve stimulation in a yohimbine-sensitive fashion.

**5** Phenylephrine (0.1 – 100 μM) and methoxamine (1 – 300 μM) also induced an inhibitory effect on transmural nerve stimulation. The effect of phenylephrine was antagonized by yohimbine (1 μM) more efficiently than by prazosin (0.5 μM).

**6** These results are in keeping with the presence of inhibitory prejunctional $\alpha_2$-adrenoceptors on cardiac sensory nerve endings which modulate the efferent function of capsaicin-sensitive neurones.

**Keywords:** Noradrenaline; α-adrenoceptors; capsaicin; sensory neurones; guinea-pig heart

## Introduction

Capsaicin-sensitive sensory neurones are present in the mammalian heart (Papka *et al.*, 1981; Lundberg *et al.*, 1985; Saito *et al.*, 1987). Orthodromic activation of their peripheral terminals by different stimuli, is transmitted to the central nervous system (afferent function); activation of the collaterals of these sensory neurones, induced by electrical stimulation of the mammalian heart (antidromic activation), releases peptides (efferent function; for a review see Maggi & Meli, 1987) such as calcitonin gene-related peptide (CGRP), neurokinin A and substance P (Franco-Cereceda, 1988; Geppetti *et al.*, 1988). CGRP, released from peripheral terminals, produces an inotropic effect, mediated by an increase in the calcium current (Ono *et al.*, 1989) and a rise in cellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels (Ishikawa *et al.*, 1987).

The efferent function of cardiac sensory neurones can be modulated by endogenous substances such as adenosine, prostaglandins, γ-aminobutyric acid, opioids, neuropeptide Y (Giuliani *et al.*, 1989; Maggi *et al.*, 1989; Mantelli *et al.*, 1989; 1990a,b; Rubino *et al.*, 1990). A noradrenergic control of the function of these sensory neurones has been demonstrated in different peripheral preparations. Noradrenaline (NA) and neuropeptide Y, co-stored in sympathetic nerve terminals, and released together by electrical field stimulation (Lundberg *et al.*, 1982; Fried *et al.*, 1985), suppress the non-adrenergic non-cholinergic contractions evoked by antidromic nerve stimulation in guinea-pig bronchi and rat gastric fundus through an action on specific pre-junctional receptors (Grundström *et al.*, 1984; Grundström & Anderson, 1985; Matran *et al.*, 1989; MacDonald *et al.*, 1990).

The influence of NA on the efferent function of capsaicin-sensitive neurones in the heart has not been investigated; therefore in the present study we have evaluated the effect of NA and other adrenergic agonists and antagonists on the response of these neurones to electrical field stimulation in guinea-pig isolated atria.

Correspondence: Dr Sandra Amerini, Department of Pharmacology, University of Florence, Viale G.B. Morgagni 65, 50134 Florence, Italy.

## Methods

The method used has been detailed by Mantelli *et al.* (1989). Isolated atria were obtained from male guinea-pigs (200–300 *g*) pretreated with reserpine (5 mg $kg^{-1}$, i.p.) 24 h beforehand. Atria were mounted vertically in an organ bath containing Tyrode solution of the following composition (mM): NaCl 115, KCl 4.7, $CaCl_2$ 1.8, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25, glucose 10, oxygenated with 95% $O_2$ and 5% $CO_2$, at 30°C. The preparations were stretched until the maximum force of contraction was reached and then maintained at this length throughout the experiment. The isometric contraction was measured with an isometric transducer and a d.c. preamplifer and recorded on a pen recorder (Battaglia Rangoni KV 135) and displayed on a dual beam oscilloscope (Tektronix D 13). The preparations were stimulated at a constant rate (4 Hz; 5V; 0.5 ms) by punctate Ag electrodes connected to a pulse generator (Tektronix type 161 pulse generator). Transmural nerve stimulation was obtained by applying trains of pulses (75–100 mA; 1 ms) through two Pt plates, parallel to the preparations, connected to a second pulse generator (MARB 82/2/200). Trains of two pulses for each of 40 consecutive contractions, were applied during the absolute refractory period to avoid interference with the normal rhythm of contractility. To verify whether or not reserpine pretreatment was successful, a maximally effective concentration of tyramine was tested; tyramine 100 μM induced only a negligible positive inotropic effect ($+ 10.7 \pm 1.2\%$; $n = 30$). Therefore CGP 20712A 1 μM, a $\beta_1$ adrenoceptor selective antagonist (Dooley *et al.*, 1986), was

added to the Tyrode solution to eliminate this residual adrenergic component. Moreover the parasympathetic component of the response to field stimulation was eliminated by the addition to the solution of atropine, 1 μM. After a period of equilibration of at least 60 min, trains of pulses were applied at 15 min intervals. The response to transmural nerve stimulation consisted of a transient inotropic effect and was evaluated as the difference between the maximum force of contraction developed after transmural nerve stimulation and the basal contractility (Δ Fc). The control response to transmural nerve stimulation was taken as 100%; the effects of the agonists tested alone or in the presence of antagonists were evaluated as percentage inhibition or percentage of the control Δ Fc. Since the response to each train remained reproducible for many consecutive tests, it was possible to obtain cumulative dose-response curves for the agonists. Increasing concentrations of the agonists were added to the bathing solution 8 min after the control train and transmural nerve stimulation was then applied again after 7 min contact with the agonists. At the end of the first concentration-effect curve the agonist was washed out and a second curve was constructed in the presence of the selected antagonist. The antagonists were added 30 min before application of the agonist. Generally two concentration-effect curves were obtained in each preparation.

## Drugs

The drugs used in this study were: reserpine (Sigma. The injectable solution was prepared by the University Pharmacy Hospital by dissolving 100 mg reserpine plus 250 mg anhydrous citric acid in 2 ml benzyl alcohol plus 10 ml polysorbate '80' and water for injection to 100 ml), atropine sulphate (BDH), CGP 20712A (2-hydroxy-5-{2-[hydroxy-3-(4-[(1-methyl-4-trifluoromethyl) 1H-imidazol-2-yl-]phenoxy) propyl]amin-oethoxyl}-benzamide; kindly supplied by Ciba Geigy), (±)-noradrenaline hydrochloride, prazosin hydrochloride, yohimbine hydrochloride, tyramine hydrochloride, calcitonin-gene related peptide (all supplied by Sigma), (R)-(−)-phenylephrine hydrochloride (Aldrich), methoxamine hydrochloride (Burroughs Wellcome & Co), BHT 920 ([2-amino-6-allyl-5,6,7,8-tetrahydro-4H-thiazolo, (4,5-d)azepine] hydrochloride, Boehringer Ingelheim), phenoxybenzamine hydrochloride (kindly supplied by S.K. & F), ICI 118551 (erythro- (±)-1-(7-methylindan-4-yloxy) -3-isopropylamino-batan-2-ol; kindly supplied by ICI Pharmaceuticals). Fresh stock solutions were prepared daily in distilled water and subsequently diluted with Tyrode solution to achieve the desired concentration. Prazosin was dissolved in HCl (0.01 N) and 20% ethanol.

### *Statistical analysis of results*

Values were expressed as means ± s.e. of mean. Statistical analysis was performed by Student's *t* test for comparison between two groups of data; analysis of variance followed by Tuckey's test was used when a comparison among three or more groups was made.

## Results

### *Effect of transmural nerve stimulation*

Guinea-pig atria, obtained from reserpine pretreated animals and electrically driven at 4 Hz, developed a basal tension of 453 ± 24 mg ($n = 30$). Transmural nerve stimulation, in the presence of atropine 1 μM and CGP 20712A 1 μM, induced a positive inotropic effect (Figure 1). An early slight increase in contractile force was observed during the application of transmural nerve stimulation which was due to the synchronization of the contraction of the different parts of the

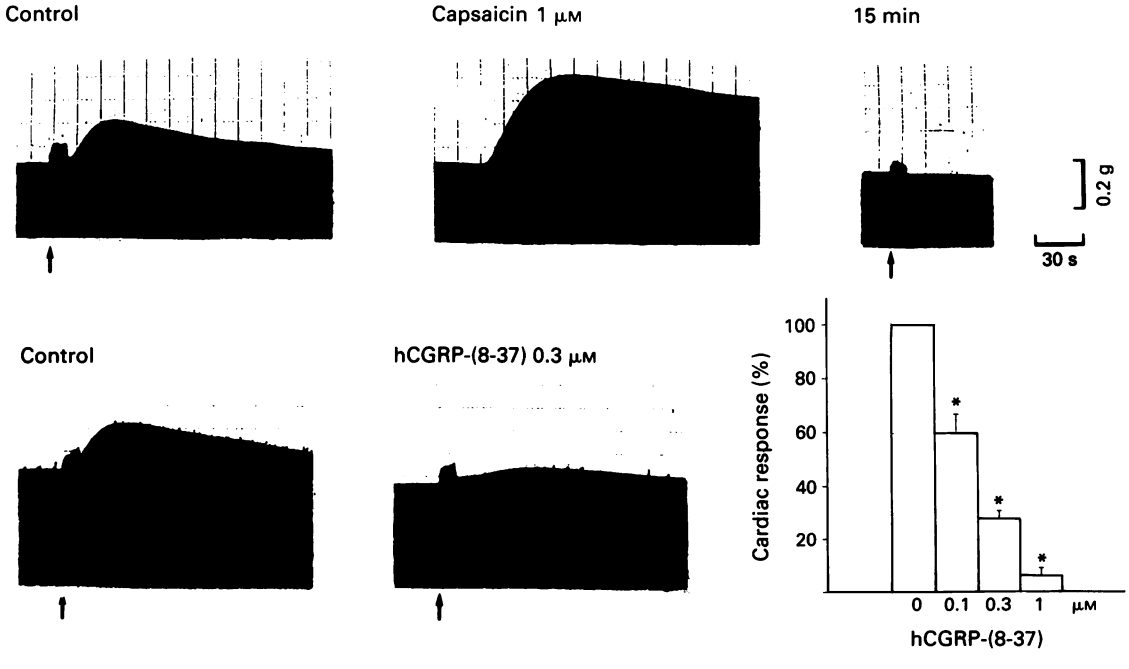


**Figure 1** Upper row: The effect of transmural nerve stimulation on atria obtained from guinea-pigs pretreated with reserpine (5 mg kg$^{-1}$, i.p.), in the presence of atropine 1 μM and CGP 20712A 1 μM, before and after 15 min treatment with capsaicin 1 μM. Lower row: records from a typical experiment showing the response to transmural nerve stimulation before and after treatment with the calcitonin gene-related peptide (CGRP) competitive antagonist hCGRP-(8–37). In these and in the experiments shown in the following figures the arrows indicate the beginning of transmural nerve stimulation that consisted of two field pulses (75–100 mA, 1 ms, 100 Hz) for each of 40 consecutive contractions.

The histograms show the statistical evaluation of the hCGRP-(8-37) antagonistic effect: the response induced by transmural nerve stimulation before drug addition was taken as 100% response. Means of 4 experiments; vertical bars show s.e.mean. *$P < 0.05$ vs control.

preparation, as previously described by Blinks (1966). This phase was followed by an increase in cardiac contractility to about 36% above the control value (615 ± 31 mg, Δ Fc 162 ± 12 mg, $n = 30$), reaching a maximum about 40 s after the beginning of field stimulation, and then declining to control values in about 7 min. This response was constant and reproducible. In the same preparation, 1 μM capsaicin induced a potent inotropic effect (Figure 1) which gradually declined despite the continuous presence of the drug. When the contractility returned to the control value, electrical field stimulation of the same intensity and duration failed to produce any inotropic response.

As shown in the lower part of Figure 1, the inotropic response to transmural nerve stimulation was antagonized in a dose-dependent manner by hCGRP-(8-37), a competitive antagonist of CGRP (Chiba *et al.*, 1989), indicating that the positive inotropic effect induced by field stimulation was due to the release of CGRP from capsaicin-sensitive neurones.

### *Effect of noradrenaline on transmural nerve stimulation*

NA (0.01–10 μM) induced a concentration-dependent reduction in the response to transmural nerve stimulation (Figure 2). In the presence of the $\beta_1$-adrenoceptor antagonist, CGP 20712A (1 μM), NA (0.1 and 1 μM) reduced the response to transmural nerve stimulation in a concentration-dependent manner, leaving the basal contractile tension of the preparations unaffected. The degree of inhibition observed with 1 μM NA was about 50% of the control response. Slight increases in the basal force of contraction were induced only by higher concentrations of the agonist: 3 and 10 μM NA increased the basal tension by 11.7 ± 3.7 and 21.7 ± 4.7% of the control value. NA, at the highest concentration tested (10 μM), reduced the response to transmural nerve stimulation by about 80% of the control value. This degree of inhibition could have been overestimated because of the increase in the basal contractile force induced by the amine. However, CGRP 10 nM, which produced a positive inotropic effect comparable to that induced by transmural nerve stimulation, produced, in the presence of 10 μM NA, a positive inotropic effect insignificantly different from that of untreated preparations (Table 1).

### *Effect of noradrenaline in the presence of α-adrenoceptor antagonists*

Yohimbine (0.5 and 1 μM) significantly reduced the basal contractile tension from 403 ± 24 mg to 331 ± 26 mg ($n = 7$) and 312 ± 14 mg ($n = 8$) respectively but failed to modify the increase in contractility induced by transmural nerve stimulation. The concentration-inhibitory effect curve for NA was significantly shifted to the right in a concentration-dependent and parallel manner by yohimbine 0.5 and 1 μM (Figure 3a).

Prazosin (0.5–1 μM) did not modify either basal contractility or the response induced by transmural nerve stimulation; prazosin 0.5 μM significantly antagonized the inhibitory effect of concentrations of NA up to 3 μM (Figure 3b). Prazosin (up to 1 μM) did not further shift the concentration-inhibitory response curve to NA (Figure 3b). However, prazosin 0.5 and 1 μM completely prevented the direct positive inotropic effect induced by 3 and 10 μM NA (not shown).

Moreover, prazosin (1 μM) significantly shifted, to the right, the lower part of the concentration inhibitory-response curve for NA obtained in the presence of 1 μM yohimbine. At higher concentrations of the agonist (3 and 10 μM) the curve was not affected by the α-adrenoceptor antagonist (Figure 4). Phenoxybenzamine (1 μM), a non competitive antagonist of both α-adrenoceptor subtypes, antagonized the effect of NA comparably to that observed after yohimbine plus prazosin (Figure 4).

To evaluate whether β-adrenoceptors were involved in the inhibitory effect of NA, the effect of the selective $\beta_2$- antagonist, ICI 118551 (Bilski *et al.*, 1983) on the response to transmural nerve stimulation was tested: neither the response to transmural nerve stimulation nor the inhibitory effect of NA were modified by up to 50 nM of this compound (data not shown, $n = 4$).

**Table 1** Changes in contractile tension (mg) induced by calcitonin gene-related peptide (CGRP, 10 nM) in the absence and presence of noradrenaline (10 μM) after 10 min contact

| | *Basal tension* | *CGRP* | *Δ Fc* |
|---|---|---|---|
| Control ($n = 11$) | 406 ± 31 | 587 ± 35 | 190 ± 18 |
| Noradrenaline ($n = 5$) | 492 ± 42 | 673 ± 57 | 181 ± 27 |

The values are expressed as means ± s.e.mean, and the number of experiments is shown in parentheses. The difference from the control value is not statistically significant according to Student's *t* test.

### *Effect of α-adrenoceptor agonists*

More selective agonists for $\alpha_1$ and $\alpha_2$-adrenoceptors, were used to characterize better the receptors involved in the

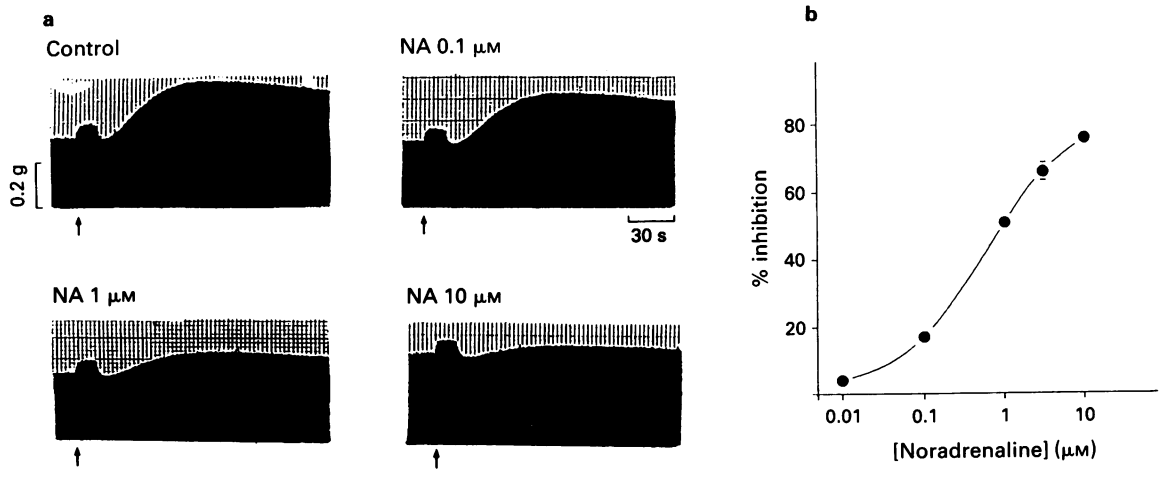


**Figure 2** (a) The inhibitory effect of increasing concentrations of noradrenaline (NA, 0.1–10 μM) on the response to transmural nerve stimulation in guinea-pig atria from reserpine-pretreated animals (5 mg kg$^{-1}$, i.p.), in the presence of atropine 1 μM and CGP 20712A 1 μM. (b) Concentration-inhibitory response curve for NA ($n = 13$). In this and in the following figures each point represents the mean value. Vertical bars indicate standard errors; where not drawn they are included in the symbols.

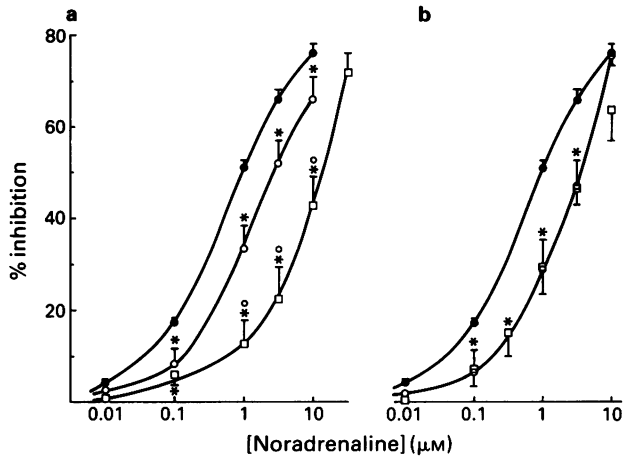


**Figure 3** Concentration-inhibitory response curves for noradrenaline (NA) alone and in the presence of different concentrations of antagonists, in preparations obtained from reserpinized animals (5 mg kg$^{-1}$, i.p.), in the presence of atropine (1 μM) and CGP 20712A (1 μM). (a) (●) NA alone ($n = 13$); (○) NA plus yohimbine (0.5 μM, $n = 6$); (□) NA plus yohimbine (1 μM, $n = 7$). (b) (●) NA ($n = 13$); (○) NA plus prazosin 0.5 μM ($n = 7$), (□) NA plus prazosin 1 μM ($n = 4$). *$P < 0.05$ vs NA alone, ° $P < 0.05$ vs NA plus yohimbine 0.5 μM.

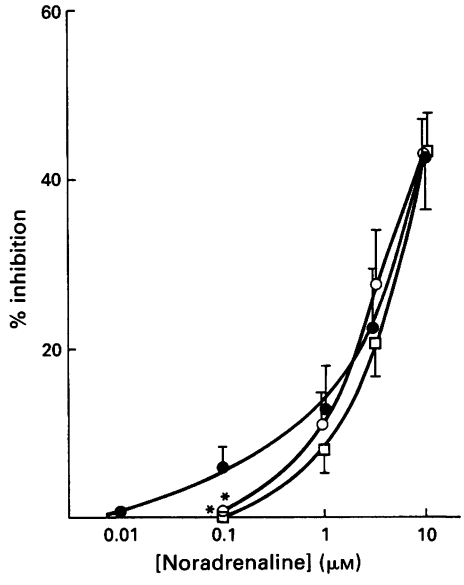


**Figure 4** Concentration-inhibitory response curves for noradrenaline (NA) in the presence of yohimbine 1 μM (●, $n = 7$), NA in the presence of yohimbine (1 μM) plus prazosin (1 μM, ○, $n = 7$) and NA in the presence of phenoxybenzamine 1 μM (□, $n = 4$). Experiments were performed in preparations obtained from reserpinized animals (5 mg kg$^{-1}$, i.p.), in the presence of atropine (1 μM) and CGP 20712A (1 μM). *$P < 0.05$ vs NA plus yohimbine.

inhibitory response to NA. Clonidine and BHT 920 were chosen as predominantly $\alpha_2$- and phenylephrine and methoxamine as predominantly $\alpha_1$-adrenoceptor agonists. Each of the substances tested reduced the response to transmural nerve stimulation to different degrees. BHT 920 and clonidine, at concentrations ranging between 0.01 and 1 μM (Figure 5), which were devoid of any effect on basal cardiac contractility, reduced the cardiac response to transmural nerve stimulation. The inhibitory effects induced by the highest concentration of both the agonists were completely antagonized by yohimbine 1 μM (Figure 5).

Phenylephrine showed an inhibitory effect quantitatively similar to that of clonidine even though higher concentrations (1–100 μM; Figure 6) were required. The highest concentration of the amine also increased the contractile tension by 16.4 ± 3.3%. The cardiac response to transmural nerve stimulation was also reduced by methoxamine (1–300 μM) (Figure 6), which showed no effect on basal cardiac contractility. The selective antagonists prazosin and yohimbine antagonized the inhibitory effect of phenylephrine (Figure 7). As in the case of NA, the concentration-response curve for phenylephrine was significantly shifted to the right more by 1 μM yohimbine than by 0.5 μM prazosin.

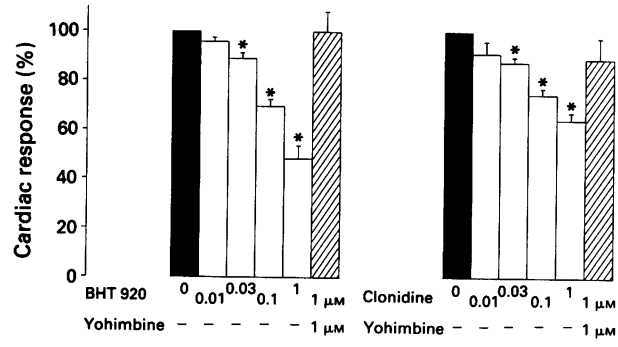


**Figure 5** Effect of BHT 920 and clonidine (0.01–1 μM) alone and in the presence of yohimbine (1 μM) on the cardiac contractile response to transmural nerve stimulation of guinea-pig isolated atria obtained from reserpinized animals (5 mg kg$^{-1}$, i.p.), in the presence of atropine (1 μM) and CGP 20712A (1 μM). Solid columns: control responses taken as 100%; open columns: cardiac responses in the presence of increasing concentrations of BHT 920 and clonidine; hatched columns: antagonistic effect of yohimbine (1 μM) against BHT 920 (1 μM) and clonidine (1 μM). Mean values of at least 6 experiments; s.e.mean shown by vertical bars. *$P < 0.05$ vs control.

## Discussion

The present study confirms that the excitatory response to transmural nerve stimulation is due to CGRP released from capsaicin-sensitive sensory neurones (Saito *et al.*, 1986; Mayauchi *et al.*, 1987; Maggi *et al.*, 1991) and demonstrates that NA, in the presence of a β-adrenoceptor blocking drug (CGP 20712A), reduces the response to transmural nerve stimulation in a concentration-dependent manner. A qualitatively similar inhibitory effect is observed with the α-adrenoceptor agonists, BHT 920, clonidine, phenylephrine and methoxamine.

The inhibitory effect of NA on the response to transmural nerve stimulation is observed both at concentrations (up to

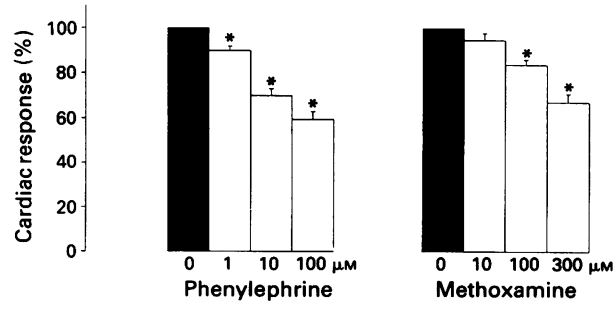


**Figure 6** Effect of phenylephrine (1–100 μM; $n = 14$) and methoxamine (10–300 μM; $n = 5$) on the cardiac contractile response to transmural nerve stimulation in guinea-pig isolated atria obtained from reserpinized animals (5 mg kg$^{-1}$, i.p.), in the presence of atropine (1 μM) and CGP 20712A (1 μM). Solid columns: control responses taken as 100%; open columns: cardiac responses in the presence of increasing concentrations each of phenylephrine and methoxamine. *$P < 0.05$ vs control.

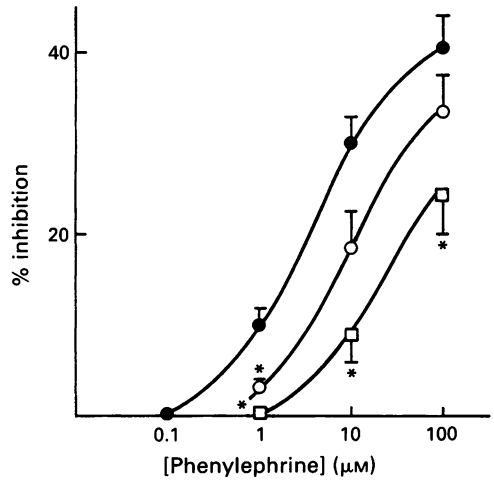


**Figure 7** Inhibitory concentration-response curves for phenylephrine alone (●, $n = 14$), phenylephrine plus prazosin 0.5 μM (○, $n = 10$) and phenylephrine plus yohimbine 1 μM (□, $n = 10$). Experiments were performed in preparations obtained from reserpinized animals (5 mg kg$^{-1}$, i.p.), in the presence of atropine (1 μM) and CGP 20712A (1 μM). *$P < 0.05$ vs phenylephrine alone.

1 μM) devoid of any direct positive inotropic effect and at higher concentrations, which also produce a positive inotropic effect. This latter effect could have led to an overestimation of the inhibitory action of the amine but this seems improbable; exogenous CGRP produces the same inotropic effect both in the absence and in the presence of the highest NA concentration tested, indicating that the preparations, notwithstanding the inotropic effect induced by NA, are still able to develop an increase in contractile tension in response to CGRP. This observation is not surprising since it is conceivable that the increase in contractile tension elicited by the two inotropic agents, NA and CGRP, is due to different transduction mechanisms. In fact, the inotropic effect of CGRP is mediated by an increase in the calcium current due to the activation of adenylate cyclase (Ishikawa *et al.*, 1987; Ono *et al.*, 1989). On the other hand, the effect of the higher concentrations of NA, in our experiments, is due to a stimulation of postjunctional $\alpha_1$-receptors since it is completely prevented by prazosin. $\alpha_1$-Receptor stimulation, in mammalian heart, is associated with an inositol trisphosphate increase (Schmitz *et al.*, 1987; Scholz *et al.*, 1988). In any case, the observation that the inotropic response to exogenous CGRP is unaffected by NA provides indirect evidence for a prejunctional inhibitory action of the amine.

Yohimbine, in spite of its negative inotropic effect, which is probably attributable to an unspecific membrane effect (Rosen *et al.*, 1984), failed to modify the response to transmural nerve stimulation but shifted the inhibitory-response curve for NA to the right in a concentration-dependent manner. This suggests that the inhibitory effect of NA is mediated by prejunctional $\alpha_2$-adrenoceptors. Similarly, in guinea-pig main bronchi and in rat gastric fundus, the non-adrenergic non-cholinergic component of the excitatory response to field stimulation is inhibited by NA and by the $\alpha_2$-adrenoceptor selective agonists, BHT 920 and clonidine (Grunström *et al.*, 1984; Grundström & Andersson, 1985; Matran *et al.*, 1989; MacDonald *et al.*, 1990). This indicates that prejunctional $\alpha_2$-adrenoceptors are present on non-adrenergic, non-cholinergic nerve endings. BHT 920 and clonidine also reduced the cardiac response to transmural nerve stimulation, although they failed to stimulate basal contractility. The inhibitory effects of the highest concentrations of BHT 920 and clonidine were completely reversed by yohimbine; this finding confirms that their action is mediated through $\alpha_2$-adrenoceptor stimulation.

The inhibitory effect of noradrenaline was also slightly antagonized by the predominantly $\alpha_1$-receptor antagonist, prazosin. Prazosin caused a further shift of the lower part of the concentration-inhibitory response curve for NA in the presence of yohimbine. A degree of inhibition similar to that observed with prazosin plus yohimbine was also induced by the mixed $\alpha_1$-, $\alpha_2$-non-competitive antagonist phenoxybenzamine. Furthermore, phenylephrine and methoxamine, predominantly $\alpha_1$-adrenoceptor agonists, also reduced the cardiac response to transmural nerve stimulation. Again, the effect of phenylephrine was antagonized by prazosin, although less efficiently than by yohimbine. Thus, as well as in the cardiac adrenergic terminals of several animal species (Kobinger & Pichler, 1980; Docherty, 1983; Ledda & Mantelli, 1984; Uchida *et al.*, 1984), both $\alpha_1$- and $\alpha_2$-prejunctional receptors are present in cardiac sensory nerve endings. The antagonistic effect of prazosin observed in the present study cannot be taken as strictly indicative of the presence of $\alpha_1$-adrenoceptors. Prazosin binds with a relatively high affinity to [$^3$H]-rauwolscine binding sites in several membrane preparations (Neylon & Summers, 1985; Harrison *et al.*, 1991) including those of rat submandibular gland tissue slices and rat brain slices (Turner *et al.*, 1984; Nasseri & Minneman 1987).

Even if the presence of prejunctional $\alpha_1$-adrenoceptors on the cardiac sensory terminals is acknowledged, they probably represent a small population compared to that of $\alpha_2$-receptors. The finding that the degree of inhibition induced by low (0.5 μM) concentrations of prazosin was not increased by the rise in the concentration of the antagonist, suggests that a small number of $\alpha_1$-receptors are present, which are easily saturatable by prazosin. This hypothesis is confirmed by the observation that prazosin antagonized only the lower part of the concentration-inhibitory effect curve to NA in the presence of yohimbine. This indicates that $\alpha_1$-adrenoceptors may be of functional significance only in the inhibition of the effect of lower NA concentrations.

Finally, differences exist between the inhibitory effects of the imidazolines (BHT 920 and clonidine) and those of the phenylethylamines (noradrenaline and phenylephrine) as $\alpha_2$-adrenoceptor agonists. While the inhibitory effect of the highest concentrations of BHT 920 and clonidine is abolished, that of the highest concentration of NA is only partially reversed by yohimbine, 1 μM. The residual response to NA in the presence of yohimbine is unaffected by prazosin; a degree of antagonism similar to that induced by prazosin plus yohimbine is also observed in the presence of phenoxybenzamine. These observations seem to exclude the involvement of $\alpha_1$-receptors and also of spare $\alpha_2$-receptors in the response to high NA concentrations. The effect of NA on sensory neurotransmission does not seem due to stimulation of inhibitory prejunctional $\beta_1$- or $\beta_2$-adrenoceptors, since a high concentration of CGP 20712A, a $\beta_1$-antagonist, was present in Tyrode solution, and since NA shows the same inhibitory effect in the presence of ICI 118.551, a selective $\beta_2$-antagonist. Indeed the results of the present study appear to be very similar to those obtained in the guinea-pig ileum where the twitch response induced by electrical field stimulation is inhibited by NA and by the $\alpha_2$-agonists, clonidine and UK-14,304-18. Only the effect of the imidazolines however is completely antagonized by $\alpha_2$-blockers, while noradrenaline shows a 'remaining effect' which is insensitive to $\alpha_1$-, $\alpha_2$- or $\beta$-adrenoceptor blockers (Bond *et al.*, 1986a;b). Those effects of NA insensitive to $\alpha$- and $\beta$-blockade are consistently observed in other cardiac preparations, such as rabbit papillary muscle (Schümann, 1980), rat atria (Williamson & Broadley, 1989) and chick atria (Tayo, 1984). It is tempting to speculate that the residual effect of NA is due to the presence of $\beta_3$-adrenoceptors, or 'gamma ($\gamma$) receptors' (Hirst & Neild, 1980; Kaumann, 1989; Zaagsma & Nahorski, 1990).

The present findings confirm that, in reserpine pretreated preparations, electrical field stimulation induces an excitatory response, due to CGRP release, from cardiac sensory neurones, and indicate that NA produces an inhibitory effect on the efferent function of capsaicin-sensitive sensory terminals through stimulation of prejunctional $\alpha_2$-adrenoceptors.

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# Correlation between airway epithelium-induced relaxation of rat aorta in the co-axial bioassay and cyclic nucleotide levels

D.W.P. Hay, R.M. Muccitelli, *C.P. Page & [1]*D. Spina

Department of Pharmacology, L-532, SmithKline Beecham Pharmaceuticals, P.O. Box 1539, King of Prussia, PA 19406-930, U.S.A. and *Department of Pharmacology, King's College London, Manresa Road, London SW3 6LX, U.K.

**1** In co-axial bioassays, in the presence of indomethacin, addition of histamine (100 μM) or methacholine (100 μM) to guinea-pig trachea produced an epithelium-dependent relaxation of precontracted rat aorta which was associated with an approximately 2 fold elevation in tissue levels of guanosine 3′:5′-cyclic monophosphate (cyclic GMP). Removal of the airway epithelium abolished the histamine-induced relaxation of rat aorta and the associated increase in intracellular cyclic GMP.

**2** Epithelium-dependent relaxation was not associated with altered adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels in rat aorta. Unstimulated intact or denuded guinea-pig trachea also did not affect the levels of cyclic AMP or cyclic GMP in rat aorta.

**3** Methylene blue (10 μM) abolished the methacholine-induced, endothelium-derived relaxing factor (EDRF)-mediated rise in intracellular cyclic GMP in rat endothelium-intact aorta alone. In contrast, methylene blue (10 μM) did not affect the methacholine-induced epithelium-dependent rise in intracellular cyclic GMP in rat endothelium-denuded aorta in the co-axial bioassay.

**4** Relaxation of the rat aorta without endothelium was associated with increased levels of cyclic GMP (but not cyclic AMP) in response to sodium nitroprusside (5 nM) and of cyclic AMP (but not cyclic GMP) in response to isoprenaline (1 μM).

**5** These results provide evidence that the postulated epithelium-derived inhibitory factor (EpDIF) may produce relaxation of vascular tissue via elevation in cyclic GMP levels. Furthermore, some data suggest that EpDIF may act by stimulation of the particulate, rather than the soluble form of guanylate cyclase.

**Keywords:** Epithelium-derived inhibitory factor (EpDIF); cyclic GMP; co-axial bioassay; guanylate cyclase

## Introduction

A variety of studies have shown that the sensitivity of airway smooth muscle preparations from various animal species (Barnes *et al.*, 1985; Flavahan *et al.*, 1985; Goldie *et al.*, 1986; Hay *et al.*, 1986) including man (Raeburn *et al.*, 1986; Aizawa *et al.*, 1988; Fernandes *et al.*, 1990; Knight *et al.*, 1990) to several bronchoconstrictor agents is significantly increased by removal of the epithelium. These studies have led to the hypothesis that the epithelium can release an inhibitory factor(s) other than prostaglandins (designated as epithelium-derived inhibitory or relaxing factor, EpDIF/EpDRF; Farmer, 1988; Fedan *et al.*, 1988; Vanhoutte, 1988; Goldie *et al.*, 1990; Raeburn, 1990). The release of a vasoactive EpDIF in response to histamine and methacholine has been detected in several studies using a co-axial bioassay technique (Ilhan & Sahin, 1986; Fernandes *et al.*, 1989; Fernandes & Goldie, 1990; Spina & Page, 1991). Extensive pharmacological assessments of this factor have failed to define its nature (Fernandes & Goldie, 1990; Spina & Page, 1991).

Similarly, the mechanism by which EpDIF produces relaxation of vascular tissue is not known. A number of agents including β-adrenoceptor agonists and forskolin are thought to mediate relaxation by activation of adenylate cyclase resulting in an increase in adenosine 3′:5′ cyclic monophosphate (cyclic AMP) formation (Murray, 1990), while sodium nitroprusside, nitroglycerin and endothelium-derived relaxing factor (EDRF) are thought to mediate relaxation by activating guanylate cyclase and raising the levels of guanosine 3′:5′ cyclic monophosphate (cyclic GMP) (Waldman & Murad, 1987). In an attempt to elucidate the mechanism whereby EpDIF induces relaxation we have investigated whether EpDIF increases the levels of either or both of these two second messenger cyclic nucleotides in a co-axial bioassay. A preliminary account of these studies was presented by Hay *et al.* (1989).

[1] Author for correspondence.

## Methods

### *Tissue preparation*

Male Wistar rats (200–300 g) were killed by stunning and exsanguination and the thoracic aorta removed and spiral preparations (7–8 mm, in length) were prepared. In most of the studies, the endothelium was removed by gentle rubbing of the mucosal surface with a cotton wool coated probe, while in some experiments care was taken not to damage the endothelium. For each experiment, pharmacological verification of the presence or absence of a functional endothelium, as assessed by the presence or absence of relaxation in response to histamine (100 μM) or methacholine (100 μM), was performed. Spiral preparations were suspended under 1 g tension in an organ bath containing Krebs-Henseleit solution aerated with 95% $O_2$ and 5% $CO_2$ at 37°C and equilibrated for 45 min with changes in the bath fluid every 15 min. In all experiments, the Krebs-Henseleit solution also contained the cyclo-oxygenase inhibitor, indomethacin (5 μM) and the β-adrenoceptor antagonist, propranolol (1 μM). Changes in the isometric tension were measured with a Grass force-displacement transducer (FTO3C) and recorded on a multi-channel Grass polygraph. The use of a special drop-away bath chamber enabled spiral strips to be frozen quickly and isometric force was recorded until freezing.

### *Experiments with rat aorta alone*

*Endothelium-intact preparations* Spiral aortic preparations were initially exposed to phenylephrine (0.2 μM) and then

washed 5 times over a 15 min period. After a further 30 min equilibration period some tissues were contracted with phenylephrine (0.05 μM) in control and methylene blue (10 μM; 30 min) pretreated preparations. The contraction was allowed to plateau for 10 min then some tissues were flash-frozen with Wollenberger tongs that had been precooled in liquid $N_2$. In other, paired tissues, 10 min after plateau, methacholine (100 μM) was added to the bath in control and methylene blue-treated preparations for 30 s before the tissues were flash-frozen.

*Endothelium-denuded preparations* Spiral aortic preparations were initially exposed to phenylephrine (0.2 μM) and then washed 5 times over a 15 min period. After a 30 min equilibration period, tissues were contracted with phenylephrine (0.05 μM) and the contraction was allowed to plateau for 10 min; then isoprenaline (1 μM) or sodium nitroprusside (5 nM) was added to the bath for 3 and 2 min, respectively. These times were chosen as initial experiments indicated that the maximum relaxation occurred at these times after addition of the drugs. Paired tissues were flash-frozen either 10 min after phenylephrine addition, or at the appropriate times after addition of isoprenaline or sodium nitroprusside.

### *Co-axial bioassay experiments*

Male Dunkin-Hartley guinea-pigs weighing 450–500 g were killed by cervical dislocation and the trachea removed, dissected free of surrounding tissue and cut into tube segments approximately 7 mm in length. Following initial pharmacological verification of the absence of endothelium, aortic preparations were set-up in co-axial assemblies within guinea-pig tracheal tube segments as previously described (Fernandes *et al.*, 1989).

To examine the time-course of histamine-induced effects, rat aorta surrounded by epithelium-intact guinea-pig trachea were contracted with phenylephrine (0.05 μM) and the tension allowed to plateau for 10 min. Following this, histamine (100 μM) was added to the bath and the tracheal tubes were quickly removed from the aorta and the aorta was flash-frozen 0, 0.5, 1, 4 and 10 min after incubation with histamine.

In other experiments, rat aorta was surrounded with either epithelium-intact or epithelium-denuded guinea-pig trachea. The aorta was contracted with phenylephrine (0.05 μM) and the tension allowed to plateau for 10 min. Histamine (100 μM) was added to some of the baths for 10 min. In appropriate preparations, the tracheal tubes were quickly removed from the aorta and the aorta flash-frozen either just before addition of phenylephrine or histamine (10 min after phenylephrine addition) or 10 min after histamine was added.

In some experiments, rat aorta surrounded by epithelium-intact trachea was contracted with phenylephrine (0.05 μM) in control and methylene blue (10 μM; 30 min) pretreated preparations. Ten minutes after the contractile response had reached plateau, methacholine (100 μM) was added to the bath in control and methylene blue-treated preparations for 10 min. In appropriate preparations, the trachea was removed from the aorta and the aorta flash-frozen just before or 10 min after addition of methacholine (100 μM).

### *Cyclic nucleotide determination*

Frozen tissue was stored at −70°C until required for the determination of vascular cyclic nucleotides. Briefly, 1.5 ml of cold (0°C) 10% trichloroacetic acid, containing approximately 4000 c.p.m. [$^3$H]-cyclic AMP added as a tracer for recovery determinations, was added to a cold ground glass homogenizing tube containing frozen tissue. The tissue was then homogenized with a motor-driven ground glass pestle. Precipitated protein was separated from the soluble extract by centrifugation at 3000 *g* for 20 min at 4°C and the supernatants collected. Trichloroacetic acid was removed from the sample with 5 successive ether extractions (Brooker *et al.*, 1979). Concentrations of cyclic nucleotides in the tissue extracts were determined with commercially available assay kits (DuPont, New England Nuclear, Boston, MA, U.S.A.) after acetylation by the radioimmunoassay method of Brooker *et al.* (1979). Cyclic nucleotide content was corrected for percentage of recovery (70–95%) and expressed as fmol (cyclic GMP) or pmol (cyclic AMP) of cyclic nucleotide per mg of protein. A separate standard curve was run in duplicate with each set of samples.

### *Statistical analysis*

Results expressed as arithmetic mean ± s.e.mean. The magnitude of the relaxation response was expressed as a % of the spasmogen-induced tone. Two-way analysis of variance was used to analyze the effects of methylene blue treatment on EDRF- and EpDIF-induced responses. Kruskal-Wallis one-way analysis of variance by ranks was used to analyze the time course data due to the heterogeneous nature of the variances. Unpaired Student's *t* test was used to analyze differences in means and where multiple comparisons were made, a Bonferroni correction was used (Wallenstein *et al.*, 1980) and considered significant at the 0.05 level.

### *Drugs*

Drugs used in this study were obtained from the following sources: histamine hydrochloride, indomethacin, isoprenaline hydrochloride, methacholine hydrochloride, sodium nitroprusside, propranolol hydrochloride (Sigma). Krebs-Henseleit solution consisted of (mM): NaCl 117.6, KCl 5.4, $NaHCO_3$ 25, $KH_2PO_4$ 1.03, $MgSO_4$ 0.57, D-glucose 11.1 and $CaCl_2$ 2.5. Isoprenaline was dissolved in 0.9% NaCl containing ascorbic acid 20 μg ml$^{-1}$. All other drugs were dissolved in Krebs-Henseleit solution.

## Results

### *Co-axial bioassay*

*Relaxation studies* In the presence of indomethacin, addition of histamine (100 μM; Figure 1) or methacholine (100 μM) to guinea-pig trachea produced an epithelium-dependent reversal of phenylephrine-induced tone in rat aorta (% maximum relaxation ($\%E_{max}$) = 44.0 ± 3.2%, $n$ = 12 and 30.0 ± 6.0%, $n$ = 16, respectively). The time course for relaxation induced by histamine (100 μM) is shown in Figure 2. The histamine-mediated relaxation was abolished by removal of the airway epithelium ($\%E_{max}$ = 6.2 ± 1.3%, $n$ = 12, $P<0.05$ cf. epithelium-intact). Pretreatment of the co-axial bioassay tissue with methylene blue (10 μM) failed to inhibit the methacholine-induced reversal of phenylephrine-induced tone

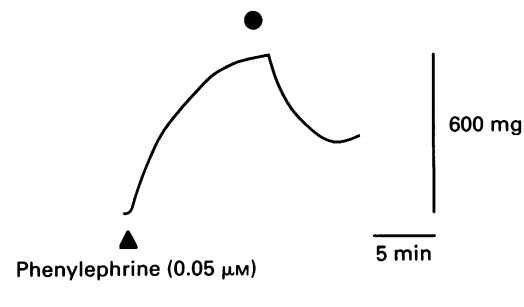


**Figure 1** Response induced by histamine (100 μM; ●) in phenylephrine (0.05 μM; ▲)-precontracted endothelium-denuded spiral-cut aorta of the rat mounted co-axially within the lumen of an epithelium-intact guinea-pig tracheal tube segment.

compared with control (+methylene blue: $\%E_{max} = 20.0 \pm 6.0\%$, $n = 14$; vs −methylene blue: $\%E_{max} = 30.0 \pm 6.0\%$, $n = 16$, $P > 0.05$).

*Cyclic nucleotide levels* Histamine- and methacholine-induced epithelium-dependent reversal of phenylephrine-contracted aorta was associated with a 2.3 and 2.1 fold increase in intracellular levels of cyclic GMP, respectively, in rat aorta smooth muscle (Figures 2 and 3, Table 1). No significant increase in the level of intracellular cyclic AMP was observed (Figures 2 and 3).

Following the addition of histamine (100 μM) significant increases in cyclic GMP but not cyclic AMP occurred after 0.5 min (the first time point measured) and continued over a 10 min experimental period ($P < 0.05$; Figure 2). The elevation in cyclic GMP levels preceded the induction of relaxation by histamine. Removal of the epithelium abolished the histamine-induced elevations in cyclic GMP (Figure 3).

Pretreatment with methylene blue (10 μM) failed to alter the methacholine-induced increase in cyclic GMP (Table 1; $F_{1,59} = 1.2$, $P = 0.6$).

## *Rat aorta alone*

In the presence of endothelium, methacholine (100 μM) produced a 54.0 ± 7.0% ($n = 8$) reversal of phenylephrine-induced tone which was associated with a 27 fold increase in cyclic GMP ($F_{1,28} = 12$, $P = 0.002$, Table 2). Pretreatment of tissues with methylene blue (10 μM) produced a significant inhibition of both the methacholine-induced relaxation ($\%E_{max} = 3.0 \pm 3.0\%$, $n = 8$, $P < 0.05$) and methacholine-induced increase in cyclic GMP (239 ± 93 fmol mg$^{-1}$ protein; $F_{1,28} = 24$, $P < 0.001$, Table 2).

In the absence of endothelium, addition of isoprenaline

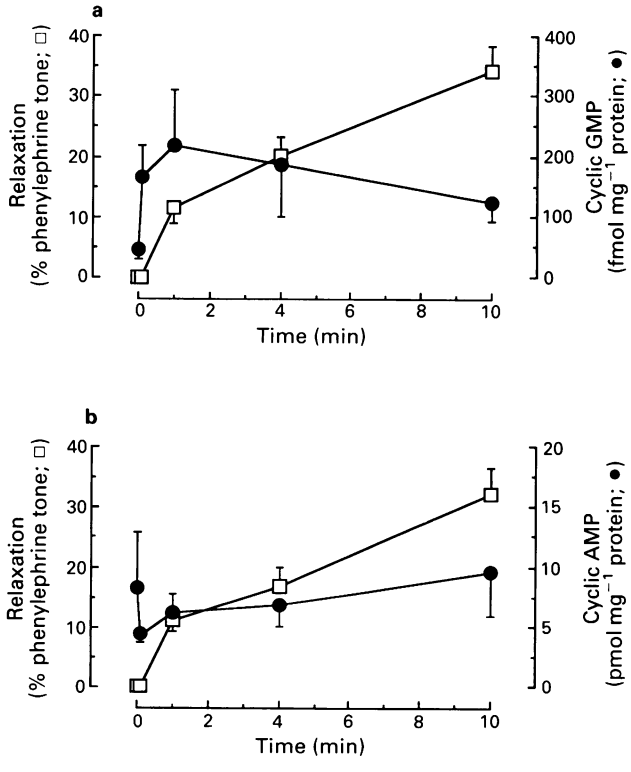


**Figure 2** Time course of the effects of histamine (100 μM) on (a,b) tension (□) and (a) cyclic GMP (●) or (b) cyclic AMP (●) levels in phenylephrine-contracted endothelium-denuded aorta of the rat surrounded by epithelium-intact guinea-pig trachea. Results are expressed as % relaxation (a,b) and cyclic nucleotide levels for cyclic GMP (fmol mg$^{-1}$ protein) (a) and cyclic AMP (pmol mg$^{-1}$ protein) (b). Results are the mean of 7–9 experiments; s.e.mean shown by vertical bars.

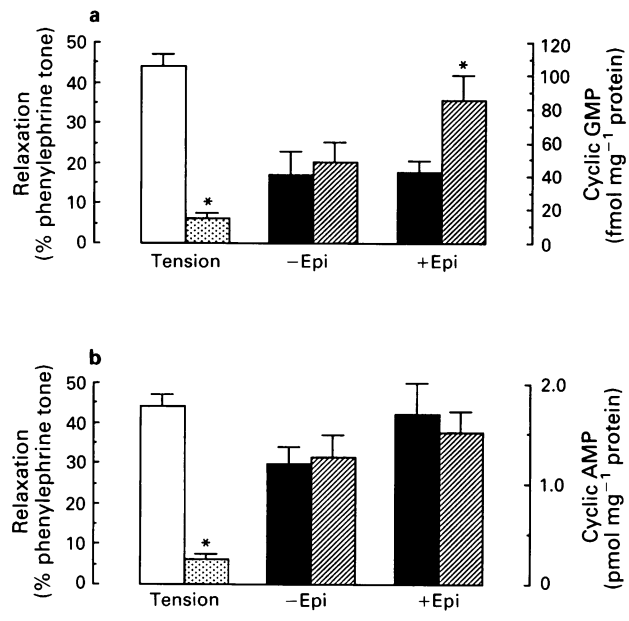


**Figure 3** Effect of histamine (100 μM) on tension (% relaxation; epithelium-intact, open column; epithelium-denuded, stippled column) and on the levels of (a) cyclic GMP (fmol mg$^{-1}$ protein) and (b) cyclic AMP (pmol mg$^{-1}$ protein) just before (solid column) or 10 min after addition of histamine (hatched columns) in phenylephrine-contracted endothelium-denuded aorta of the rat surrounded by epithelium-denuded (−Epi) or epithelium-intact (+Epi) guinea-pig trachea. Results are expressed as mean of 12 experiments; vertical bars shown s.e.mean.

**Table 1** Effect of methylene blue (MB, 10 μM) on methacholine (MCh, 100 μM)-induced, epithelium-dependent increase in cyclic GMP content of rat endothelium-denuded aorta in co-axial bioassay

| *Stimulus* | *Treatment* | *Cyclic GMP* (fmol mg$^{-1}$ protein) | |
|---|---|---|---|
| None (control) | None | 352 ± 87 | 16 |
| None | MB (10 μM) | 152 ± 32 | 15 |
| MCh (100 μM) | None | 746 ± 182* | 16 |
| MCh (100 μM) | MB (10 μM) | 489 ± 168# | 16 |

Results expressed as mean ± s.e.mean of $n$ observations.
Significant increase in cyclic GMP to methacholine ($F_{1,59} = 7.2$, $P = 0.009$).
* Significant increase in cyclic GMP levels in the presence cf. absence (control) of methacholine ($P < 0.01$).
# Significant increase in cyclic GMP levels in the presence cf. absence of methacholine in methylene blue-treated preparations ($P < 0.01$).

**Table 2** Effect of methylene blue (MB, 10 μM) treatment on methacholine (MCh, 100 μM)-induced increase in cyclic GMP content of endothelium-intact rings of rat aorta.

| *Stimulus* | *Treatment* | *Cyclic GMP* (fmol mg$^{-1}$ protein) | |
|---|---|---|---|
| None (control) | None | 325 ± 59 | 8 |
| None | MB (10 μM) | 102 ± 28 | 8 |
| MCh (100 μM) | None | 8842 ± 2534* | 8 |
| MCh (100 μM) | MB (10 μM) | 239 ± 93$^{NS}$ | 8 |

Results expressed as mean ± s.e.mean of $n$ observations.
Significant interaction between methacholine and methylene blue treatment ($F_{1,28} = 10.6$, $P = 0.003$).
* Significant increase in cyclic GMP levels in the presence cf. absence (control) of methacholine ($P < 0.01$).
$^{NS}$ No significant increase in cyclic GMP levels in the presence cf. absence of methacholine in methylene blue treated-preparations ($P < 0.05$).

(Iso, 1 μM) for 3 min produced a 35.0 ± 3.0% ($n = 6$) reversal of tone which was associated with a significant increase in cyclic AMP (control: 2.6 ± 0.5 pmol $mg^{-1}$ protein; +Iso: 6.3 ± 1.0 pmol $mg^{-1}$ protein, $n = 6$, $P < 0.05$) but not in cyclic GMP (control: 265.5 ± 94.3 fmol $mg^{-1}$ protein; +Iso: 300.8 ± 95.9 fmol $mg^{-1}$ protein, $n = 6$, $P > 0.05$). Addition of sodium nitroprusside (SNP, 5 nM) for 2 min produced a 53.3 ± 9.0% ($n = 6$) reversal of tone which was associated with an 8 fold increase in cyclic GMP (control: 22.3 ± 6.4 fmol $mg^{-1}$ protein; +SNP: 184.5 ± 23.4 fmol $mg^{-1}$ protein, $n = 8$, $P < 0.005$). In contrast, SNP-induced relaxation was not associated with changes in cyclic AMP (control: 1.42 ± 0.07 pmol $mg^{-1}$ protein; +SNP: 1.30 ± 0.18 pmol $mg^{-1}$ protein, $n = 6$, $P > 0.05$).

## Discussion

This study indicates that agonist-induced vascular relaxation in co-axial bioassay assemblies mediated by EpDIF (Ilhan & Sahin, 1986; Fernandes *et al.*, 1989; Fernandes & Goldie, 1990) was associated with increases in the levels of intracellular cyclic GMP but not in cyclic AMP. The increase in intracellular cyclic GMP following exposure to histamine was time-dependent, preceded smooth muscle relaxation and was not observed when the epithelium had been removed from the guinea-pig trachea. These data are also consistent with previous studies which examined the role of cyclic nucleotides in mediating EDRF-induced relaxation in blood vessels. Thus, EDRF-induced relaxations of rat aorta (Rapoport & Murad, 1983) and bovine intrapulmonary artery (Ignarro *et al.*, 1984) were associated with elevations in intracellular cyclic GMP but not cyclic AMP, were time-dependent, preceded smooth muscle relaxation and were not observed in endothelium-denuded preparations.

Many studies have shown that the guanylate cyclase inhibitor, methylene blue, inhibits nitrovasodilator- and EDRF-induced relaxation and the associated elevation of intracellular cyclic GMP (Gruetter *et al.*, 1981; Ignarro *et al.*, 1984; Martin *et al.*, 1985). We confirmed that methylene blue inhibited EDRF-induced relaxation and the associated increase in intracellular cyclic GMP. In contrast, the EpDIF-induced relaxation was not inhibited by methylene blue, consistent with earlier observations (Fernandes *et al.*, 1989; Fernandes & Goldie, 1990; Spina & Page, 1991). Furthermore, the EpDIF-induced rise in intracellular cyclic GMP was not inhibited by pretreatment with methylene blue. These findings suggest that the EpDIF and EDRF may activate different populations of guanylate cyclase. It has been demonstrated by various groups that EDRF increases cyclic GMP following activation of the soluble form of guanylate cyclase (Rapoport & Murad, 1983; Ignarro *et al.*, 1984; Martin *et al.*, 1985; Waldman & Murad, 1987). In contrast, a number of studies have demonstrated that atriopeptin, an atrial natriuretic peptide, activates particular guanylate cylase resulting in vascular smooth muscle relaxation and increases in intracellular cyclic GMP. The slightly larger than 2 fold increase in cyclic GMP produced by histamine (44% relaxation) and methacholine (30% relaxation) in endothelium-denuded rat aorta in the co-axial preparation was markedly less than that produced by methacholine (27 fold increase) in endothelium-containing rat aorta alone. However, the EpDIF-induced increase was similar to that reported for atriopeptin which caused a 3 to 10 fold increase in cyclic GMP and a greater than 80% reversal of tone in both rat and rabbit aorta (Waldman *et al.*, 1984; Winquist *et al.*, 1984; Rapoport *et al.*, 1985; Martin *et al.*, 1986). However, EpDIF does not appear to be an atrial naturietic factor since EpDIF (Spina & Page, 1991) unlike atrial naturietic factor (Erdos & Skidgel, 1989) is not metabolised by neutral endopeptidase. The activation of particulate guanylate cyclase by this peptide in rat aorta is not inhibited by methylene blue (Rapoport *et al.*, 1985).

Based on the differential effect of methylene blue on EDRF- and EpDIF-induced relaxation and associated increases in cyclic GMP, in addition to the smaller but similar increase in cyclic GMP levels for EpDIF and atriopeptin compared with endothelium-dependent vasorelaxants, it is therefore possible that EpDIF may activate the particulate rather than the soluble form of guanylate cyclase. However, this must be tempered in light of the finding that methylene blue appears not to be a selective inhibitor of soluble guanylate cyclase and may possibly inhibit EDRF-induced effects via the generation of superoxide anions (Marshall *et al.*, 1988; Wolin *et al.*, 1990). Furthermore, based on the findings that methylene blue may be a source of superoxide anions, these data indicate that EpDIF, unlike EDRF, is not sensitive to oxygen-derived radicals, a finding that has been demonstrated previously (Fernandes *et al.*, 1989; Fernandes & Goldie, 1990; Spina & Page, 1991). Thus, notwithstanding the mechanism of action of methylene blue, the different sensitivity of EpDIF- and EDRF-induced responses to the inhibitory effects of this agent provides evidence that they produce relaxation via different mechanisms.

It has recently been suggested that the epithelium-dependent relaxant responses observed in co-axial bioassay are attributable to hypoxia (Gunn & Piper, 1989a,b). However, these data are inconsistent with this finding, since the hypoxia-induced reversal of tone in rat aorta, unlike the epithelium-dependent relaxation, was not associated with elevations in cyclic GMP (Spina *et al.*, 1991).

The present study suggests that airway EpDIF-induced relaxation of vascular smooth muscle is associated with a selective increase in intracellular levels of cyclic GMP perhaps via stimulation of particulate guanylate cyclase.

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# Synergism between the contractile effect of epidermal growth factor and that of des-Arg$^9$-bradykinin or of α-thrombin in rabbit aortic rings

[1]Denis deBlois, Guy Drapeau, Eric Petitclerc & [2]François Marceau

Centre de recherche de l'Hôtel-Dieu de Québec (Université Laval), Québec P.Q., Canada G1R 2J6

**1** Rabbit aortic rings were used to test the possible contractile effects of growth factors and their interaction with other stimuli. A rapid potentiation of kinin-induced contraction by epidermal growth factor (EGF) has been previously observed in this preparation.

**2** EGF (5–1500 ng ml$^{-1}$) and the isoform BB of platelet-derived growth factor (PDGF-BB; 1–126 ng ml$^{-1}$) exerted modest but sustained contractile effects in rabbit aortic rings.

**3** EGF pretreatment (100 ng ml$^{-1}$) potentiated the contractile responses to des-Arg$^9$-bradykinin (des-Arg$^9$-BK), an agonist of the $B_1$ receptors for kinin found in this preparation, and to human α-thrombin but not to several other contractile stimuli. The interaction appeared also relatively selective for the growth factor, because PDGF-BB pretreatment potentiated neither des-Arg$^9$-BK nor α-thrombin-induced contraction.

**4** EGF, applied on a contraction plateau induced by des-Arg$^9$-BK or α-thrombin, exerted a synergistic contractile effect, with a time course and a half-maximal concentration for EGF-induced contraction similar to the ones recorded in resting tissues (between 67 and 220 ng ml$^{-1}$, depending on the series of experiments).

**5** The direct or synergistic contractile effects of EGF were not modified by the removal of the endothelium or by treatment with indomethacin. However, the tyrosine kinase inhibitors, erbstatin or genistein, inhibited the synergistic effect of EGF with des-Arg$^9$-BK. The small direct contractile effect of EGF was significantly reduced by genistein. The synergistic effect of EGF with α-thrombin was comparatively more resistant to the tested tyrosine kinase inhibitors.

**6** An inhibitor of the catalytic activity of α-thrombin, D-Phe-Pro-Arg-$CH_2Cl$, prevented the contractile effect of α-thrombin in the aortic rings. In this system, a tetradecapeptide derived from a recently cloned α-thrombin receptor was a contractile stimulus at and above 10 μM. Consistent with the hypothesis that this peptide could behave as an α-thrombin receptor agonist, its contractile effect was potentiated by EGF pretreatment. Pharmacological evidence was provided to show that the receptors for α-thrombin were distinct from the $B_1$ receptors for kinins. Together, these findings suggest that a model of a cleavable receptor recently elaborated to account for α-thrombin effects on human platelets is valid in blood-free vascular smooth muscle preparations such as the rabbit isolated aorta.

**7** The synergism between EGF and kinin- or α-thrombin-induced contractions constitutes a novel mode of myotropic action for growth factors. The synergism is probably dependent on the tyrosine kinase activity of receptors for EGF. These combinations of stimuli could occur in various types of vascular disease and account for abnormal vascular reactivity often associated with atheroma lesions or vascular wound healing.

**Keywords:** des-Arg$^9$-bradykinin; $B_1$ receptors for kinins; rabbit aorta; α-thrombin receptor; epidermal growth factor

## Introduction

Epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) are two potent mitogenic principles for cultured vascular cells (Schwartz *et al.*, 1990). Both factors also elicit mechanical responses when applied to isolated blood vessels *in vitro* (reviewed by Berk & Alexander, 1989). These findings have generated interest because adhering platelets present in advanced atheroma lesions can secrete both PDGF and transforming growth factor-α (TGF-α), a material stimulating the same receptors as EGF (Oka & Orth, 1983; Ross, 1986). In addition, activated macrophages within the lesion constitute a potential source of TGF-α (Jonasson *et al.*, 1986; Madtes *et al.*, 1988). Injuries to the vessel wall, such as coronary atherosclerosis or angioplasty performed with a balloon catheter, are accompanied by platelet deposition (Ross, 1986; Cowley *et al.*, 1987) and may be complicated by vasospasm in man (Kalsner & Richards, 1984; Fischell *et al.*, 1988). Thus, the vascular reaction to injuries is a possible application for growth factor-induced vascular contractility.

The mechanisms of the contractile responses to EGF or PDGF are not fully understood and may vary from one vascular model to another. Proposed mechanisms include the increased production of vasoactive prostaglandins (Muramatsu *et al.*, 1985) or the direct activation of membrane signalling mechanisms by the activated receptors for the growth factors. These factors may activate diacylglycerol and inositol triphosphate production and calcium mobilization (Berk & Alexander, 1989), just as other conventional contractile agonists do (Campbell *et al.*, 1985). Stimulation of intracellular signalling pathways by EGF or PDGF is associated with the intrinsic tyrosine kinase activity of the activated growth factor receptors (Ullrich & Schlessinger, 1990).

A possible link between this activity and the phosphatidylinositol cycle is the tyrosine phosphorylation, accompanied with increased activity, of the isoform γ1 of phospholipase C by EGF or PDGF receptors (Nishibe *et al.*, 1990; Goldschmidt-Clermont *et al.*, 1991). This common effector pathway for both EGF- and PDGF-BB-activated receptors constitutes

[1] Present address: Dept. of Pathology SJ-60, School of Medicine, University of Washington, Seattle WA 98195 U.S.A.
[2] Author for correspondence.

the rationale for comparing the vasomotor effects of the growth factors in the present study.

We have previously observed that EGF potentiates the contractile effect of bradykinin (BK) and of des-Arg$^9$-bradykinin in the rabbit isolated aorta (Bouthillier *et al.*, 1987; deBlois *et al.*, 1989). This preparation possesses the $B_1$ type receptors for kinins, which implies that des-Arg$^9$-BK is more potent than BK, and that both peptides are antagonized by the analogue [Leu$^8$]des-Arg$^9$-BK (Regoli & Barabé, 1980). The potentiation of the effect of kinins by EGF was a very rapid event that could be observed within 15 min of exposure to EGF. This phenomenon differs from the slow up-regulation of $B_1$ receptors under the effects of immunological stimuli such as lipopolysaccharide and interleukin-1 (deBlois *et al.*, 1989; 1991). Noradrenaline-induced contractions were not potentiated by EGF (Bouthillier *et al.*, 1987; deBlois *et al.*, 1989), suggesting a possible selectivity of EGF for the contractile stimulus. This observation may indicate a novel mechanism for growth factor-induced vasoconstriction: the potentiation of the contractile responses to other specific agents.

One of the objectives of the present study was to determine the selectivity of the interaction between kinins and EGF in the rabbit aortic preparation. A number of other stimuli have been screened and we have also tried to substitute the growth factor PDGF-BB for EGF. The isoform BB of PDGF was used because it does not discriminate between the various receptor subtypes for this growth factor (Seifert *et al.*, 1989). Possible small direct contractile effects of the growth factors were also investigated. The role of the tyrosine kinase activity of the EGF receptor has been evaluated by use of selective inhibitory drugs for this enzyme activity. Other possible mechanisms of EGF action involving the endothelium or the arachidonate cascade were also examined.

As the contractile effect of α-thrombin was also shown to be potentiated by EGF, new concepts on thrombin pharmacology have been verified. α-Thrombin is a known contractile stimulus of the rabbit aortic preparation (Haver & Namm, 1984), and a catalytically active protease is required for this action. A cleavable receptor for α-thrombin from megakaryocyte-like cell lines has been cloned and sequenced recently (Vu *et al.*, 1991). This receptor is a member of the rhodopsin superfamily of signalling proteins. It was proposed that a typical thrombin cleavage site present in the N-terminal region of the chain would expose a 'new amino terminus' (NAT) domain that would act as a 'tethered' agonist of the receptor. Vu *et al.* (1991) tested this model by showing that a synthetic tetradecapeptide ($NAT_{14}$) corresponding to the NAT domain was an agonist of the cellular receptor for α-thrombin in human platelets and in *Xenopus* oocytes expressing the cloned receptor. In the present experiments, we used $NAT_{14}$ to test whether it would behave as a contractile agonist in a blood-free vascular bioassay and whether the relatively selective potentiation of α-thrombin effects by EGF would apply to this oligopeptide.

## Methods

### *Pharmacological preparation*

The thoracic aorta was isolated from New Zealand White rabbits of either sex (1.5–2 kg). The vessels were cut into rings (2–3 mm width), suspended between a metal hook and a thread loop under a basal tension of 2 g in 5 ml organ chambers containing oxygenated (95% $O_2$: 5% $CO_2$) and warmed (37°C) Krebs solution (composition as in Marceau *et al.*, 1991). Their responses to agents were isometrically recorded as described previously (Bouthillier *et al.*, 1987).

### *Protocols*

The direct contractile effects of EGF and PDGF-BB were verified on tissues equilibrated for 1 h. Single concentration or a cumulative scale of concentrations (cumulative concentration-effect curve) were applied.

Various agents were screened for potentiation of their contractile effects by EGF (100 ng ml$^{-1}$). Several agents known to contract the rabbit aortic preparation were applied approximately at half-maximal concentrations, except for des-Arg$^9$-BK which was applied at a nearly maximal concentration (1.7 μM) and for α-thrombin, which was applied at two concentration levels. The effect of EGF on the whole concentration-effect curve to this kinin has been described previously (Bouthillier *et al.*, 1987; deBlois *et al.*, 1989). The screening of agonists was performed on control tissues or tissues continuously exposed to EGF (100 ng ml$^{-1}$). After 1 h of equilibration, up to 4 agonists were injected at 90 min intervals and following a random sequence. An exception was des-Arg$^9$-BK, which was injected at the time 6 h, because the maximal effect of this agent changes considerably as a function of the incubation time. The mechanism for the increase of tissue response to $B_1$ receptor agonists has been studied previously (Bouthillier *et al.*, 1987; deBlois *et al.*, 1988; 1989; 1991).

Cumulative concentration-effect curves for EGF were obtained after 4.5 h of tissue incubation *in vitro* in aortic rings precontracted or not with des-Arg$^9$-BK. The kinin was applied at a threshold (1.7 nM) or a maximal (1.7 μM) concentration. Tissues from the same aorta and stimulated with des-Arg$^9$-BK alone were used as controls to evaluate quantitatively the stability of the kinin-induced plateau of contraction over time (control plateau). The rate of tone loss from control plateaus, expressed as a percentage over the period required for the completion of the concentration-effect curve for EGF, was used to determine the baseline upon which the contraction to EGF were superimposed in the paired aortic ring. Similar experiments were conducted with another contractile agonist potentiated by EGF, α-thrombin.

The direct or synergistic contractile responses of growth factors and other agonists were analyzed in terms of dependence on tyrosine kinase activity, on arachidonate cascade activation, or on the presence of endothelium. Some tissues were treated for 1 h before stimulation with the tyrosine kinase inhibitors, erbstatin (Imoto *et al.*, 1987), genistein (Akiyama *et al.*, 1987) or tyrphostin-51 (Gazit *et al.*, 1989), or with the cyclo-oxygenase blocker, indomethacin. Because erbstatin was not available in large quantities, several tissues were grouped together and exposed for 1 h to this inhibitor in a small volume of Krebs solution. The tissues were then mounted separately in tissue baths and allowed to equilibrate for an additional hour before recording contractility. In some experiments, the endothelial lining of the aortic rings was removed by gently rubbing the intimal surface with a round wooden stick; the loss of acetylcholine-induced relaxation was monitored as an indication of a successful procedure as endothelial cells are necessary for this response in the rabbit isolated aorta (Furchgott & Zawadzki, 1980).

As α-thrombin interactions with EGF were found to be important, the oligopeptide $NAT_{14}$, which behaves as a thrombin receptor agonist on human platelets (Vu *et al.*, 1991), was tested as a contractile agonist in the isolated aortic rings in combination or not with EGF. The requirement for a proteolytically active form of α-thrombin for the myotropic effects was verified with an irreversible inhibitor of the enzyme.

Finally, pharmacological criteria have been used to show that receptors for α-thrombin and $B_1$ receptors for kinins are distinct pharmacological entities.

### *Drugs*

Des-Arg$^9$-BK was purchased from Bachem (Torrance, CA, U.S.A.). Epidermal growth factor (EGF; receptor grade, from mouse submaxillary glands) was from Sigma Chemicals (St-Louis, MO, U.S.A.), as well as cycloheximide, [Leu$^8$]des-Arg$^9$-BK, (−)-noradrenaline, 5-hydroxytryptamine (creati-

nine sulphate complex), histamine dihydrochloride, indomethacin, angiotensin II and phorbol 12-myristate 13-acetate (PMA). Human recombinant interleukin-1β was a gift from Biogen S.A. (Geneva, Switzerland). Highly purified human α-thrombin (2863 NIH units per mg) was purchased from Calbiochem (La Jolla, CA, U.S.A.). Human recombinant PDGF (BB isoform, produced in yeast) was a gift from Abbott Laboratories. Erbstatin (Institute of Microbial Chemistry, Tokyo, Japan) was purified from *Streptomyces*. Tyrphostin-51, one of the most potent available inhibitors of the tyrosine kinase activity of EGF receptors (Gazit *et al.*, 1989), was purchased from Biomol (Plymouth Meeting, PA, U.S.A.). D-Phe-Pro-Arg-$CH_2Cl$ (PPACK), an irreversible blocker of the protease activity of α-thrombin (Kettner & Shaw, 1979), and genistein, a tyrosine kinase inhibitor (Akiyama *et al.*, 1987) were purchased from Calbiochem.

The tetradecapeptide $NAT_{14}$ (H-Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe-OH) was synthesized and purified in our laboratory by use of a solid-phase method (general methods outlined by Drapeau & Regoli, 1988). Analytical high performance liquid chromatography (h.p.l.c.) profile and mass spectroscopy indicated a pure compound of the expected molecular weight (1740 Da).

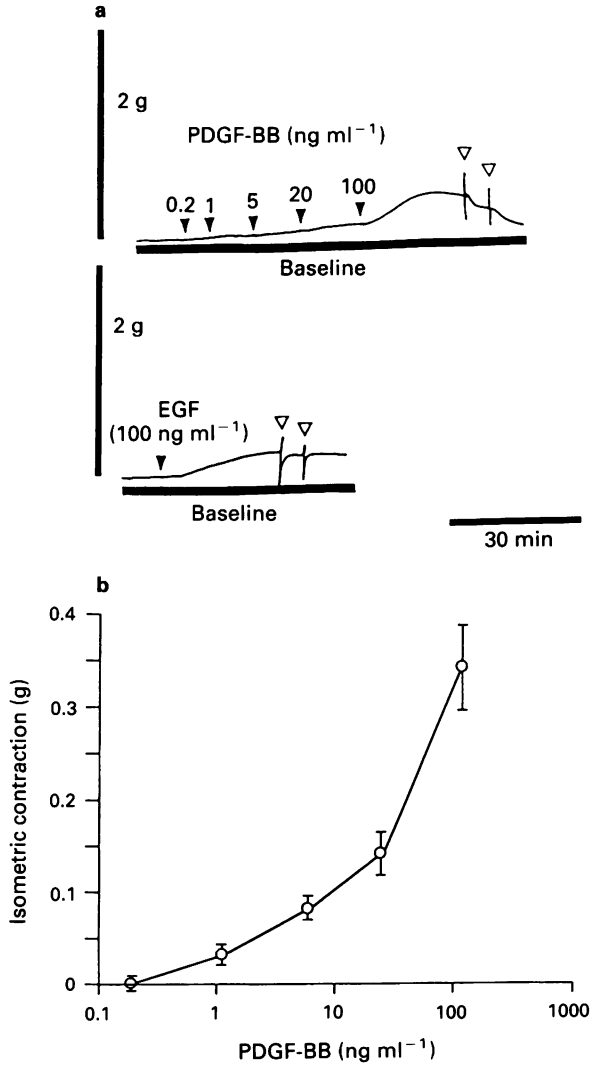


**Figure 1** Contractile effect of the isoform BB of platelet-derived growth factor (PDGF-BB) and epidermal growth factor (EGF) on rabbit aortic rings. Vascular tissues were stimulated after 1 h of *in vitro* equilibration. (a) Tracing showing the building of a cumulative concentration-effect curve for PDGF (0.2–100 ng $ml^{-1}$) and the time course of the development of a contraction induced by a single concentration of EGF (100 ng $ml^{-1}$). Abscissa scale: time (min). Ordinate scale: isometric contraction (g). Closed symbols indicate the application of agents and open symbols the washout of stimulants. (b) Concentration-effect curve for PDGF-BB. Results are the means of 11 determinations; s.e. shown by vertical bars

## Results

### *Direct contractile effects of growth factors*

Both EGF and PDGF elicited contractile responses in rabbit aortic rings (Figure 1), but these responses were comparatively very slow to develop and of modest amplitude when compared with many other vasoactive agents. The contractile response to a single application of EGF (100 ng $ml^{-1}$) was $0.19 \pm 0.045$ g ($n = 6$). The effect of PDGF was concentration-dependent in the range of concentration 1–126 ng $ml^{-1}$ (Figure 1b).

### *Screening of various agonists for synergism with EGF*

Various contractile agents effective in the rabbit aortic preparations were tested for potentiation by EGF. Responses of tissues continuously exposed to EGF 100 ng $ml^{-1}$, were compared to control tissues from the same animals. As found previously, (Bouthillier *et al.*, 1987), exposure to EGF increased the contractile effect of des-$Arg^9$-BK (at 1.7 μM: from $1.75 \pm 0.26$ g to $3.90 \pm 0.90$ g, $n = 4$, $P < 0.001$ by Student's $t$ test), but not that of noradrenaline (at 100 nM: $1.95 \pm 0.11$ g without EGF; $1.84 \pm 0.43$ g with EGF, $n = 8$). Other stimuli, the effects of which were not significantly modified by EGF treatments, were angiotensin II (2 nM), histamine (10 μM), 5-hydroxytryptamine (400 nM), KCl (30 mM) and PMA (700 ng $ml^{-1}$) (not shown). Human α-thrombin (6 or 60 nM) was the other contractile agent besides des-$Arg^9$-BK that was significantly potentiated by the EGF treatment. Responses to thrombin at 6 nM were $0.46 \pm 0.05$ without EGF, and $0.64 \pm 0.06$ g in the presence of EGF ($n = 6$, $P < 0.01$ by Student's $t$ test). With 60 nM thrombin, contractile responses of $1.02 \pm 0.10$ g were recorded in control tissues, and of $1.50 \pm 0.09$ g in EGF-treated tissues ($n = 6$, $P < 0.01$). In this series of experiments, the baseline tension presumably included the direct contractile effect of EGF, as the growth factor was continuously present.

The agents potentiated by EGF, des-$Arg^9$-BK and α-thrombin, were not significantly potentiated in tissues continuously exposed to PDGF-BB (Table 1), indicating a relative selectivity for the growth factor in this system. The concentration of PDGF used in these experiments was biologically active in the rabbit isolated aorta (Figure 1b).

### *Concentration-effect relationship for EGF*

The cumulative concentration-effect curve for the contractile effect of EGF has been established with resting tissues and also with tissues precontracted with either des-$Arg^9$-BK or α-thrombin (Figures 2–4). The responses attributed to EGF were subtracted from contraction plateaus induced by des-$Arg^9$-BK (1.7 nM or 1.7 μM) or by α-thrombin (6 or 60 nM) and were plotted in Figures 3 and 4, respectively. The small contractile effect of EGF was increased significantly in tissues pretreated with the higher concentration level of either stimulus (Figures 3a and 4a), but the time course of the contraction was similar, as seen from the tracings (Figure 2). It can be seen that the amplitude of the EGF-induced contraction increased concentration-dependently in the presence of either des-$Arg^9$-BK or α-thrombin (Figures 3a, 4a), but when these data were plotted as a percentage of maximal EGF-induced effects, the half-maximal concentration of EGF did not change significantly when the other contractile stimulus was present (approximately 220 ng $ml^{-1}$ in the series of experiments with des-$Arg^9$-BK and 70 ng $ml^{-1}$ in the thrombin series; Figures 3b, 4b).

**Table 1** Lack of effect of PDGF-BB on contractions of the rabbit aorta induced by a kinin or α-thrombin receptor agonist

| *Contractile agent* (concentration) | | *n* | *Isometric contraction* (g)[a] *Control* | *PDGF-BB* (100 ng ml$^{-1}$) |
|---|---|---|---|---|
| α-Thrombin | (6 nM) | 9 | 0.17 ± 0.036 | 0.20 ± 0.02 |
| | (60 nM) | 5 | 1.11 ± 0.09 | 1.33 ± 0.09 |
| des-Arg$^9$-bradykinin | (1.7 μM) | 5–6 | 1.44 ± 0.37 | 1.48 ± 0.44 |

[a]Results are the means ± s.e. of the number of determinations indicated by *n*. PDGF-BB-treated tissues and controls were exposed to α-thrombin at 1 or 1.5 h of incubation, and to des-Arg$^9$-BK at 6 h. No value was significantly different from control as calculated by Student's *t* test.

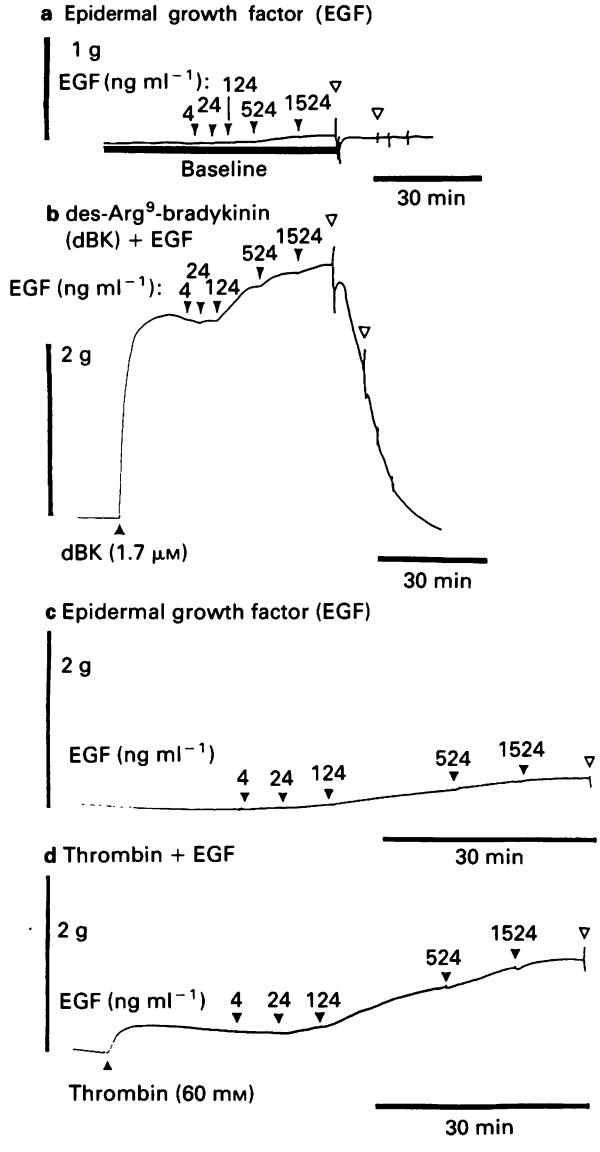


**Figure 2** Tracings illustrating the construction of a cumulative concentration-effect curve for the contractile effect of epidermal growth factor (EGF) on rabbit aortic rings. The preparation was resting (tracings a,c) or precontracted with des-Arg$^9$-BK (1.7 μM; tracing b) or with α-thrombin (60 nM, tracing d). Each pair of tissues was derived from the same animal. Abscissa scale: time (min). Ordinate scale: isometric contraction(g). Closed symbols indicate the application of agents and open symbols the washout of stimulants. The cumulative concentration (ng ml$^{-1}$) of EGF is indicated

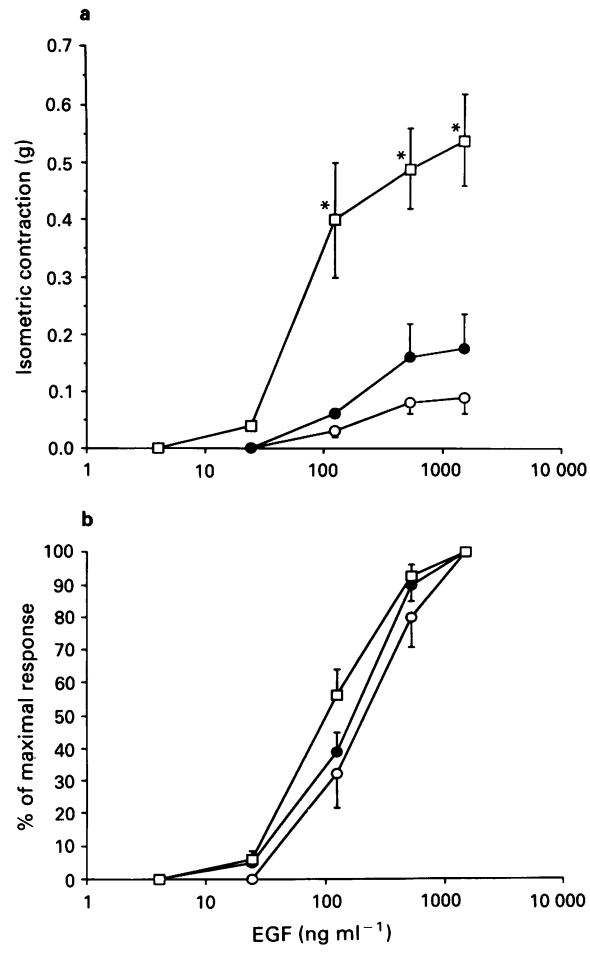


**Figure 3** Effect of des-Arg$^9$-BK pre-stimulation on the contractile responses to epidermal growth factor (EGF) in rabbit aortic rings. (a) EGF concentration-effect curves expressed as grams of developed tension in control preparations (○) or in tissues precontracted with des-Arg$^9$-BK (1.7 nM, ●; 1.7 μM, □). (b) The same data presented as percentage of the maximal EGF-induced effect. In each case, the contraction plateau induced by des-Arg$^9$-BK was subtracted, as described in Methods. The peak contractile responses averaged 0.006 ± 0.005 g for the 1.7 nM concentration of des-Arg$^9$-BK ($n = 9$), and 1.20 ± 0.22 g at 1.7 μM of the peptide. Results are the mean of 5–9 determinations; vertical bars show s.e.. In (a), the contractile responses at each concentration of EGF were compared by one-way analysis of variance, followed by Dunnett's test. Significant differences from the control responses are indicated by $*P < 0.05$

### *Mechanism of EGF direct or synergistic effects*

The synergism between EGF (100 ng ml$^{-1}$) and des-Arg$^9$-BK appears to be dependent on the tyrosine kinase activity of the EGF receptors, as suggested by the inhibitory effect of erbstatin and genistein (Table 2). The same drugs, plus tyrphostin-51, another tyrosine kinase inhibitor, partially decreased the potentiating effect of EGF on α-thrombin-induced contraction (to a non-significant level for erbstatin and tyrphostin-51; Table 2), but this system was more resistant than the EGF-des-Arg$^9$-BK synergy to the action of these metabolic inhibitors. Erbstatin significantly inhibited the direct contrac-

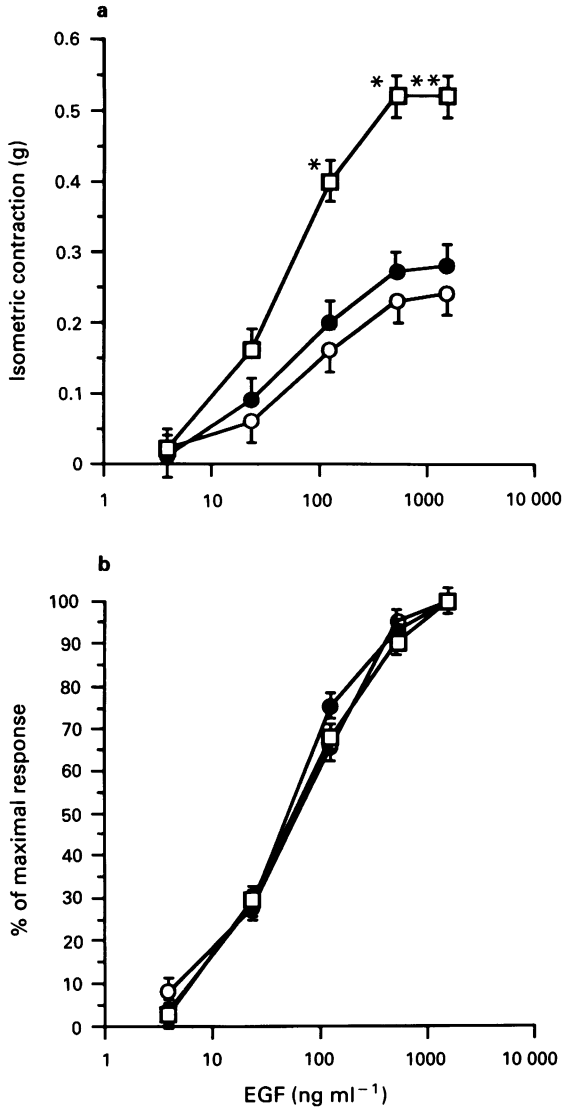


**Figure 4** Effect of α-thrombin pre-stimulation on the contractile responses to epidermal growth factor (EGF) in rabbit aortic ring. (a) EGF concentration-effect curves expressed as grams of developed tension in control preparations (○) or in tissues precontracted with thrombin (6 nM, ●; 60 nM, □). (b) The same data presented as percentage of the maximal EGF-induced effect. In each case, the contraction plateau induced by α-thrombin was subtracted, as described in Methods. The peak contractile responses averaged 0.37 ± 0.08 g for the 6 nM concentration of α-thrombin ($n = 5$), and 0.86 ± 0.12 g at 60 nM of the protease. Results are the mean of 5–10 determinations; vertical bars show s.e. In (a), the contractile responses at each concentration of EGF were compared by one-way analysis of variance, followed by Dunnett's test. Significant differences from the control responses are indicated by $*P<0.01$; $**P<0.001$

tile response to PDGF (100 ng ml$^{-1}$: 0.22 ± 0.04 g of contraction in controls, 0.09 ± 0.02 g in drug-treated tissues, $n = 16$, $P<0.01$ by Student's $t$ test). However, erbstatin exerted no significant effect against the direct effect of EGF (100 ng ml$^{-1}$: 0.08 ± 0.01 g in controls, 0.07 ± 0.03 g in drug-treated tissues, $n = 16$). Tyrphostin-51 also failed to modify the small contractile effect of EGF (not shown), and only genistein was active in this respect (0.12 ± 0.02 g in controls, 0.06 ± 0.02 g in drug-treated tissues, $n = 11$, $P<0.01$). The contractions induced by des-Arg$^9$-BK (Table 2), α-thrombin (Table 2) or noradrenaline (not shown) were not affected by the tyrosine kinase inhibitors, suggesting the selectivity of these drugs for effects related to growth factors.

Treatment of aortic rings with the cyclo-oxygenase inhibitor, indomethacin, failed to inhibit either the synergistic effects of EGF (Table 2) or its direct contractile effect (0.13 ± 0.03 g in controls, 0.17 ± 0.05 g in drug-treated tissues, $n = 15$). The removal of the endothelium from the aortic rings also failed to modify the effects of EGF (Table 2).

### *Effect of α-thrombin on vascular smooth muscle*

The concentration-effect relationship for the contractile action of human α-thrombin on the rabbit aortic preparation has been described elsewhere (Haver & Namm, 1984). We established a cumulative concentration-effect curve for $NAT_{14}$, which was found to be active at and above 10 μM (Figure 5). The contraction induced by $NAT_{14}$ exhibited a temporal profile similar to the one elicited by α-thrombin. The $NAT_{14}$ contractile effect was not tachyphylactic when stimulations were applied at 20–30 min intervals and the contraction amplitude did not vary as a function of incubation time *in vitro* (not shown). A partial tachyphylaxis has been reported when α-thrombin was repeatedly applied to this preparation (Haver & Namm, 1984). The $NAT_{14}$-induced contraction (100 μM) was significantly potentiated by EGF (100 ng ml$^{-1}$): 0.94 ± 0.20 g of contraction was recorded in EGF-treated tissues, as compared to 0.51 ± 0.12 in paired controls ($n = 8$; $P<0.01$ by Student's $t$ test). This is in agreement with the hypothesis that the peptide is a α-thrombin receptor agonist.

Although α-thrombin and kinins that are agonist for $B_1$ receptors share the capability of contracting the aortic rings in synergism with EGF, they probably do not stimulate the same population of receptors. This conclusion is supported by several experimental approaches (Table 3). The inhibitor of α-thrombin catalytic activity, PPACK, prevented completely the contractile effect of α-thrombin (6 nM) without influencing that of des-Arg$^9$-BK (1.7 μM). Incidentally, PPACK could also partially relax a tissue precontracted with α-thrombin, indicating the need for a continuous proteolytic action for the maintenance of a contraction plateau (Figure 5b). PPACK had no effect on contractions induced by $NAT_{14}$ at 100 μM (Figure 5b; statistical analysis: contraction of 0.59 ± 0.10 in tissues pretreated with PPACK, compared to a control of 0.51 ± 0.12, $n = 8$).

The competitive agonist of $B_1$ receptors, [Leu$^8$]des-Arg$^9$-BK (Regoli & Barabé, 1980), did not inhibit α-thrombin-induced contraction (Table 3). In addition, two types of treatment that increase the level of response to $B_1$ agonists over several hours, namely pulse exposure to cycloheximide or to interleukin-1β (deBlois *et al.*, 1991), failed to modify the contractile effect of α-thrombin.

## Discussion

In this study, we investigated the possible interactions between the growth factors EGF or PDGF-BB and other stimuli in the regulation of vascular smooth muscle tone. Our data indicate a novel mode of myotropic action for EGF and, possibly, other growth factors: the potentiation of the contractile responses to other specific agents. When applied alone, EGF and PDGF-BB exerted only modest direct contractile effects on the rabbit aortic preparation. The temporal profile of growth factor-induced contraction consisted of a relatively slow onset, a sustained plateau and a slow relaxation after washing. All these features were observed previously on the rat isolated aorta (Berk *et al.*, 1985; Berk & Alexander, 1989). The concentrations of EGF needed to contract the rabbit aorta are similar to those needed to induce contraction in the rat aortic preparation (Berk *et al.*, 1985) or proliferation in calf aortic smooth muscle (Bhargava *et al.*, 1979). The concentrations of PDGF-BB needed to elicit a contractile response in the rabbit aorta are somewhat higher than those reportedly active in contracting the rat

**Table 2** Studies on the mechanism of epidermal growth factor (EGF) potentiation of des-Arg$^9$-BK-induced or of α-thrombin-induced contraction in rabbit aortic rings

| | *Response to des-Arg$^9$-BK*, 1.7 μM (g) | | *Response to α-thrombin*[b] (g) | |
|---|---|---|---|---|
| *Treatment*[a] | *Paired control without EGF* | *EGF* (100 ng ml$^{-1}$) | *Paired control without EGF* | *EGF* (100 ng ml$^{-1}$) |
| *Experiment A* | | | | |
| Control | 1.08 ± 0.15 (12)[c] | 1.59 ± 0.20 (12)**[d] | 0.46 ± 0.05 (9) | 0.64 ± 0.05 (9)** |
| Erbstatin (5 μg ml$^{-1}$) | 0.82 ± 0.13 (12) | 0.95 ± 0.19 (12) | 0.36 ± 0.05 (9) | 0.51 ± 0.07 (9) |
| *Experiment B* | | | | |
| Control | 1.47 ± 0.24 (6) | 2.31 ± 0.25 (6)* | 0.64 ± 0.18 (5) | 1.00 ± 0.21 (5)** |
| Genistein (50 μg ml$^{-1}$) | 1.01 ± 0.14 (6) | 0.97 ± 0.09 (6) | 0.61 ± 0.10 (5) | 0.87 ± 0.16 (5)* |
| *Experiment C* | | | | |
| Control | NT[e] | NT | 1.26 ± 0.14 (5) | 1.70 ± 0.21 (5)* |
| Tyrphostin-51 (5 μM) | NT | NT | 1.20 ± 0.08 (5) | 1.57 ± 0.21 (5) |
| *Experiment D* | | | | |
| Control | 1.19 ± 0.29 (10) | 1.63 ± 0.38 (10)* | 1.04 ± 0.12 (5) | 1.53 ± 0.11 (5)* |
| Indomethacin (2.8 μM) | 1.13 ± 0.26 (10) | 1.49 ± 0.33 (10)* | 0.95 ± 0.05 (5) | 1.54 ± 0.17 (5)* |
| *Experiment E* | | | | |
| Endothelium removal | 2.16 ± 0.55 (4) | 3.07 ± 0.14 (4)** | 1.60 ± 0.11 (9) | 1.87 ± 0.15 (9)* |

[a]EGF was applied 15 min before the application of the major contractile stimulus, des-Arg$^9$-BK (applied in tissues preincubated for 6 h) or thrombin (applied either at 6 or 60 nM in tissues preincubated for 1.5 h).
Tyrosine kinase inhibitors (erbstatin, genistein or tyrphostin-51) were applied 1 h before stimulation with the contractile agent and indomethacin, 30 min before. Tissues with the endothelium removed did not relax when acetylcholine (100 nM) was applied on a contraction induced by noradrenaline or phenylephrine (not shown).
[b]Thrombin concentration was 6 nM in experiment A and 60 nM in the other ones.
[c]Values are the means ± s.e.mean of the number of determinations indicated in parentheses.
[d]EGF potentiation was evaluated by comparing the values from EGF-treated tissues from the paired controls using Student's *t* test for paired data. Levels of significance: $*P<0.05$; $**P<0.01$.
[e]NT, not tested.

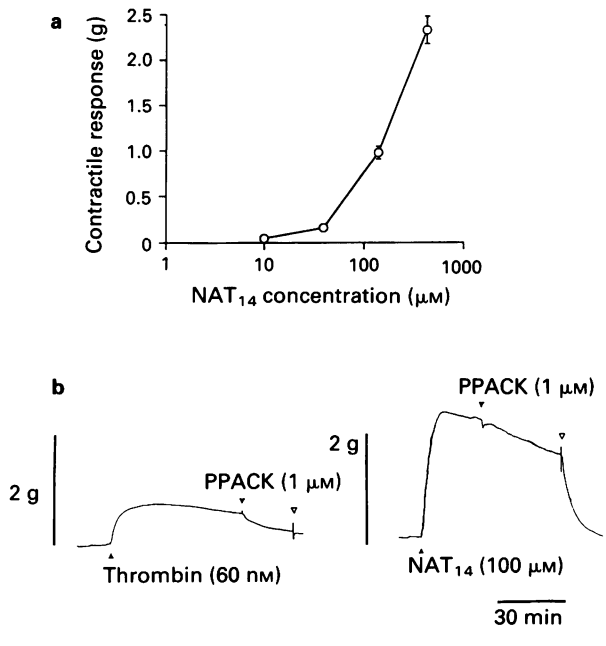


**Figure 5** Effect of stimuli related to receptors for α-thrombin on rabbit aortic rings. (a) Concentration-effect relationship for $NAT_{14}$, a peptide derived from a cloned receptor for α-thrombin. Values are expressed as the means of 5 determinations. (b) Representative tracings of tension changes induced by α-thrombin (60 nM) or $NAT_{14}$ (100 μM) and acute effect of the α-thrombin inhibitor, D-Phe-Pro-Arg-$CH_2Cl$ (PPACK; 1 μM), on the contraction induced by these agents. Abscissa scale: time (min). Ordinate scale: isometric contraction (g). Closed symbols indicate the application of agents and open symbols the washout of stimulants

aorta (Berk *et al.*, 1985) or stimulating proliferation in human fibroblasts (Seifert *et al.*, 1989).

In addition, and as previously observed (deBlois *et al.*, 1989), the kinin-induced contractility in the rabbit aortic preparation was rapidly potentiated by EGF. In this study the interaction with EGF was shown to be relatively selective, being shared only by α-thrombin. The order of application of the growth factor and the other agonist was not important, as EGF applied on a plateau induced by des-Arg$^9$-BK or by α-thrombin also resulted in a contractile effect greater than the sum of the responses to each agent (Figures 2–4). Considering the small direct contractile effect of EGF and the fact that the concentration of either des-Arg$^9$-BK or α-thrombin resulted in a further increase in the contractile effect of EGF, the interaction of EGF with des-Arg$^9$-BK or α-thrombin could be called synergism.

The rat superior mesenteric artery responded to EGF by an apparent prostaglandin-mediated contraction (Muramatsu *et al.*, 1985). However, in the present study, indomethacin did not inhibit direct or synergistic contractile effects of EGF, excluding the role of cyclo-oxygenase products. Endothelium removal did not influence the direct contractile effect of the growth factor, nor its synergistic effect with α-thrombin. This was attempted because some of the effects of α-thrombin in various vascular preparations are endothelium-dependent (DeMey & Vanhoutte, 1981; Hatake *et al.*, 1990; Boulanger & Lüscher, 1991). The contractile effect of des-Arg$^9$-BK was not influenced by endothelium removal in the rabbit aortic preparation (Bouthillier *et al.*, 1987).

The primary signalling mechanism of EGF and of PDGF is believed to be the activation of a protein tyrosine kinase domain present in their respective receptors (Ullrich & Schlessinger, 1990). Therefore appropriate blockers of tyrosine kinase activity could theoretically inhibit the effects of growth factors. We observed that the three competitive inhibitors used, erbstatin, genistein and tryphostin-51, behaved differently in reference to different effects of growth factors on the rabbit aorta at the concentrations tested. Erbstatin inhibited the direct contractile effect of PDGF-BB and the EGF/des-Arg$^9$-BK synergism, but was a weak inhibitor of the thrombin/EGF synergism and failed to inhibit the direct contractile effect of EGF. Genistein inhibited all effects of EGF, but again, the inhibition of EGF/thrombin synergism was less extensive. Tyrphostin-51, one of the most potent inhibitors of EGF receptor tyrosine kinase in cell-free

**Table 3** Differential effects of several treatments on the contractile response to des-Arg$^9$-BK and to α-thrombin

| *Treatment* (concentration; duration)[a] | n | *Contractile response* (g) *des-Arg$^9$-BK* (1.7 μM) | *α-Thrombin* (6 nM) |
|---|---|---|---|
| *Experiment A* | | | |
| Control | 4 | 0.58 ± 0.02 | 0.39 ± 0.06 |
| PPACK (1 μM, 0–6.5 h) | 4 | 0.61 ± 0.05 | 0**[b] |
| [Leu$^8$]des-Arg$^9$-BK (17 μM, 20 min) | 4 | NT[c] | 0.42 ± 0.11 |
| *Experiment B* | | | |
| Control | 7 | 1.84 ± 0.30 | 0.47 ± 0.05 |
| Cycloheximide (17 μM, 0–3 h) | 7 | 2.88 ± 0.28* | 0.58 ± 0.09 |
| *Experiment C* | | | |
| Control | 4 | 0.78 ± 0.26 | 0.23 ± 0.07 |
| Interleukin-1β (5 ng ml$^{-1}$, 0–3 h) | 4 | 1.72 ± 0.20* | 0.19 ± 0.03 |

[a]Rabbit aortic rings were challenged with des-Arg$^9$-BK after 6 h, or with α-thrombin after 1 h of *in vitro* incubation in Krebs solution. Treatments consisted of exposing tissues to a drug for a definite period of time: cycloheximide and interleukin-1β were given as a 'pulse' during the first 3 h of incubation, [Leu$^8$]des-Arg$^9$-BK was given 20 min before the 6 h recording and D-Phe-Pro-Arg-$CH_2Cl$ (PPACK) was applied continuously to treated tissues. The statistical weight of each animal involved in these experiments was the same in control and treated tissue groups.
[b]Values from treated groups were compared to controls by Student's *t* test: $*P<0.05$; $**P<0.01$.
[c]Not tested.

systems (Gazit *et al.*, 1989), was only marginally active against EGF/thrombin synergism and inactive against the direct contractile effect of EGF. The tyrosine kinase activities of various growth factor receptors are sufficiently distinct to be inhibited differentially by tyrosine analogues, such as erbstatin and tyrphostins, but both types of drugs are reported to inhibit cell responses to EGF (Gazit *et al.*, 1989; Powis, 1991). These compounds are known to be relatively unstable in biological systems (Powis, 1991). The inhibitors may be much less potent in our system than in cell-free biochemical assays (Gazit *et al.*, 1989) and it is plausible that different concentration levels are required to inhibit the phosphorylation of different substrates (Enright & Booth, 1991). If this is the case, it is possible that the thrombin/EGF synergism is dependent on the action of tyrosine kinase on a high affinity substrate, because the competitive kinase inhibitors are comparatively less efficient to prevent it.

Growth factor-associated tyrosine kinase activities also exhibit some substrate selectivities, and there is indication that some kinase substrates are recognized by only one (or few) of these activities (Pandiella *et al.*, 1989; Powis, 1991). The synergism that we observed between EGF and two other agonists must fall in this category, because PDGF-BB exhibited no such interactions. The activation of phospholipase Cγ1 is a common effect of EGF and PDGF (Goldschmidt-Clermont *et al.*, 1991) and consequently, it is not a likely explanation of the observed synergisms in the rabbit aortic rings. It is of interest that the synergism between EGF and α-thrombin has been documented previously: the mitotic rate of human cultured endothelial cells under stimulation with EGF or fibroblast growth factor was markedly increased by α-thrombin (Gospodarowicz *et al.*, 1978). EGF is known to increase the duration of the plateau of the cytosolic calcium increase induced by BK in various cell lines (Olsen *et al.*, 1988; Pandiella & Meldolesi, 1988; Marks *et al.*, 1988). A rapid and selective reinforcement of the transmembrane signalling at BK receptors was postulated; in these systems the $B_2$ receptor type was involved. Some undetermined interaction of intracellular second messenger pathways was postulated to occur at a level proximal to the kinin receptor. A proposed mechanism was the phosphorylation of the $B_2$ type receptor, or of a G protein that couples this (but not all) receptor to phospholipase C, by the kinase activity of EGF receptors (Pandiella & Meldolesi, 1988). Such an interaction between $B_1$ receptors for kinins or α-thrombin receptors and EGF receptors could constitute a reasonable basis to explain the relative selectivity of the EGF synergistic effect in the rabbit aortic tissue. The C-terminal cytosolic domain of the cloned receptor for α-thrombin is rich in tyrosine residues: 6 are present, including a tyrosine triplet apparently unique in the rhodopsin family of receptors (Vu *et al.*, 1991). These are potential sites for phosphorylation by activated growth factor receptors.

α-Thrombin (EC 3.4.21.5) is a serine protease, the principal function of which is to convert the soluble plasma protein, fibrinogen, into insoluble fibrin (Fenton, 1986). Therefore α-thrombin plays a central role in the mechanism of blood coagulation. Incidentally, the concentrations of α-thrombin used in this study were less than the ones found in spontaneously clotting human blood (circa 140 nM; Aronson *et al.*, 1977). In addition, this enzyme also exerts hormone-like effects on a large number of cell types (Shuman, 1986). Both endothelium-dependent and independent actions of α-thrombin have been reported on vascular tissue (DeMey & Vanhoutte, 1981; Hatake *et al.*, 1990; Boulanger & Lüscher, 1991). In the rabbit aortic preparation, we have reproduced the findings of Haver & Namm (1984), which could be summarized as follows: α-thrombin is an endothelium-independent contractile agent (Table 2) the activity of which is abolished by inhibitors of its catalytic function (see the effect of PPACK, Table 3), but not influenced by inhibitors of arachidonate metabolism (lack of effect of indomethacin on the direct effect of α-thrombin, Table 2). The recently cloned receptor for α-thrombin from megakaryocyte-like cell lines also requires a proteolytically active enzyme. A tetradecapeptide from the receptor N-terminal region, $NAT_{14}$, behaves as an agonist (Vu *et al.*, 1991). We show here that this peptide, a postulated stimulatory domain of α-thrombin receptor, also behaves as a contractile agonist on the rabbit aorta at concentrations similar to those used previously (Vu *et al.*, 1991). Therefore it seems that the cleavable receptor model of Vu *et al.* (1991) also applies to the myotropic effect of α-thrombin in the aortic system. Consistent with an effect of $NAT_{14}$ on the receptors for α-thrombin, the contraction induced by this peptide was also potentiated by EGF pretreatment of the tissues. A noticeable difference between $NAT_{14}$ and α-thrombin is that the effect of the former is not inhibited by PPACK. This is consistent with the model of Vu *et al.* (1991): the peptide is not a protease.

In human platelets, the action of α-thrombin on the cleavable receptor results in the activation of phospholipase C (Rittenhouse-Simmons, 1979), an event that could take place in the rabbit aorta. Proteolytically active α-thrombin exerts several effects on rat vascular smooth muscle cells, including the increased synthesis of DNA and proteins, increased cytosolic concentration of calcium, protein kinase C

activation and $Na^+/H^+$ exchange (Berk *et al.*, 1990).

The synergism between the contractile effects of EGF and of other agents is a novel mode of the vasomotor actions of growth factors. The relatively selective interaction between the effects of EGF and mediators that are formed during coagulation, namely kinins and α-thrombin, may occur in a wide range of vascular pathologies. For example, in partially occluded human coronary arteries (Fischell *et al.*, 1988), as well as in rat carotid arteries (Clowes *et al.*, 1983), chronic spasm may occur early after mechanical dilatation of the lumen with a balloon catheter.

Removal of the endothelium *in vivo* with a balloon catheter initiates a thrombogenic reaction and platelet adhesion at the rabbit aorta luminal surface (Hatton *et al.*, 1989). Although α-thrombin is a vasodilator in several vascular beds, such as the perfused coronary artery of the dog, the protease becomes a potent vasoconstrictor after the destruction of the endothelium (Ku *et al.*, 1987). Thus, the combination of α-thrombin and EGF-like material derived from platelets (e.g. TGF-α) may contribute to vasospasm, especially in vessels where the endothelium is absent. In contrast, pig isolated coronary artery incubated *in vitro* contracts in response to des-Arg$^9$-BK independently of the presence of the endothelium (Beny *et al.*, 1987). It is tempting to speculate that vascular injury *in vivo* may also lead to the expression of kinin $B_1$ receptors mediating vascular contraction and capable of interacting with receptors for EGF. Finally, this type of interaction may not be limited to mechanical responses and may extend to muscle proliferation in response to injury, with pathological remodelling of the vascular wall as a consequence (neointima formation; Schwartz *et al.*, 1990). This hypothesis will be tested by use of a balloon injury model.

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# Bronchodilatation by tachykinins and capsaicin in the mouse main bronchus

[1]Stefano Manzini

Menarini Ricerche Sud, Pharmacology Department, via Tito Speri 10, 00040 Pomezia (Roma), Italy

**1** The effect of sensory neuropeptides and capsaicin on basal and stimulated tone of mouse bronchial smooth muscle has been evaluated.

**2** In basal conditions neither sensory neuropeptides (subtance P, neurokinin A or calcitonin gene-related peptide (CGRP)) nor capsaicin exerted any contractile effects. However, when a tonic contraction was induced with carbachol (1 μM) a prompt relaxation was induced by substance P (1– 100 nM) and by neurokinin A (1– 100 nM), with substance P being more potent. A second application of substance P was without effect. CGRP (10 nM) produced only a very small and erratic relaxation. Relaxation was also induced by capsaicin (1 μM), and this response could be evoked only once in each preparation. In 4 out of 6 preparations a cross-desensitization between substance P and capsaicin was observed.

**3** The selective $NK_1$ tachykinin agonist, [$Pro^9$]-SP sulphone (1 μM), exerted potent bronchodilator actions on carbachol-contracted mouse bronchial preparations. In contrast, neither [β $Ala^8$]-NKA (4–10) nor [$MePhe^7$]-NKB (both at a concentration of 1 μM), selective synthetic agonists for $NK_2$ and $NK_3$ receptors, exerted significant relaxant effects. Furthermore, the selective $NK_1$ tachykinin antagonist, (±)-CP 96,345 (1 μM), abolished substance P (1 nM)- but not isoprenaline (0.1 μM)-induced relaxations.

**4** Application of electrical field stimulation (EFS) (20 Hz, supramaximal voltage, 0.5 ms for 10 s) to carbachol-contracted preparations evoked a transient contraction followed by a relaxation. The tetrodotoxin-sensitive slow component of this relaxation was reduced following capsaicin desensitization.

**5** In the presence of indomethacin (5 μM) the relaxation induced by substance P, capsaicin or EFS was suppressed.

**6** In conclusion, the mouse main bronchus appears to be a monoreceptorial tissue containing only $NK_1$ receptors which subserve bronchodilator functions. Such receptors could be activated by exogenous or endogenously (capsaicin or EFS) released tachykinins and the consequent relaxation is probably mediated by the generation of prostanoids.

**Keywords:** Sensory neuropeptides; $NK_1$ receptors; synthetic tachykinin agonists

## Introduction

The local release of sensory neuropeptides (mainly tachykinins and calcitonin gene-related peptide, CGRP) from nerve endings of capsaicin-sensitive primary afferents is thought to play a relevant role in asthma pathophysiology (Lundberg & Saria, 1987). Indeed, tachykinins exert prominent bronchomotor, inflammatory and secretory actions, both in animals and in man (for a review, see Maggi, 1990). Furthermore, depletion of sensory neuropeptide affords partial protection against antigen-induced respiratory distress (Saria *et al.*, 1983; Manzini *et al.*, 1987). However, sensory neuropeptides exhibit additional biological actions which might counteract their powerful pro-asthmatic effects. Thus, it has been proposed that stimulation of $NK_1$ receptors localized on epithelial cells is linked with the generation and release of an epithelium-derived inhibitory factor (EpDIF) with relaxant actions on airways smooth muscle (Tschirhart & Landry, 1986; Rangachari *et al.*, 1987; Frossard *et al.*, 1989). Most of these studies have been performed on guinea-pig airways, and it has been recently demonstrated that, in this species, $NK_1$ receptors are localized not only in the epithelium, but also on smooth muscle cells (Devillier *et al.*, 1988). Therefore, exposure of whole tissues to exogenous tachykinins leads to a mixed response comprising both contractile and relaxant components. It is possible to separate the two activities by using normal and epithelium-denuded preparations, even though the effectiveness of the procedure to remove the epithelium completely remains uncertain.

In view of the above it might be useful to establish whether there are airway preparations in which tachykinins might produce solely contraction or relaxation. Consequently, this study has assessed the motor effects of natural and synthetic tachykinins as well as of capsaicin in the main bronchus of the mouse. Although the mouse tracheobronchial tree contains high amounts of sensory neuropeptides (Wharton *et al.*, 1979; Brodin & Nilsson, 1981), no functional data are available about their possible motor effects. In this paper we present evidence that in the mouse right main bronchus, both exogenous tachykinins and capsaicin exert no contractile effect while they produce indomethacin-sensitive relaxations which are mediated by the stimulation of $NK_1$ receptors, thought to be located on epithelial cells.

## Methods

Male albino Swiss mice weighing 30–50 g were used throughout the study. Animals were stunned and bled and the trachea and the right main bronchus were quickly excised and dissected free from adhering tissues. Tracheal or bronchial rings were mounted on two 'L'-shaped stainless steel holders and placed in a 5 ml organ bath containing physiological salt solution (at 37°C and oxygenated with 95% $O_2$ and 5% $CO_2$) of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.5, $MgCl_2 \cdot 6H_2O$ 0.5, $NaH_2PO_4 \cdot H_2O$ 1.0,

[1] Address for correspondence: Menarini Ricerche Sud S.p.A., P.O. Box 15302, 00143 Roma EUR Laurentino, Italy.

$NaHCO_3$ 25, glucose 10. Contractile responses were recorded by an isometric force transducer and displayed on a Basile Unirecord 7050 polygraph. An optimal resting tension of 400 mg was applied to the preparations. Each preparation was allowed to equilibrate for at least 45 min before the beginning of the experiments. In some experiments bronchial preparations were field stimulated (20 Hz, supramaximal voltage, 0.5 ms for 10 s) by a pair of platinum electrodes placed at the top and the bottom of the organ bath and connected to a Grass Instruments S11 stimulator.

To avoid desensitization, concentration-response curves to tachykinins were performed in a non-cumulative manner. In each tissue different randomized concentrations of substance P or neurokinin A were tested after washing and re-establishment of the carbachol contraction.

In experiments with (±)-CP 96,345, this antagonist was added to the organ bath (when the response to carbachol had reached a steady-state) 10 min before the administration of substance P or isoprenaline.

### *Statistics*

All data are mean ± s.e.mean of the indicated number of experiments. Statistical analyses were performed with Student's *t* test for paired or unpaired data, when appropriate.

### *Drugs*

Substance P, capsaicin and indomethacin (Serva, Heidelberg, Germany); atropine sulphate, tetrodotoxin (TTX), papaverine HCl and isoprenaline (Sigma, St. Louis, U.S.A.); calcitonin gene-related peptide (CGRP) (Bachem, Budendorf, Switzerland); carbachol (Merck, Darmstadt, Germany); neurokinin A (NKA) (Peninsula, Belmont, U.S.A.). The selective tachykinin analogues [$Pro^9$]- SP sulphone, [β $Ala^8$]-NKA (4–10), [$MePhe^7$]-NKB and (±)-CP 96,345 were a generous gift of Dr C.A. Maggi and Dr P. Rovero from the Research Laboratories of Menarini Pharmaceuticals, Florence, Italy. CP 96,345 was synthesized as a racemic mixture containing both [2**R**, 3**R**]-*cis*- and [2**S**, 3**S**]-*cis*-2-(diphenylmethyl)-N-(2-methoxyphenyl)-methyl]-azabicyclo[2.2.2] octan-3-amine, according to the method described by J.A. Lowe III (1990). The identity of the compound was assessed by its $^1H$ and $^{13}C$ nuclear magnetic resonance (n.m.r.) spectra. The purity of (±)-CP 96,345, as verified by high performance liquid chromatography (h.p.l.c.), was estimated about 99% according to its adsorbance at 214 nm.

## Results

### *Effect of sensory neuropeptides and capsaicin on basal tone and on carbachol-induced contraction*

In basal conditions, mouse tracheal or bronchial rings were quiescent ($n = 7$ and 40, respectively). Administration of substance P (1 μM, $n = 4$), neurokinin A (10 μM, $n = 4$), CGRP (0.1 μM, $n = 4$) or capsaicin (2 μM, $n = 4$) had no motor (contraction or relaxation) effects. However, the exposure of the preparations to carbachol (1 μM, $n = 36$) or high-$K^+$ medium (20–80 mM, $n = 5$) resulted in a prompt contraction which reached a steady state within 10–15 min and remained stable for at least 1 h. The amplitude of carbachol-induced tonic contraction was of 82 ± 7 and 208 ± 17 mg $mg^{-1}$ tissue weight in tracheal ($n = 12$) or in bronchial ($n = 32$) preparations, respectively ($P < 0.001$). Atropine (1 μM) suppressed the carbachol-induced contraction ($n = 5$). In the same bronchial preparation at least four similar contractile responses to carbachol could be easily obtained, at 15 min intervals. In view of the greater amplitude and reproducibility of motor responses, further studies were only carried out in bronchial preparations.

The exogenous administration of substance P (1–100 nM) on carbachol-induced tonic bronchomotor response elicited a fast and steep relaxation which started after about 20 s, lasted for 1–2 min and then slowly returned toward the precontracted tone (Figures 1 and 2). Substance P-induced relaxation was an almost all-or-none phenomenon and a modest difference was observed between the effect obtained with 1 nM (178 ± 33 mg; 46 ± 9% inhibition; $n = 5$), as compared to 1 μM substance P (226 ± 28 mg, 63 ± 3% inhibition; $n = 9$). Substance P concentrations lower than 1 nM were ineffective. A second application of substance P (1 μM) after recovery from or during the relaxation phase of a first challenge with substance P (1 μM), had no further relaxant effect, indicating complete desensitization ($n = 8$). When in the same preparation, following several washings, a further response to carbachol was re-obtained, the relaxant action of substance P was instead fully recovered.

Neurokinin A (1–100 nM) also evoked relaxation of carbachol-precontracted bronchial rings, although with a significantly lower activity than substance P (Figure 2). A consistent relaxation (6 out of 6 experiments) amounting to 176 ± 29 mg, with an inhibition of 57 ± 9% ($n = 6$) was obtained only at the maximal concentration tested (100 nM).

The administration of CGRP (10 nM) exerted a very small (22 ± 9 mg) relaxant effect in 4 out of 6 preparations.

In 15 out of 20 preparations capsaicin (1 μM) administration resulted in a relaxation of carbachol-induced contractions. These relaxations ensued after a lag time of 30–60 s and their amplitude amounted to 176 ± 25 mg, corresponding to an inhibition of 41 ± 4% ($n = 15$, Figure 3). A second challenge with capsaicin (1 μM) in the same preparation did not result in a further relaxation ($n = 7$, Figure 3), indicating complete desensitization. Furthermore, when capsaicin (1 μM) was administered in the presence of substance P (1 μM) its relaxant effect was almost abolished in 4 out of 6 preparations, suggesting a cross-desensitization.

### *Effect of synthetic tachykinin analogues on carbachol-induced contraction*

To investigate which neurokinin receptor(s) could be involved in the substance P-induced relaxation of carbachol-induced contraction of mouse bronchus, the relaxant properties of some synthetic tachykinin analogues proposed as selective agonists for $NK_1$, $NK_2$ and $NK_3$ receptors, (i.e. [$Pro^9$]-SP sulphone, [β $Ala^8$]-NKA(4–10) and [$MePhe^7$]-NKB, respectively (Drapeau *et al.*, 1987; Rovero *et al.*, 1989)) were assessed.

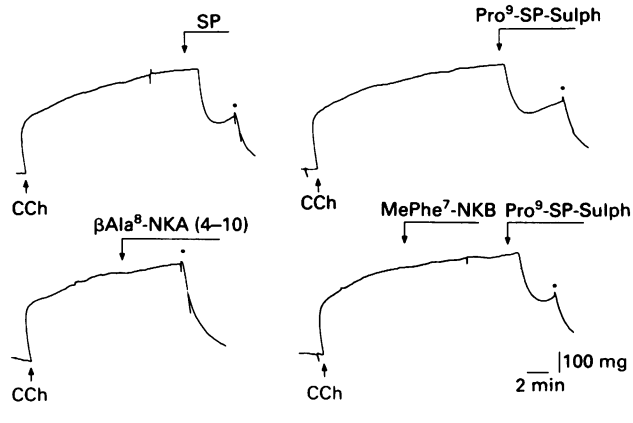


**Figure 1** Typical tracings showing the response of carbachol (CCh)-precontracted mouse bronchus to substance P (SP) and the selective synthetic tachykinin analogues [$Pro^9$]- SP sulphone ($NK_1$ selective), [β $Ala^8$]-NKA (4–10) ($NK_2$ selective) and [$MePhe^7$]-NKB ($NK_3$ selective). All drugs were administered at the concentration of 1 μM. Only substance P and the selective $NK_1$ agonist produced a relaxation. Drugs were administered at the arrows. The black dots indicate washing out.

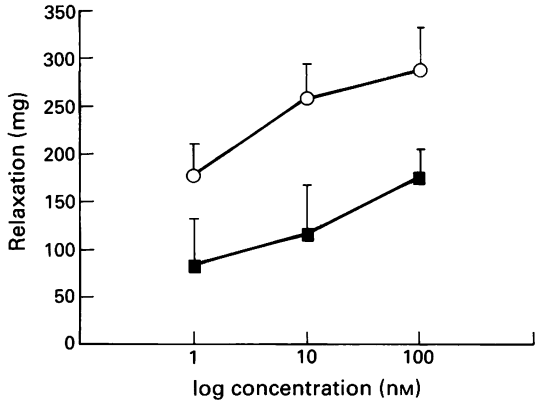


**Figure 2** Concentration-response curve for substance P (○) and neurokinin A (■) for their ability to induce a relaxation of carbachol-induced tonic contraction of mouse bronchi. The tachykinins were added in a randomized non-cumulative manner.

Each value is the mean of at least 5 experiments; s.e.mean shown by vertical bars.

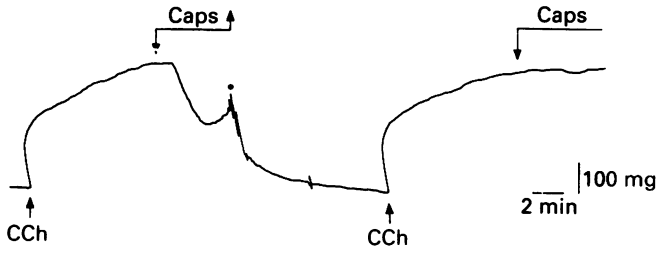


**Figure 3** Typical tracings showing the response to capsaicin (Caps, 1 μM) of carbachol (CCh)-precontracted mouse bronchus. After a lag time of about 1 min, a marked relaxation ensued. In the same preparation a second application of capsaicin on a further carbachol-induced contraction was without effect.

Drugs were administered at the arrows. The black dot indicates washing out.

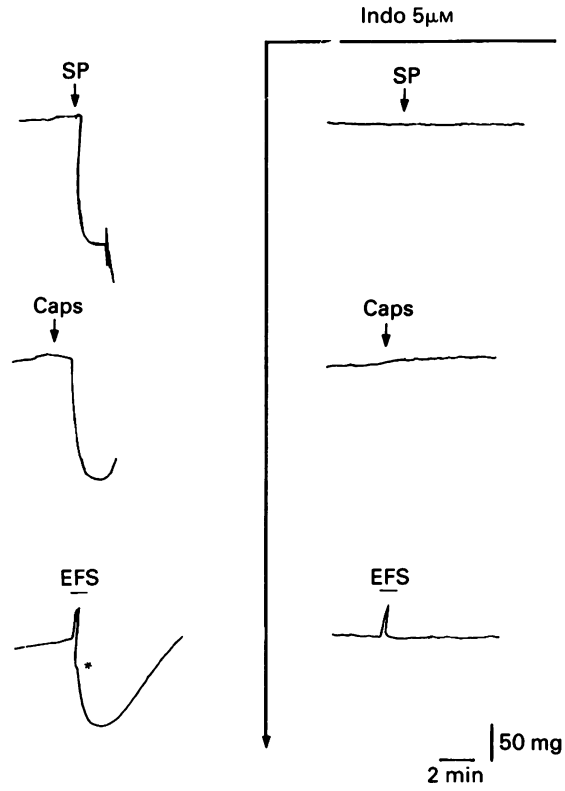


**Figure 4** Typical tracings showing the bronchodilatation induced by substance P (SP, 1 μM), capsaicin (Caps, 1 μM) or electrical field stimulation (EFS, 20 Hz, supramaximal voltage, 0.5 ms for 10 s) in control conditions or following incubation for 30 min with indomethacin (Indo). In the case of capsaicin the responses were obtained in two different tissues. On the other hand, the responses to substance P and EFS were obtained in the same bronchial preparation, but on two different responses to carbachol elicited at about 60 min interval with repetitive washing out during the first 30 min.

Drugs were administered at the arrows (thin bar for EFS). The asterisk indicates the notch dividing the two phases of EFS-induced relaxation.

[Pro$^9$]-SP sulphone (1 μM) elicited a marked relaxation of carbachol-induced contraction, which amounted to 162 ± 30 mg ($n$ = 5, Figure 1). The amplitude of such relaxations was not significantly different from that obtained with substance P, in the same preparations.

On the other hand, neither [β Ala$^8$]-NKA (4–10) nor [MePhe$^7$]-NKB (both at 1 μM) exerted significant relaxant effects ($n$ = 5 or 6, respectively, see Figure 1).

The selective $NK_1$ receptor antagonist, (±)- CP 96,345 (1 μM) had no effect *per se* on carbachol-induced contraction, but almost abolished the bronchodilatation induced by substance P (1 nM) ($n$ = 5). At this concentration, this compound had no inhibitory effect on isoprenaline (0.1 μM)-induced relaxation of carbachol-precontracted preparations ($n$ = 5).

### *Effect of electrical field stimulation on carbachol-induced contraction: modification by capsaicin*

The application of EFS (20 Hz, supramaximal voltage, 0.5 ms for 10 s) to bronchi previously contracted with carbachol (1 μM), produced a short-lived contraction (99 ± 39 mg, $n$ = 9) immediately followed by a relaxation (Figure 4). Often in the rising phase of the relaxation response there was a rapid first component evident, then a notch followed by a more slowly ensuing further relaxation (see Figure 4, the notch is indicated by an asterisk). As a whole the amplitude of EFS-induced relaxations amounted to 99 ± 14 mg ($n$ = 9). Such relaxations were transient in nature and the tone usually recovered within 10–15 min (Figure 4).

When EFS was repeated in the presence of capsaicin (10 μM, $n$ = 9), (which *per se* elicited a transient relaxation of 211 ± 39 mg), the amplitude of EFS-induced contractions was unchanged while EFS-induced relaxations were significantly reduced, especially in the second slowly rising components (Table 1). A similar inhibition was observed even when capsaicin pretreatment (3 μM for 30 min) was performed before carbachol addition ($n$ = 4).

In the presence of TTX (3 μM, $n$ = 9) EFS-induced contractions were virtually abolished, while relaxations were markedly reduced, although in some preparations (6 out of 9) the first rapid component of the relaxation was still evident (Table 1).

**Table 1** Amplitude of electrical field stimulation (20 Hz, supramaximal voltage, 0.5 ms for 10 s)-induced contraction and relaxation on carbachol-precontracted mouse bronchi, in control conditions and in presence of capsaicin (10 μM) or tetrodotoxin (TTX, 3 μM)

| | *Control* | *Capsaicin* | *TTX* |
|---|---|---|---|
| Contraction (mg) | 99 ± 39 | 75 ± 21 | 10 ± 5* |
| Relaxation (mg) | 99 ± 14 | 39 ± 9* | 32 ± 10* |

Each value is the mean ± s.e.mean of 9 experiments. *$P$ < 0.01 as compared to control values.

*Effect of indomethacin-pretreatment on relaxation elicited by substance P, capsaicin and electrical field stimulation*

Pretreatment of the mouse isolated bronchus preparation with indomethacin (5 μM for 30 min) abolished the relaxation induced by substance P (1 μM, $n = 7$), capsaicin (1 μM, $n = 6$) or EFS (20 Hz, 0.5 ms, 10 s; $n = 7$) (Figure 4). In the presence of indomethacin, the amplitude of the carbachol-induced contraction was increased from $146 \pm 20$ to $225 \pm 26$ mg mg$^{-1}$ tissue weight ($n = 6$, $P < 0.01$), while the ability of papaverine (0.5 mM) to relax such contractions ($86 \pm 6\%$ inhibition) was similar to that observed in control preparations ($n = 8$).

## Discussion

The presence of substance P-like immunoreactive nerves around bronchi and running just below the epithelium in the mouse tracheobronchial tree, has been well established (Wharton *et al.*, 1979; Brodin & Nilsson, 1981). The present findings indicate that exogenous administration of substance P to mouse bronchial rings produces no contractile effects, while, when a steady bronchomotor tone is induced, it elicited a significant, albeit transient, relaxation. Similar findings were obtained with other sensory neuropeptides such as neurokinin A and, to a far lesser extent, CGRP. Substance P-induced relaxation was mimicked by exogenous administration of capsaicin and by electrical field stimulation. Both capsaicin and EFS-induced relaxations were significantly reduced by a previous capsaicin exposure, indicating desensitization. It is widely accepted that capsaicin selectivity stimulates a subset of sensory nerves leading to a centripetal sensory excitation along with a local release of sensory neuropeptides such as tachykinins and CGRP (Maggi & Meli, 1988; Holzer, 1988). A typical hallmark of this specific pharmacological effect of capsaicin is its rapid desensitization, i.e. it can be evoked only once in each preparation (Maggi & Meli, 1988). In other mammalian respiratory tissues (such as guinea-pig, hamster, rabbit and cat), it has been demonstrated that capsaicin administration had prominent bronchomotor effects largely due to a local release of tachykinins with potent constrictor effects (Lundberg & Saria, 1982; 1987; Manzini *et al.*, 1989). As a whole, our functional findings suggest that a similar model can apply also to the mouse bronchus, although the released tachykinins instead of producing a contraction cause a solely relaxant response.

Tachykinins exert their biological effects through the stimulation of at least three kinds of receptor, namely $NK_1$, $NK_2$ and $NK_3$ (Regoli *et al.*, 1988). The present results suggest that $NK_1$ receptors mediate the relaxant response to tachykinins in the mouse isolated bronchus since (a) [Pro$^9$]-SP sulphone, one of the most selective $NK_1$ selective agonist known to date (Drapeau *et al.*, 1987), had about the same potency as substance P, (b) two selective agonists for $NK_2$ and $NK_3$ receptors, (i.e. [β Ala$^8$]-NKA(4–10) and [MePhe$^7$]-NKB respectively (Drapeau *et al.*, 1987; Rovero *et al.*, 1989)) did not exert any relaxant effect and (c) the selective non-peptide $NK_1$ receptor antagonist, (±)- CP 96,345 (Snider *et al.*, 1991) antagonized substance P- but not isoprenaline-induced bronchodilatation. It has been suggested that $NK_2$ receptors located on smooth muscle cells might explain the motor effect of tachykinins in various mammalian respiratory tissues such as guinea-pig bronchus (Devillier *et al.*, 1988), hamster trachea (Maggi *et al.*, 1989) and human bronchus (Advenier *et al.*, 1987; Naline *et al.*, 1988). In guinea-pig airways a contribution of $NK_1$ receptors to tachykinin-induced contraction has also been demonstrated (Devillier *et al.*, 1988; Ireland *et al.*, 1991; Maggi *et al.*, 1991). Since in mouse bronchus no contraction was observed with substance P, neurokinin A or capsaicin, the hypothesis could be advanced that no tachykinin receptors are present on smooth muscle cells of this tissue. On the other hand, various lines of evidence suggest that tachykinins can release a relaxant factor from the airway epithelium (Tschirhart & Landry, 1986). Indeed, epithelium removal markedly enhanced the motor effects of substance P in guinea-pig airways (Fine *et al.*, 1989; Maggi *et al.*, 1990). By use of selective synthetic agonists it has been demonstrated that this release is mediated by the stimulation of $NK_1$ receptors (Frossard *et al.*, 1989; Devillier *et al.*, 1989). Studies have indicated that this response is abolished by the cyclo-oxygenase inhibitor, indomethacin and is probably mediated by the formation of prostaglandin $E_2$ in the epithelium (Frossard *et al.*, 1989; Deviller *et al.*, 1989). It is intriguing to speculate that a similar mechanism might also explain substance P-, capsaicin and (partially) EFS-induced relaxation obtained in the mouse isolated bronchus. For technical reasons it is impossible to remove the epithelium from a very small preparation such as the mouse bronchial ring. However, the following considerations are in favour of such an hypothesis: (a) substance P-induced relaxation is completely abolished by cyclo-oxygenase inhibition, (b) the phenomenon is apparently mediated by the stimulation of $NK_1$ receptors and (c) a lag time of 20–30 s exists between substance P administration and the beginning of relaxation (30–60 s for capsaicin) suggesting an indirect action.

Species differences in the response to activation of capsaicin-sensitive nerves have already been presented for urinary bladder motor function (Maggi *et al.*, 1987) and nerve excitation (Baranowski *et al.*, 1986). Our findings indicate that such heterogeneity also exists for airways motor responses. Indeed, there is the hamster trachea in which only a $NK_2$-mediated contraction is evident (Maggi *et al.*, 1989), the guinea-pig airways with contractile (mediated by $NK_1$ and $NK_2$ receptors) and relaxant (mediated by $NK_1$ receptors) responses (Devillier *et al.*, 1988) and the mouse bronchus in which only an $NK_1$-mediated relaxation is clearly evident (this study). Obviously it would be of interest to assess the position of human bronchial tissues in this array of responses. Tachykinins exert powerful contractile actions in human bronchi, through selective stimulation of $NK_2$ receptors (Naline *et al.*, 1988). However, in one isolated human bronchus, capsaicin elicited only a slight and slowly-rising contraction (Lundberg *et al.*, 1983) suggesting that a bronchodilator effect might accompany the bronchoconstrictor response (Fuller, 1990). Indeed, in normal subjects the intravenous administration of substance P and neurokinin A caused bronchoconstriction and bronchodilatation, respectively (Evans *et al.*, 1988). Furthermore inhalation of capsaicin in normal and asthmatic subjects can lead to a non-adrenergic, non-cholinergic bronchodilatation (Lammers *et al.*, 1988; 1989).

In conclusion the mouse isolated bronchus is a new preparation in which tachykinins exert a selective relaxant effect mediated by stimulation of the $NK_1$ receptor, possibly located on epithelial structures. This preparation appears to be a monoreceptorial organ for $NK_1$ responses and therefore might be useful for assessing the efficacy of selective $NK_1$ agonists and/or antagonists. Furthermore, this preparation might be used to evaluate tachykinin-mediated bronchodilator effects (epithelium-mediated?) without the interference of additional contractile effects. Finally, these findings further underline how postjunctional factors might account for remarkable species differences in the response of mammalian airways to stimulation of capsaicin-sensitive sensory nerves. It is becoming clear that the overall response to capsaicin might be the result of both bronchoconstrictor and bronchodilator mechanisms and the final biological output might be different from species to species and, possibly, from physiological to pathological states.

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# The 5-$HT_4$ receptor subtype inhibits $K^+$ current in colliculi neurones via activation of a cyclic AMP-dependent protein kinase

[1]L. Fagni, A. Dumuis, M. Sebben & J. Bockaert

Centre de Pharmacologie et d'Endocrinologie, Rue de la Cardonille, 34094 Montpellier, Cedex 05 France

**1** The aim of the present study was to examine the effect of 5-hydroxytryptamine (5-HT) on $K^+$ current in primary culture of mouse colliculi neurones and to identify the 5-HT receptor subtype that could be involved in this effect.

**2** The voltage-activated $K^+$ current of the neurones was partially blocked by 8-bromo adenosine 3':5'-cyclic monophosphate (8-bromo-cyclic AMP). This effect was mimicked by 5-HT and the action of 5-HT could be antagonized by H7, a non specific protein kinase inhibitor, and by PKI, the specific cyclic AMP-dependent protein kinase blocker.

**3** A similar cyclic AMP-dependent blockade of the $K^+$ current was found with renzapride (BRL 24 924) and other 5-$HT_4$ receptor agonists such as cisapride, BIMU 8, zacopride and 5-methoxytryptamine (5-MeOT). ICS 205 930, the classical 5-$HT_4$ receptor blocker, could not be used in this study because it inhibited the studied $K^+$ current by itself. However, the novel 5-$HT_4$ receptor antagonist, DAU 6285 blocked the effects of 5-HT and renzapride on the $K^+$ current.

**4** The current was insensitive to the 5-$HT_1$ and 5-$HT_3$ receptor agonists (8-hydroxy-2-(di-n-propylamino) tetralin, RU 24 969, carboxamidotryptamine, 2-$CH_3$-5-HT) as well as to 5-$HT_1$, 5-$HT_2$ and 5-$HT_3$ antagonists (methiothepin, ketanserin, ondansetron [GR 38 032]). Moreover, these antagonists did not affect the actions of the tested 5-$HT_4$ receptor agonists.

**5** The present results show that part of the voltage-activated $K^+$ current in mouse colliculi neurones is cyclic AMP-sensitive and the blockade of the current by 5-HT involves the 5-$HT_4$ receptor subtype. The putative implication of 5-$HT_4$ receptors in neuronal plasticity, via a blockade of $K^+$ channels, is discussed.

**Keywords:** 5-Hydroxytryptamine; 5-$HT_4$ receptor; $K^+$ current; 'patch-clamp'; neuronal plasticity

## Introduction

A variety of mechanisms have been described to trigger modulation of $K^+$ channels by neurotransmitters. Phosphorylation/dephosphorylation of the channel itself or some modulatory intermediate events are the most common mechanisms (Rudy, 1988; Brown, 1990). Phosphorylation is the result of protein kinase C (PKC; Akerman *et al.*, 1988; Doerner *et al.*, 1988), adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase (PKA; Siegelbaum *et al.*, 1982; Deterre *et al.*, 1982; Ewald *et al.*, 1985) or other $Ca^{2+}$-activated protein kinase or phosphatase (Rudy, 1988; Hoger *et al.*, 1991). Other mechanisms have been described, including direct interaction of Gi/Go proteins (Yatani *et al.*, 1987; Von Dongen *et al.*, 1988) or arachidonic acid metabolites (Piomelli *et al.*, 1987).

The physiological roles of such $K^+$ channel regulations by second messengers and transmitters are not always known. However, in *Aplysia californa* a cellular biological approach has demonstrated that short-term and long-term sensitizations of the gill withdrawal reflex is due in part to a 5-HT receptor-activated cyclic AMP cascade (Kandel & Schwartz, 1982; Greenberg *et al.*, 1987). 5-HT receptors localized on sensory neurones increase cyclic AMP production and therefore PKA activation, leading to closure of $K^+$ channels, prolonged depolarization, opening of $Ca^{2+}$ channels and then enhanced transmitter release. These 5-HT-inhibited $K^+$ channels in *Aplysia californa* neurones are specific $K^+$ channels called S-$K^+$ channels. They are active at resting membrane potential, do not inactivate and do not participate in the fast (A-current), delayed and $Ca^{2+}$-activated $K^+$ currents (Klein *et al.*, 1982). This model of presynaptic facilitation is quite interesting because it may represent a cellular model of short- or long-term synaptic plasticity. However, such a model has not yet been described in vertebrates.

Recently, we have found in colliculi and hippocampal neurones a novel 5-HT receptor subtype having a pharmacology that differs strictly from those of the already described 5-$HT_1$, 5-$HT_2$ and 5-$HT_3$ receptors. We designated this new receptor as the 5-$HT_4$ receptor (Dumuis *et al.*, 1988; 1989; Bockaert *et al.*, 1990). It stimulates cyclic AMP production and has now been described in guinea-pig ileum and colon, rat oesophagus and human heart (Craig & Clarke, 1990; Craig *et al.*, 1990; Kaumann *et al.*, 1990; Ouadid *et al.*, 1991). In guinea-pig ileum, 5-$HT_4$ receptors are likely localized on myenteric cholinergic neurones and trigger acetylcholine release (Pfeuffer-Friederich & Kilbinder, 1984; Buchheit *et al.*, 1985).

It is possible that 5-$HT_4$ receptors in vertebrates have similar physiological roles in neuronal plasticity as other 5-HT receptors do in *Aplysia californa*. In order to investigate this question, the first step was to analyze the possibility that 5-$HT_4$ receptors trigger a cyclic AMP-mediated inhibition of $K^+$ current in colliculi neurones.

## Methods

Colliculi were dissected from 14–15 days old mouse embryos. Cultures were prepared as previously described for striatal

[1] Author for correspondence.

neurones (Weiss *et al.*, 1986). Briefly, neurones were dissociated and plated in 35 mm diameter dishes containing a defined culture medium supplemented with 2% serum and hormone mixture. They were maintained at 37°C in a humidified atmosphere and in the presence of 5% $CO_2$/95% $O_2$ for 5–10 days before electrophysiological recording.

Patch-clamp experiments were performed at room temperature. The culture medium was replaced by a solution containing (mM): $Cl^-$ 139, $K^+$ 5, $Na^+$ 130, $Ca^{2+}$ 2, $Mg^{2+}$ 2, HEPES 10 and tetrodotoxin 0.3 μM (pH 7.4). The whole-cell recording pipettes were filled with a solution of the following composition (mM): $Cl^-$ 137, $K^+$ 130, $Na^+$ 8.5, $Mg^{2+}$ 2, $Ca^{2+}$ 0.5, EGTA 5, HEPES 10, ATP 4 and GTP 0.5 (pH 7.2). Under these conditions, the impedances of our recording electrodes were between 3 and 5 MΩ. Voltage-activated $K^+$ currents were recorded using a LIST EPC7 amplifier and digitized at 1 kHz for storage and analysis on a PC compatible computer. They were digitized at 100 Hz for iconography. Linear leak and capacitive currents were substracted. All these computerized procedures were performed with the pClamp 5.5.1 programme of Axon Instruments.

Drug solutions were prepared in these media (either external or internal medium, as indicated in the text and figure legends) and pH of the solutions was adjusted at the appropriated value (see here above). External drug applications were performed by use of an 8 barreled fast perfusion system. One mM isobutylmethylxanthine (IBMX) and 0.1 μM forskolin were applied before (control conditions) and during perfusion of all the tested 5-HT receptor agonists. Pargyline (10 μM) and ascorbic acid (1%) were added to the external solutions in order to prevent oxidative degradation of 5-HT. At the concentrations used, neither IBMX nor forskolin nor the antioxidative agents, applied alone or together, modified the studied $K^+$ current, in the absence of agonist. These observations corroborate our previous studies showing that 0.1 μM forskolin, in the presence of 1 mM IBMX, does not modify basal cyclic AMP concentrations but increases neurotransmitter efficacy in cyclic AMP production, whereas potency remains unaffected (see Figure 1 in article by Weiss *et al.*, 1985).

The following drugs were purchased from SIGMA: ATP-$Na_2$, GTP-Na, 5-HT, the protein kinase inhibitor peptide (PKI), 8-bromo-cyclic- AMP, H7 (1-[5-isoquinolinylsulphonyl]-2-methylpiperazine) , tetrodotoxin, IBMX, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) forskolin and 5-MeOT. The following drugs were generously donated: ICS 205 930 ([3-α-tropanyl]-1H-indole-3-carboxylic acid ester and 2-$CH_3$-5-HT; (Sandoz, Basel, Switzerland), methiothepin (Hoffmann-Laroche, Basel, Switzerland), cisapride ([*cis*]-4-amino-5-chloro-N-[1- [3- (4-fluorophenoxy) propyl]-3-methoxy- 4-piperidinyl]-2-methoxy-benzamide) and ketanserin (Janssen Pharmaceutica, Beerse, Belgium), 5-carboxamidotryptamine (5-CT) and ondansetron (or GR 38 032; Glaxo Group Research, Hertfordshire, UK), RU 24 969 (Roussel-Uclaf, Romainville, France), zacopride (4-amino-N-[1-azabicyclo [2.2.2]oct-3yl]-5-chloro-2-methoxy-benzamide HCl; Delalande, Paris, France), BIMU 8 ([endo-N-8-methyl-8-azabicyclo-[3.2.1]-oct-3-yl] -2,3-dihydro-3-iso-propyl- 2- oxo- 1H-benzimid- azol - 1-carboxamide HCl) and DAU 6285 (endo-6-methoxy-8-methyl-8-azabicydo[3.2.1]-oct. 3-yl-2,3-dihydro-2-oxo-1H-benzimidazole-1 carboxylate hydrochloride quinuclidinyl benzilate Boehringer Ingelheim, Italy), renzapride (or BRL 24 924; ([ ± ]-endo)-2-meth oxy-4-amino-5-chloro-N-(1-azabicyclo-[3.1.1]- non-4-y)-benzamide HCl; Beecham Pharmaceutica, Harlow, U.K.).

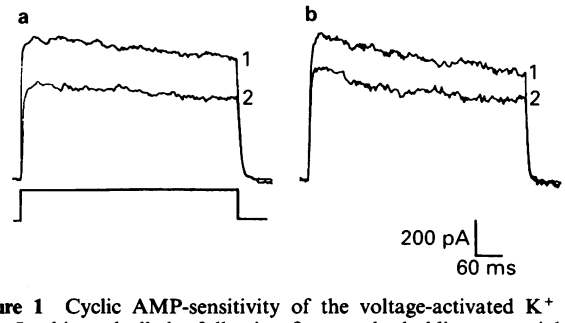


**Figure 1** Cyclic AMP-sensitivity of the voltage-activated $K^+$ current. In this and all the following figures, the holding potential was −60 mV and depolarizing steps to +30 mV (lower trace in a) served to evoke the recorded outward currents. In (a and b) trace 1 represents the control recordings and trace 2 is the current obtained in the presence of 100 μM external 8-bromo-cyclic AMP in (a) and in the presence of 5 μM external forskolin in (b). (a) and (b) are from two different cells. Similar results were obtained in 11 other cells recorded in the presence of both drugs.

## Results

### *Inhibition of a $K^+$ current in colliculi neurones by cyclic AMP*

We examined membrane currents of cultured colliculi neurones using the whole-cell configuration of the patch-clamp technique. Membrane potential was clamped at −60 mV and outward currents were evoked by depolarizing steps of various amplitude. The current-voltage relationship shifted to the right with increasing external $K^+$ concentration ($[K^+]_o$). Under symmetrical $K^+$ concentration ($[K^+]_o/[K^+]_i$ = 130 mM/130 mM) the current reversed at 0 mV, which is the theoretical equilibrium potential for $K^+$ ions, showing that the outward current studied was mainly carried by $K^+$ ions. This conclusion was further strengthened by the fact that the current was completely blocked by replacing internal $K^+$ ions (130 mM) by $Cs^+$ ions in the whole-cell recording pipette solution. It was also sensitive to tetraethylammonium ions and to 4-aminopyridine (data not shown).

The membrane-permeant analogue of cyclic AMP, 8-bromo-cyclic AMP, applied outside the cell, decreased the recorded current (Figure 1a). At concentrations of 50 and 100 μM, the decreases were of 13 ± 3% and 40 ± 8% (mean ± s.d., $n$ = 6), respectively. Forskolin, an activator of adenylate

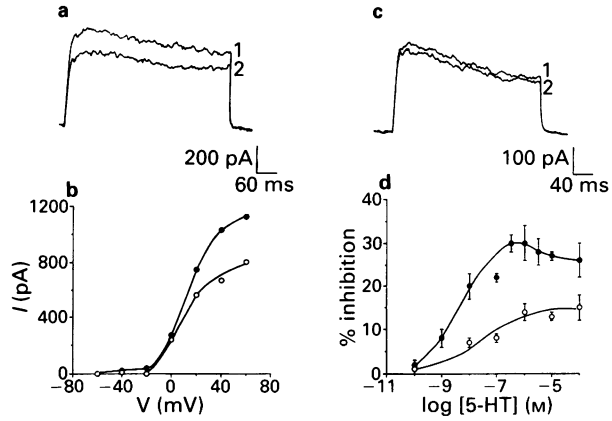


**Figure 2** Cyclic AMP-dependent protein kinase (PKA) was involved in the 5-hydroxytryptamine (5-HT)-induced inhibition of the $K^+$ current. Currents were evoked according to the procedure mentioned in Figure 1. In (a) and (c) trace 1 represents the control current obtained in two different cells; trace 2 in (a) represents the current recorded in the presence of 1 μM external 5-HT; trace 2 in (c) was obtained in the presence of 5 μM external 5-HT and 1 μM protein kinase inhibitor (PKI) in the internal solution. (b) Current-voltage relations of the outward membrane currents represented in (a) in the absence (●) and in the presence (○) of 1 μM 5-HT. Similar effects were found in 10 other cells. (d) 5-HT dose-response curves obtained from 16 cells; 8 cells recorded with normal pipette solution (●) and 8 other cells recorded with a pipette solution containing 1 μM PKI (○). Each point represents mean with s.e.mean shown by vertical bars.

cyclase, mimicked the effect of 8-bromo-cyclic AMP on the K$^+$ current (Figure 1b). At 0.5 μM, the drug inhibited the K$^+$ current by 15 ± 3%. Maximal effect was found at 5 μM where forskolin induced 43 ± 9% inhibition. External IBMX (1 mM) did not affect the action of 8-bromo-cyclic AMP but doubled the effect of threshold concentration (0.5 μM) of forskolin (data not shown). In the light of these results, it is likely that at least 40% of the outward K$^+$ current in cultured collicular neurones was regulated by cyclic AMP.

### *Inhibition by 5-HT of the K$^+$ current of colliculi neurones via a cyclic AMP-dependent protein kinase*

5-HT also decreased the K$^+$ current up to 30% (Figure 2a and b). The threshold concentration for the 5-HT effect was 1 nM and the maximal 5-HT effect (30% decrease) was obtained at an agonist concentration of 0.1 μM. Half-maximal decrease of the K$^+$ current was observed with concentrations of 5-HT close to 10 nM (Figure 2d, open circles).

PKI, a specific inhibitor of PKA, was perfused into the cell via the recording pipette at a concentration of 1 μM. Basal voltage-activated K$^+$ currents were measured in 10 cells dialyzed with PKI and compared to currents recorded in 11 other cells from the same culture that had been dialyzed with normal recording pipette solution. We found mean values (± s.e.mean) of 490 (± 48) and 350 (± 64) pA, respectively and these values were not significantly different from each other (Student's $t$-test, $P \leqslant 0.05$). We then examined the effect of PKI on the 5-HT response in the same groups of cells. As shown in Figure 2c and d, PKI reduced the 5-HT-induced inhibition of K$^+$ current. The PKI effect was higher at low 5-HT concentrations (Figure 2d). Similarly, the non-specific protein kinase inhibitor, H7 (0.1 μM), completely blocked the 5-HT effect without significantly affecting the basal K$^+$ current in the absence of agonist (data not shown).

The phosphatase inhibitor, okadaic acid (10 nM), alone did not alter the K$^+$ current. However, in the same cells ($n = 5$) the drug markedly potentiated the effects of low concentrations of renzapride ($10^{-9}$ M) and 5-HT ($5 \times 10^{-9}$ M). Examples of these effects are illustrated in Figure 3.

### *The 5-HT receptor involved in inhibition of K$^+$ current in colliculi neurones is not a 5-HT$_1$, 5-HT$_2$ or 5-HT$_3$ receptor subtype*

Several typical 5-HT receptor antagonists of the classical 5-HT receptor subtypes (5-HT$_1$, 5-HT$_2$ and 5-HT$_3$) were tested on 5-HT responses equal to approximately 50% of the maximal response. Methiothepin, a 5-HT$_{1A}$ receptor antagonist, ketanserin, a 5-HT$_2$ receptor antagonist and ondansetron, a 5-HT$_3$ receptor antagonist applied separately at a concentration of 5 μM were unable to affect the response induced by 10 nM 5-HT (data not shown). Similarly, these compounds applied altogether did not affect either the basal K$^+$ current or the inhibition of the current induced by 5-HT (10 nM; Figure 4a).

Conversely, 5-HT$_1$ and 5-HT$_3$ specific receptor agonists were tested on the K$^+$ current at concentrations (see legend of Figure 4b–e) that should have fully occupied these receptors. 8-Hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) and RU 24 969, two 5-HT$_{1A}$ receptor agonists, 5-CT which activates 5-HT$_1$ receptor types and 2-CH$_3$-5-HT, a 5-HT$_3$ receptor agonist were all inactive on this K$^+$ current (Figure 4b–e).

### *The receptor that inhibits K$^+$ current in colliculi neurones is of the 5-HT$_4$ receptor subtype*

Since these results suggested that none of the most classical receptor subtypes were involved in the observed 5-HT-induced inhibition of the K$^+$ current, we examined the possibility that 5-HT mediated its effect via the newly discovered 5-HT$_4$

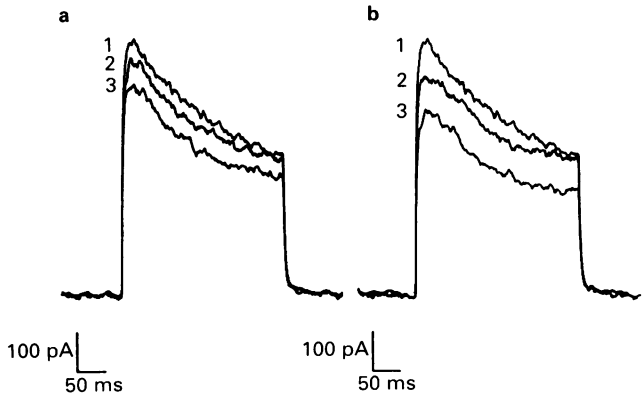


**Figure 3** The renzapride and 5-hydroxytryptamine (5-HT)-induced inhibition of the K$^+$ current involved phosphorylation processes. Currents were evoked as in Figure 1. In (a and b) trace 1 is the control current and trace 2 is obtained in the presence of renzapride ($10^{-9}$ M) in (a) and 5-HT ($5 \times 10^{-9}$ M) in (b). Trace 3 is recorded in the presence of okadaic acid (10 nM) and renzapride in (a) or 5-HT in (b) (same concentrations as above). Okadaic acid alone did not alter the control currents. Traces in (a) and (b) were obtained from the same cell.

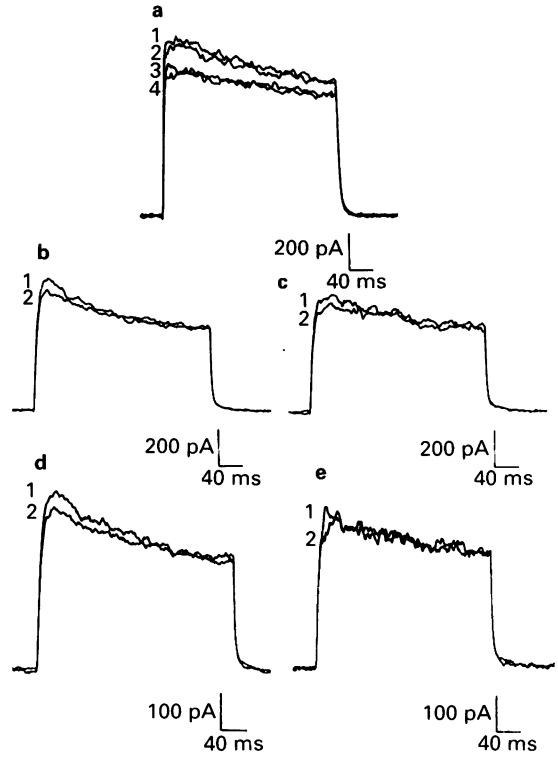


**Figure 4** Absence of 5-HT$_1$-, 5-HT$_2$- and 5-HT$_3$-mediated effects on K$^+$ current. Currents were evoked (see legend Figure 1) in 5 different cells. (a) Trace 1 represents the control current. Trace 2 (a) is obtained in the presence of a mixture of 5-hydroxytryptamine (5-HT) receptor antagonists (see text); methiothepin, ketanserin and ondansetron (all at 5 μM). Trace 3 (a) is recorded in the presence of 5-HT (10 nM) alone and trace 4 (a) in the presence of 5-HT (same concentration) in the presence of the mixture of antagonists with which trace 2 was obtained. Note the absence of effect of the 5-HT receptor antagonist mixture on the currents recorded in the absence of 5-HT and the absence of effect of these antagonists on the action of 5-HT. Similar results were obtained in 5 other cells. (b–e) Trace 1 represents control current. Trace 2 is obtained in the presence of 8-hydroxy-2-(di-n-propylamino)tetralin (1 μM; b), RU 24 969 (1 μM; c), 2-CH$_3$-5-HT (10 μM; d) and 5-carboxamidotryptamine (1 μM; e). Similar absence of drug effects was observed in 5 other cells with each tested agonist.

receptor. Renzapride (BRL 24 924), cisapride, and zacopride as well as 5-MeOT (Dumuis *et al.*, 1989; Bockaert *et al.*, 1990), which are classical 5-$HT_4$ receptor agonists, and the newly discovered 5-$HT_4$ agonist BIMU 8 (Dumuis *et al.*, 1991), when used at micromolar concentrations, reduced the outward $K^+$ current by 10 to 40% (Figure 5a and b, Figure 6 a–d). Among these drugs, the effect of renzapride was examined in more detail. The concentration of the agonist inducing half-maximal effect was found to be 0.1 μM (Figure 5d, filled circles). As observed for the 5-HT response, PKI added to the intracellular medium, blocked the effect induced by renzapride at all the concentrations tested (Figure 5c and d, open circles). The non-specific protein kinase inhibitor, H7, also completely abolished the effect of renzapride (data not shown).

The only 5-$HT_4$ receptor antagonist so far described is ICS 205 930 which is also a potent 5-$HT_3$ antagonist. The affinity of this compound for 5-$HT_3$ and 5-$HT_4$ receptors is in the nanomolar and micromolar ranges, respectively (Richardson *et al.*, 1985; Dumuis *et al.*, 1989). Therefore, we first thought to use this compound as a 5-$HT_4$ receptor antagonist. However, at micromolar concentration ICS 205 930 blocked almost all the $K^+$ current in the absence of 5-HT receptor agonist (data not shown), which corroborates the observations of Scholtysik (1987) and Scholtysik *et al.* (1988) in cardiac muscle. Therefore, this drug could not be used to test the 5-$HT_4$ nature of the effect on the $K^+$ current.

An alternative was to use the azabicycloalkyl benzimidazolone derivative, DAU 6285 (Turconi *et al.*, 1990), a drug that we found to inhibit cyclic AMP production induced by specific 5-$HT_4$ receptor agonists in cultured colliculi neurones (Dumuis *et al.*, 1992). At a concentration of 10 μM, DAU 6285 had no effect by its own on $K^+$ currents of colliculi neurones (compare traces 1 of Figure 7a and b and traces 1 of Figure 7 c and d). On the other hand, DAU 6285 (10 μM) applied to the same preparation blocked the effects of 1 μM renzapride (a specific 5-$HT_4$ receptor agonist) on the $K^+$ current (Figure 7 a and b). This compound also blocked the inhibition of the $K^+$ current induced by 5-HT (compare Figure 7 c and d). This strongly suggested that 5-$HT_4$ receptors were implicated in the observed 5-HT-induced effects.

## Discussion

The present results show that part (40%) of the outward $K^+$ current in colliculi neurones can be inhibited by activation of PKA. They also show that in these neurones 5-HT activates 5-$HT_4$ receptors (which are known to be positively coupled to adenylate cyclase; Dumuis *et al.*, 1988; 1989; Bockaert *et al.*, 1990) and blocks the cyclic AMP-sensitive $K^+$ current.

### *Existence of a cyclic AMP-sensitive $K^+$ current in colliculi neurones*

We used forskolin as a pharmacological tool to activate directly adenylate cyclase and block the cyclic AMP-sensitive $K^+$ current. In addition to its action on adenylate cyclase, forskolin directly alters the gating of voltage-dependent $K^+$ channels from cardiac muscle cells (Scholtysik, 1987) and the clonal PC12 cell line (Hoshi *et al.*, 1988), but at concentrations 5–10 fold higher than the one used here. Therefore, it seems unlikely that this unspecific effect of forskolin may have interfered with the cyclic AMP-dependent inhibition of the $K^+$ current. Moreover, inhibition of the $K^+$ current observed in the presence of 8-bromo-cyclic-AMP must have resulted from cyclic AMP-dependent processes solely. This clearly demonstrated that part of the outward $K^+$ current in colliculi neurones was cyclic AMP-sensitive.

Our results show that PKA was involved in 5-$HT_4$-mediated inhibition of $K^+$ current in colliculi neurones. However, we do not know, as is the case with other $K^+$ channels, whether the modulation is due to direct phosphorylation of

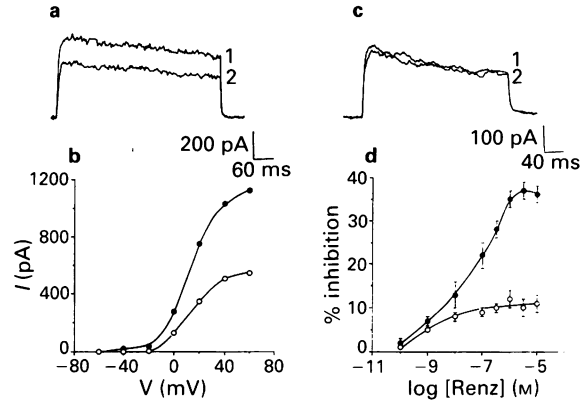


**Figure 5** Renzapride (Renz), a 5-$HT_4$ receptor agonist, like 5-hydroxytryptamine (5-HT), decreased the $K^+$ current in a cyclic AMP–dependent manner. Currents were evoked as in Figure 1. (a and c) Trace 1 is the control current obtained in 2 different cells. Trace 2 is obtained in the presence of renzapride (1 μM), without (a) and with (c) protein kinase inhibitor (PKI, 1 μM) in the recording pipette solution. (b) Current-voltage relations obtained in one cell, in the absence (●) and in the presence of renzapride (○). (d) Dose-response curve for renzapride obtained under normal intracellular recording medium (●, $n = 10$) and in the presence of PKI in the recording pipette (○, $n = 10$). Each point represents mean with s.e.mean shown by vertical bars.

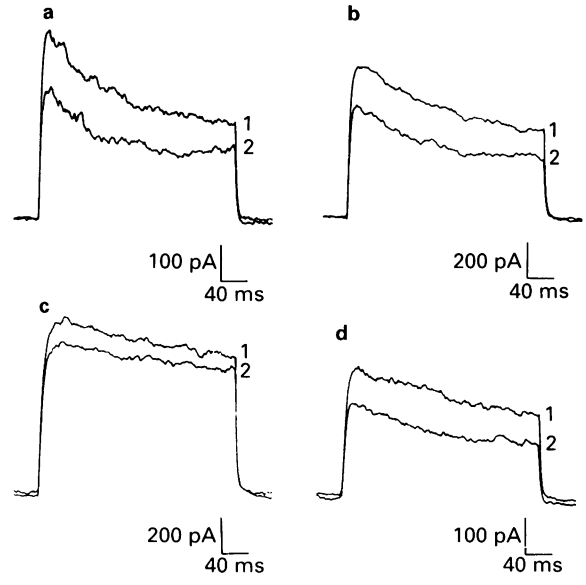


**Figure 6** Various 5-$HT_4$ agonists inhibited the $K^+$ current in colliculi neurones, as 5-hydroxytryptamine (5-HT) and renzapride did. Currents were evoked according to the protocol in Figure 1. (a, b, c and d) Trace 1 is the control recording obtained in 4 different cells. Trace 2 is obtained from the same cells, in the presence of cisapride (2 μM, a), BIMU8 (2 μM, b), zacopride (10 μM, c) and 5-methoxytryptamine (10 μM, d), respectively. Similar results were obtained for each drug in 4 other cells.

### *Pharmacology of the 5-HT-induced inhibition of the $K^+$ current*

The PKA-dependence of the 5-HT-mediated effect on the $K^+$ current suggests involvement of a 5-HT receptor that was positively coupled to adenylate cyclase. Such a 5-HT receptor does not correspond to any of the 5-HT receptor subtypes previously described in the CNS and confirms the existence

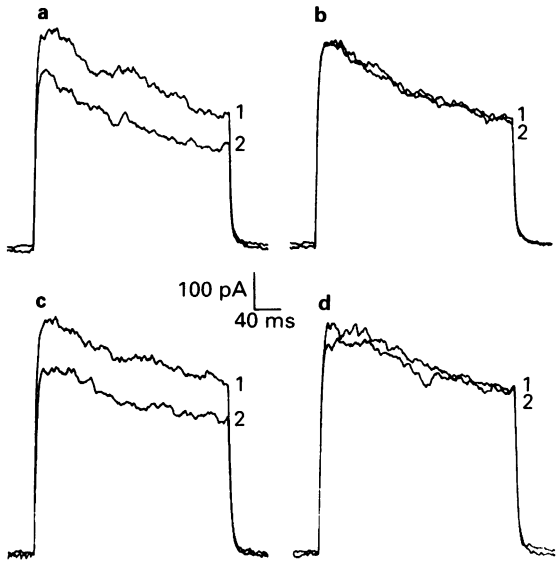


**Figure 7** The effects of 5-hydroxytryptramine (5-HT) and renzapride on the K$^+$ current were antagonized by DAU 6285, a novel 5-HT$_4$ receptor antagonist. Currents were evoked as in Figure 1. (a) Traces 1 and 2 were obtained in the same cell, in the absence and presence of 5-HT (1 μM), respectively. (b) Currents were recorded from the same cell as in (a), in the presence of DAU 6285 (10 μM) alone (trace 1) and in the presence of DAU 6285 with 5-HT (1 μM) (trace 2). (c and d) A similar protocol, except that 5-HT was replaced by renzapride (1 μM) and, was applied to the same cell as in (a) and (b). Similar results were obtained with both agonists in 5 other cells.

of the newly described 5-HT$_4$ receptor subtype in colliculi neurones (Dumuis *et al.*, 1988; 1989; Bockaert *et al.*, 1990). Several lines of evidence support this conclusion. First, the 5-HT-induced decrease in K$^+$ current was insensitive to concentrations of methiothepin, ketanserin and ondansetron sufficient to completely block all 5-HT$_1$, 5-HT$_2$ and 5-HT$_3$ receptor subtypes, respectively. Second, RU 24 969, 5-CT or 2-CH$_3$-5-HT which are active at 5-HT$_1$ and 5-HT$_3$ receptors, respectively, but not at the 5-HT$_4$ receptor subtype (Dumuis *et al.*, 1991), did not affect the K$^+$ current studied in colliculi neurones.

Third, two families of compounds are now classical agonists of the 5-HT$_4$ receptor subtype. One family consists of substituted indol derivatives including 5-substituted tryptamines such as 5-HT and 5-MeOT (Dumuis *et al.*, 1988; Bockaert *et al.*, 1990); the other family comprises the 2-methoxy-4-amino-5-chloro-substituted benzamide derivatives such as renzapride, cisapride and zacopride (Dumuis *et al.*, 1989). A newly discovered family of potent 5-HT$_4$ agonists is constituted by the azabicycloalkyl benzimidazolone derivatives such as BIMU 8 (Dumuis *et al.*, 1991). We found here that all these compounds significantly decreased the K$^+$ current in colliculi neurones, suggesting a 5-HT$_4$ receptor-mediated effect.

Moreover, in the case of renzapride, as for 5-HT, the agonist effect on the K$^+$ current was dose-dependent and was blocked by DAU 6285, a new 5-HT$_4$ antagonist, as revealed by cyclic AMP measurements in colliculi neurones (Dumuis *et al.*, 1992), that did not affect the K$^+$ current studied on its own. This observation ruled out the possibility of an unspecific effect of the agonists on the K$^+$ channels.

The 5-HT$_4$ nature of the 5-HT and renzapride effects was further supported by the fact that both drug effects were antagonized by intracellular application of PKI, a specific PKA inhibitor, and were potentiated by the phosphatase inhibitor, okadaic acid. We showed that PKI or okadaic acid alone did not significantly alter the basal activity of the studied current, suggesting two conclusions. First, the current was probably not under sustained control of cyclic AMP-dependent and phosphorylation processes in the absence of agonist. Second, the 5-HT and renzapride effects were not related to some unspecific action of the drugs but rather involved activation of adenylate cyclase via 5-HT$_4$ receptor stimulation. We found however that PKI did not completely block the effect of 5-HT or renzapride. We do not exclude the possibility that the 5-HT$_4$ effect on the K$^+$ current was in part directly mediated via a coupled-receptor G protein and thus independent of any PKA activity.

BIMU 8 (Dumuis *et al.*, 1991) and more classically renzapride (Turconi *et al.*, 1990) also display 5-HT$_3$ antagonistic activity in receptor binding assays and physiological tests. However, it seems unlikely that this property of BIMU 8 and renzapride accounted for the drug effects observed here on the K$^+$ current in colliculi neurones since another potent and specific 5-HT$_3$ antagonist, ondansetron, did not alter the current.

### *Putative physiological significance of the 5-HT$_4$ effect in colliculi neurones*

Our results indicate that the cyclic AMP-sensitive current regulated by 5-HT$_4$ receptors in colliculi neurones contributes in an apparent steady-state manner to the total voltage-dependent K$^+$ current in these cells.

It is possible that as in *Aplysia california* neurones (Klein *et al.*, 1982; Siegelbaum *et al.*, 1982; Pollock *et al.*, 1985), 5-HT decreased K$^+$ current in colliculi neurones by inhibiting specific 5-HT-sensitive K$^+$ channels (S-K$^+$ channels). Indeed, as in colliculi neurones, the action of 5-HT on S-K$^+$ channels in *Aplysia california* neurones is mimicked by cyclic AMP (Castellucci *et al.*, 1980) and is inhibited by PKI (Castellucci *et al.*, 1982). However, S-K$^+$ channels in *Aplysia* neurones are not voltage-dependent (Klein *et al.*, 1982) and the pharmacology of the 5-HT effects in this preparation are probably quite different from those presently observed in colliculi neurones. More specific studies are in progress in our laboratory to characterize further the 5-HT$_4$-sensitive K$^+$ channels in colliculi neurones.

Slowly-inactivating voltage-dependent K$^+$ currents, that can be inhibited by cyclic AMP, have also been described in hippocampal pyramidal neurones. These currents participate in the Ca$^{2+}$-activated afterhyperpolarization (AHP) that normally follows spiking activity in these cells. The currents are decreased by 5-HT application (Andrade & Nicoll, 1987) and the pharmacology of this effect corresponds to activation of a 5-HT$_4$ receptor subtype (Chaput *et al.*, 1990; Andrade & Chaput, 1991). Our results suggest that similar effect might occur in colliculi neurones.

In hippocampal pyramidal cells (Chaput *et al.*, 1990; Andrade & Chaput, 1991) and in *Aplysia california* neurones (Klein & Kandel, 1980; Pollock *et al.*, 1985), depression of K$^+$ current by 5-HT leads to depolarization, decrease in AHP and broadening of the action potential. In colliculi neurones, as in the other two cell types, such modifications should increase cell firing, induce Ca$^{2+}$ influx and thereby enhance transmitter release. This is thought to be the case also in *Aplysia california* abdominal ganglion sensory neurones following inhibition of S-K$^+$ channels by 5-HT, and this would underlie sensitization of the gill-withdrawal reflex of the animal, a simple form of learning (Kandel & Schwartz, 1982). In hippocampal pyramidal cells, blockade of K$^+$ currents by various pharmacological agents is responsible for a novel form of long-term potentiation of excitatory synaptic response of the neurones (Cherubini *et al.*, 1987; Aniksetjn & Ben-Ari, 1991), probably by enhancing transmitter (glutamate) release. On the basis of these results and in the view of our findings, it is tempting to suggest that the blockade of K$^+$ current by 5-HT is also able to increase transmitter release and thereby enhance synaptic transmission, in colliculi neurones.

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# Inhibitory effects of certain enantiomeric cannabinoids in the mouse vas deferens and the myenteric plexus preparation of guinea-pig small intestine

[1]R.G. Pertwee, L.A. Stevenson, [2]D.B. Elrick, [3]R. Mechoulam & A.D. Corbett

Division of Pharmacology, School of Biomedical Sciences, Marischal College, University of Aberdeen, Aberdeen AB9 1AS

**1** The psychoactive cannabinoids (−)-$\Delta^9$-tetrahydrocannabinol ((−)-$\Delta^9$-THC) and the 1,1-dimethylheptyl homologue of (−)-11-hydroxy-$\Delta^8$-tetrahydrocannabinol ((−)-DMH) both inhibited electrically-evoked contractions of the mouse isolated vas deferens and the myenteric plexus-longitudinal muscle preparation of the guinea-pig small intestine.

**2** Concentrations of (−)-$\Delta^9$-THC and (−)-DMH that decreased twitch heights by 50% were 6.3 and 0.15 nM respectively in the mouse vas deferens and 60 nM and 1.4 nM respectively in the myenteric plexus preparation. (−)-DMH was about 40 times more potent than (−)-$\Delta^9$-THC in both preparations, supporting the notion that their mode of action in each tissue is the same.

**3** The psychically inactive cannabinoid, (+)-DMH, had no inhibitory effect in the mouse vas deferens at a concentration of 30 nM, showing it to be at least 1000 times less potent than (−)-DMH. In the myenteric plexus preparation, (+)-DMH was about 500 times less potent than its (−)-enantiomer.

**4** The inhibitory effects of sub-maximal concentrations of (−)-$\Delta^9$-THC were not attenuated by 300 nM naloxone.

**5** The findings that (−)-$\Delta^9$-THC and (−)-DMH are highly potent as inhibitors of the twitch response of the mouse vas deferens and guinea-pig myenteric plexus preparation and that DMH shows considerable stereoselectivity suggest that the inhibitory effects of cannabinoids in these preparations are mediated by cannabinoid receptors.

**Keywords:** $\Delta^9$-Tetrahydrocannabinol; cannabinoids; mouse vas deferens; myenteric plexus; guinea-pig small intestine; stereoselectivity

## Introduction

Cannabinoids owe many of their pharmacological properties to an ability to produce functional changes in neuronal membranes and there is good evidence that this ability depends on the molecular shape of cannabinoids rather than on their marked lipophilicity (Thomas *et al.*, 1990; Pertwee, 1990). Some structure-dependent effects of cannabinoids may be produced by non-receptor-mediated processes involving, for example, the induction of conformational changes in membrane phospholipids (Hillard *et al.*, 1990; Pertwee, 1990). Other effects, however, are now thought to result from the activation of cannabinoid receptors, there being evidence that cannabinoids are highly potent, that they show marked chemical and stereochemical selectivity (Pertwee, 1990), that certain areas of the brain in which the cannabinoids are thought to initiate some of their central effects contain specific, high affinity cannabinoid binding sites (Bidaut-Russell *et al.*, 1990; Devane *et al.*, 1988; Herkenham *et al.*, 1990; 1991) and that functional cannabinoid receptors can be cloned (Matsuda *et al.*, 1990).

This investigation was directed at identifying isolated tissue preparations that would serve as models with which to elucidate further the modes of action of psychotropic cannabinoids. Our strategy was to identify tissues in which cannabinoids show a significant degree of stereoselectivity and in which (−)-$\Delta^9$-tetrahydrocannabinol [(−)-$\Delta^9$-THC], the main psychoactive constituent of cannabis, produces effects when present in the medium at concentrations yielding tissue levels no higher than those achieved following *in vivo* administration of (−)-$\Delta^9$-THC at submaximal psychotropic doses (below 1 μM; Pertwee, 1990). The present experiments compared the potency of certain enantiomeric cannabinoids as inhibitors of electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine which was selected for study because its response to electrical stimulation was already known to be readily inhibited by psychotropic cannabinoids (Pertwee, 1990) and because cannabinoids can inhibit intestinal motility *in vivo* (Anderson *et al.*, 1975). The effects of cannabinoids on evoked contractions of a second nerve-smooth muscle preparation, the mouse isolated vas deferens, were also investigated. The cannabinoids studied were (−)-$\Delta^9$-THC (see above) and the 1,1-dimethylheptyl homologues of (+)- and (−)-11-hydroxy-$\Delta^8$-tetrahydrocannabinol [abbreviated to (+)- and (−)-DMH]. (+)- and (−)-DMH were chosen because only the (−)-enantiomer is psychoactive (Little *et al.*, 1989; Pertwee & Wickens, 1991) and because the stereochemical purity of the two isomers is particularly high (Mechoulam *et al.*, 1990). The chemical structures of (−)-$\Delta^9$-THC and DMH are shown in Figure 1.

Some of the results described in this paper have been presented to the British Pharmacological Society (Pertwee *et al.*, 1991).

## Methods

### *Tissue preparations*

All tissues were mounted in 3 ml organ baths under an initial tension of 0.5 g. Isometric contractions were evoked by elect-

[1] Author for correspondence.
[2] Present address: University Department of Pharmacology, Mansfield Road, Oxford OX1 3QT.
[3] Present address: Department of Natural Products, The Hebrew University of Jerusalem, Jerusalem 91 120, Israel.

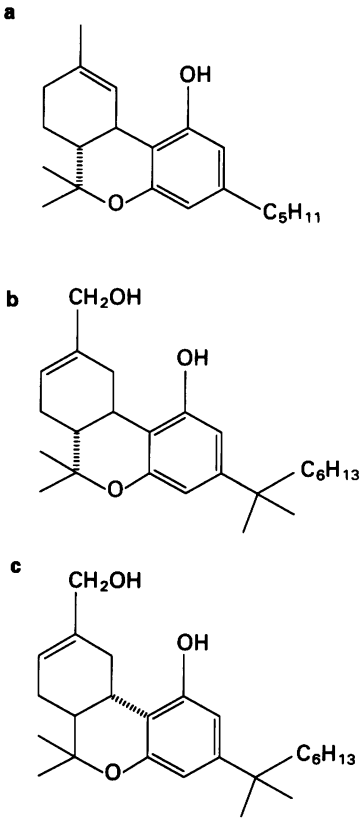


**Figure 1** The chemical structures of (a) (−)-$\Delta^9$-tetrahydrocannabinol [(−)-$\Delta^9$-THC] and of the 1,1-dimethylheptyl homologues of (b) (−)- and (c) (+)-11-hydroxy-$\Delta^8$-tetrahydrocannabinol [(−)- and (+)-DMH].

rical field stimulation through platinum electrodes attached to the upper and lower ends of each bath and were registered on a polygraph recorder (Grass model 7D) using Pye Ether UF1 transducers. The baths contained Krebs-Henseleit solution which was kept at 37°C and bubbled with 5% $CO_2$ in 95% $O_2$. They were siliconized at the beginning of each day (Sigmacote) and rinsed between experiments with ethanol followed by distilled water. Drug additions were made in volumes of 10 or 30 μl after the tissues had equilibrated. Only one dose of one drug was added to each tissue, pilot experiments having shown that the rates of onset of action of the cannabinoids used in this investigation are slow and that it is impossible to reverse their effects by perfusing the organ baths with drug-free Krebs-Henseleit solution. Once a drug had been added, tissues were incubated for up to 210 min without replacing the fluid in the bath.

Strips of myenteric plexus-longitudinal muscle were dissected from the small intestine of male albino Dunkin-Hartley guinea-pigs (180–400 g) by the method of Paton & Zar (1968) and set up for field stimulation in an inverted 'V' configuration. Each tissue was bathed in Krebs-Henseleit solution of the following composition (mM): NaCl 118.2, KCl 4.75, $MgSO_4.7H_2O$ 1.29, $KH_2PO_4$ 1.19, $NaHCO_3$ 25.0, glucose 11.0 and $CaCl_2.6H_2O$ 2.54. The solution also contained hexamethonium bromide (70 μM), mepyramine maleate (0.125 μM) and choline chloride (20 μM) (Kosterlitz *et al.*, 1970) and was stimulated with single bipolar rectangular pulses of 110% maximal voltage, 0.5 ms duration and 0.1 Hz frequency.

Vasa deferentia from TO mice (33–49 g) were bathed with $Mg^{2+}$-free Krebs-Henseleit solution (Hughes *et al.*, 1975; Corbett *et al.*, 1984). The tissue was stimulated with trains of 3 pulses of 110% maximal voltage and 0.5 ms duration at intervals of 250 ms. The trains were repeated at a frequency of 0.1 Hz. Pilot experiments showed that the amplitude of the twitch response of untreated vasa deferentia usually decreased quite markedly with time when trains of stimuli were applied continuously but remained constant when the tissue was subjected to 10 min periods of stimulation separated by 60 min periods in which there was no stimulation. Consequently, this pattern of intermittent stimulation was adopted for all the vas deferens experiments, the drug additions being made at the end of the first 11 min period of stimulation.

### *Drugs*

(+)- and (−)-DMH (code numbers HU-211 and HU-210 respectively) were synthesized at the Hebrew University, Jerusalem, Israel (Mechoulam *et al.*, 1990) and (−)-$\Delta^9$-THC was donated by the National Institute on Drug Abuse, U.S.A. Naloxone hydrochloride was obtained from Sigma and was dissolved in 0.9% w/v NaCl solution (saline). The cannabinoids were stored as ethanolic solutions which were kept in the dark at −20°C. Each cannabinoid was prepared for administration by mixing it with 2 parts of Tween 80 by weight, removing the ethanol by evaporation and then adding saline to form a 1 ml dispersion containing 30 nmol of the drug. To ensure that the dispersion would be homogeneous, the saline was added in a series of aliquots of increasing volume (50 μl × 2, 100 μl × 2, 200 μl and 500 μl), the mixture being shaken between additions with a vortex mixer. Dilutions were made serially, each dilution step involving the mixture of 1 volume of dispersion with up to 9 volumes of saline. Fresh dispersions were made up each day and were protected from light. Control experiments were performed with Tween 80.

### *Analysis of data*

Values have been expressed as means and limits of error as standard errors. The degree of inhibition of the twitch response is expressed in percentage terms and has been calculated by comparing the amplitude of the electrically-evoked twitch response immediately before drug administration with the amplitude of the twitch response at various times after drug administration. The concentration of a cannabinoid that causes 50% inhibition of the twitch response ($IC_{50}$) was used to characterize its potency. Non-linear regression analysis of the data obtained in our experiments with (−)-$\Delta^9$-THC and (−)-DMH (GraphPAD InPlot, GraphPAD Software, San Diego) showed the log concentration-response curves of these drugs in the vas deferens and myenteric plexus-longitudinal muscle preparation to be sigmoidal (correlation coefficients = 0.998 to 1.0). The log concentration at the midpoint of each of the sigmoid curves generated from our data by GraphPAD InPlot was estimated ($pD_2 \pm$ s.e.).

## Results

(−)-$\Delta^9$-THC and (−)-DMH each produced concentration-dependent decreases in the amplitude of evoked contractions in both the myenteric plexus preparation and the mouse vas deferens (Figures 2 to 4). Figure 2 shows the time courses for inhibition of the evoked twitch response of the myenteric plexus preparation by various concentrations of these drugs and indicates that they were both rather slow in their onset of action. The rate of onset of inhibition was concentration-dependent, the degree of inhibition reaching a plateau progressively more quickly as the concentration of either drug was increased. At concentrations that were approximately equi-effective, the onset of action of (−)-DMH was even slower than that of (−)-$\Delta^9$-THC (Figure 2). In the mouse vas deferens, (−)-$\Delta^9$-THC produced its maximal inhibitory effect within 70 min and (−)-DMH within 140 min (Figure 5). Log concentration-response curves (Figures 3 and 4) were constructed from measurements made after the inhibitory effects of these drugs had reached a maximum. For (−)-$\Delta^9$-THC,

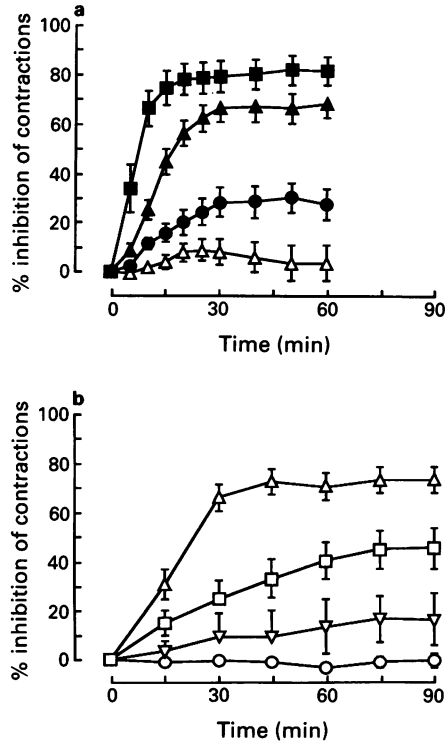


**Figure 2** Time courses of the inhibitory effects of (−)-$\Delta^9$-tetrahydrocannabinol ((−)-$\Delta^9$-THC, a) and the 1,1-dimethylheptyl homologue of (−)-11-hydroxy-$\Delta^8$-tetrahydrocannabinol ((−)-DMH, b) on the electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine. The symbols represent mean % inhibition of the twitch response by cannabinoid concentrations of $10^{-6}$ M (■), $10^{-7}$ M (▲), $3 \times 10^{-8}$ M (●), $10^{-8}$ M (△), $10^{-9}$ M (□) or $3 \times 10^{-10}$ M (▽). The effect of Tween 80 added in the amount required to produce a cannabinoid bath concentration of $10^{-6}$ M is denoted by (○). The vertical bars show standard errors ($n = 6$).

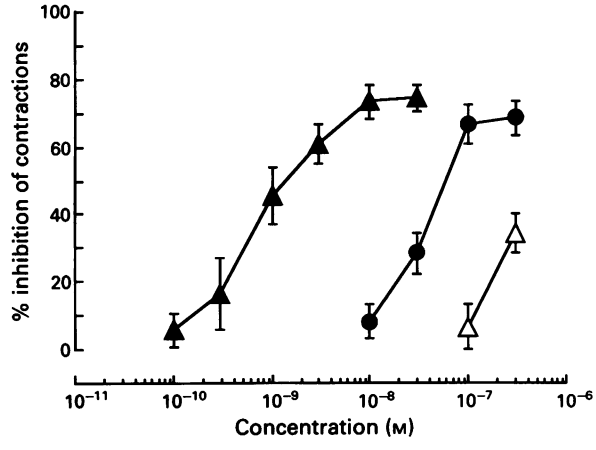


**Figure 3** Effect of (−)-$\Delta^9$-tetrahydrocannabinol ((−)-$\Delta^9$-THC, ●), the 1,1-dimethylheptyl homologue of (−)-11-hydroxy-$\Delta^8$-tetrahydrocannabinol ((−)-DMH, ▲) and (+)-DMH (△) on the size of electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine measured 30 min ((−)-$\Delta^9$-THC) or 90 min after drug administration. The symbols represent mean % inhibition of the twitch response and the vertical bars show standard errors ($n = 6$).

the measurements used were those made 30 min after its addition to the myenteric plexus preparation and 60 to 70 min after its addition to the vas deferens. For (+)- and (−)-DMH, the measurements used were those made 90 min after their addition to the first of these preparations and 130 to 140 min after their addition to the second.

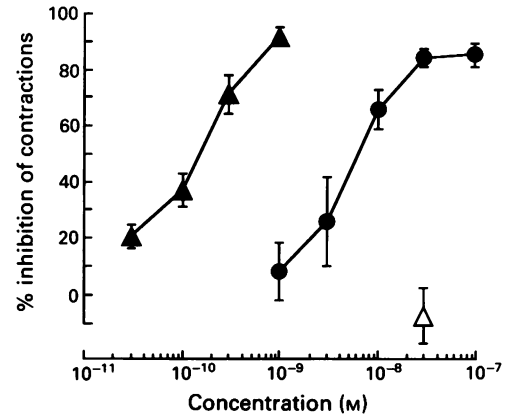


**Figure 4** Effect of (−)-$\Delta^9$-tetrahydrocannabinol ((−)-$\Delta^9$-THC, ●), the 1,1-dimethylheptyl homologue of (−)-11-hydroxy-$\Delta^8$-tetrahydrocannabinol ((−)-DMH, ▲) and (+)-DMH (△) on the size of electrically-evoked contractions of the mouse vas deferens measured 60 min ((−)-$\Delta^9$-THC) or 130 min after drug administration. The symbols represent mean % inhibition of the twitch response and the vertical bars show standard errors ($n = 6$).

By itself, the vehicle Tween 80 did not inhibit the twitch response of either preparation when it was added at a dose 3.3 times above the upper limit of its dose-range in the cannabinoid experiments. Indeed, additions of this dose of Tween 80 were followed by slight increases in twitch amplitude. More specifically, when Tween 80 was added to the mouse vas deferens in the amount (1.9 μg) that would have been required to expose the tissue to a (−)-$\Delta^9$-THC concentration of 1 μM, the mean twitch response increased by 12.2 ± 7.3%, over the first 70 min, and by 4.8 ± 4.1%, over 140 min ($n = 6$). In the myenteric plexus preparation, addition of 1.9 μg Tween 80 was followed by an increase in mean twitch amplitude of 0.7 ± 1.9%, over the first 30 min, and of 0.6 ± 2.9%, over 90 min ($n = 6$). In contrast, a dose of 5.7 μg of Tween 80, which is 10 times above the upper limit of the dose range of vehicle used in the (−)-$\Delta^9$-THC experiments, did inhibit the twitch response of the myenteric plexus preparation, the degree of inhibition after 30 and 90 min being respectively 16.8 ± 4.7% and 42.7 ± 9.5% ($n = 6$). Each of these mean changes in twitch response are significantly differ-

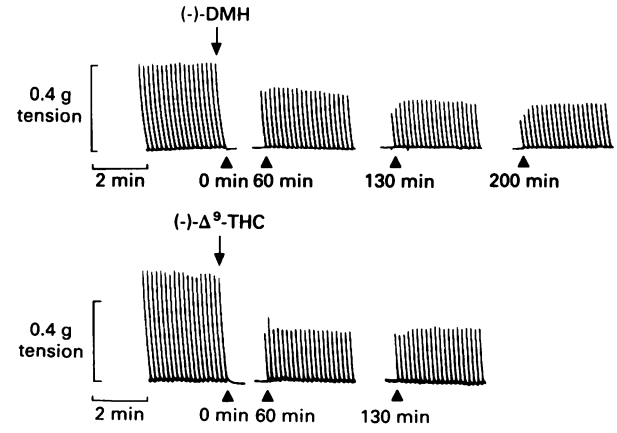


**Figure 5** The effects of the 1,1-dimethylheptyl homologue of (−)-11-hydroxy-$\Delta^8$-tetrahydrocannabinol ((−)-DMH) and of (−)-$\Delta^9$-tetrahydrocannabinol ((−)-$\Delta^9$-THC) on electrically-evoked twitch contractions of the mouse isolated vas deferens. The drugs were added at time zero. The preparations were stimulated for 11 min before drug addition and then intermittently for 10 min periods commencing 60, 130 or 200 min after drug addition. They were stimulated with trains of 3 pulses of 110% maximal voltage and 0.5 ms duration at intervals of 250 ms. The trains were repeated at a frequency of 0.1 Hz.

ent ($P<0.01$; Student's $t$ test for unpaired data) from those observed to occur at the corresponding time after the addition of 1.9 μg of Tween 80 (see above).

As shown in Figures 3 and 4, concentrations of (−)-$\Delta^9$-THC that decreased twitch heights by 50% were 60 nM in the myenteric plexus preparation ($pD_2 = 1.668 \pm 0.037$) and 6.3 nM in the vas deferens ($pD_2 = 0.73 \pm 0.03$). For (−)-DMH, the corresponding $IC_{50}$ values were respectively 1.4 nM ($pD_2 = -0.124 \pm 0.085$) and 0.15 nM ($pD_2 = -0.716 \pm 0.006$), indicating that in both preparations it is about 40 times more potent than (−)-$\Delta^9$-THC as an inhibitor of the twitch response. Although (+)-DMH also inhibited the twitch response of the myenteric plexus preparation, it was about 500 times less potent than its (−)-isomer (Figure 3). At a concentration of 30 nM, (+)-DMH had no detectable effect on the evoked twitch response of the mouse vas deferens, the results obtained demonstrating that, in this preparation, (−)-DMH is at least 1000 times more potent than (+)-DMH (Figure 4).

The ability of submaximal concentrations of (−)-$\Delta^9$-THC to inhibit the twitch response of the myenteric plexus preparation (75 nM) or the mouse vas deferens (10 nM) was not attenuated by naloxone, 300 nM, when this was added 10 min before the cannabinoid. Nor were the effects of these concentrations of (−)-$\Delta^9$-THC on either preparation reversed by naloxone (300 nM), added 60 or 70 min after the cannabinoid (data not shown).

## Discussion

The results obtained in the present study demonstrate that cannabinoids can produce a concentration-related inhibition of the electrically-evoked twitch response of the mouse isolated vas deferens and of the myenteric plexus preparation of the guinea-pig small intestine. More importantly, they demonstrate the 1,1-dimethylheptyl homologue of 11-hydroxy-$\Delta^8$-tetrahydrocannabinol (DMH) to be highly potent as an inhibitor of the twitch response of both preparations and to exhibit a remarkable degree of stereoselectivity, (−)-DMH being considerably more potent than (+)-DMH. The results from the experiments with (−)-$\Delta^9$-THC showed that this cannabinoid can also inhibit the electrically-evoked twitch response of the mouse isolated vas deferens and, in addition, confirmed its ability to inhibit the twitch response of the myenteric plexus preparation (Pertwee, 1990). Our findings are in agreement with a report, published during the preparation of this paper, that (−)-$\Delta^9$-THC is a potent inhibitor of the twitch response of the vas deferens of Swiss Webster mice (Pacheco *et al.*, 1991). Indeed, Pacheco *et al.* (1991) found the $IC_{50}$ of (−)-$\Delta^9$-THC to be 4 nM which is very close to the $IC_{50}$ of this drug determined in the present investigation with TO mice (6.3 nM). Our findings are also consistent with an earlier observation that (−)-$\Delta^9$-THC is more potent than its (+)-isomer in inhibiting the twitch response of the guinea-pig myenteric plexus preparation (Roth, 1978). However, the potency difference between (−)- and (+)-DMH observed in the present experiments with the myenteric plexus preparation (about × 500) was much greater than the potency difference between (−)- and (+)-$\Delta^9$-THC (× 25) observed by Roth (1978), an indication perhaps that the samples of (−)- and (+)-DMH used in this investigation were of a higher stereochemical purity than the samples of (−)- and (+)-$\Delta^9$-THC used in the previous study (Mechoulam *et al.*, 1990).

The electrically-evoked twitch response of the myenteric plexus preparation of guinea-pig small intestine and of the mouse vas deferens can be readily inhibited by opioid receptor agonists (Lesley, 1987). It is unlikely, however, that cannabinoids inhibit the twitch response of these preparations by acting through opioid receptors, as it was found in the present study that the inhibitory effect of (−)-$\Delta^9$-THC on the twitch response of the myenteric plexus preparation and mouse vas deferens was not attenuated by naloxone when this was applied at a concentration known to antagonize μ, δ, and κ opioid receptors (300 nM; Kosterlitz & Paterson, 1990).

An important practical difficulty associated with the study of the pharmacology of cannabinoids, is the relatively low aqueous solubility of this group of drugs (Roth & Williams, 1979). This property together with their high lipophilicity (Roth & Williams, 1979; Thomas *et al.*, 1990) may well have accounted for the rather slow onset of action of (−)-$\Delta^9$-THC and DMH that was observed in this investigation. More specifically, it is likely that the lipophilic nature of these cannabinoids will cause them to be sequestered by the solubilizing agent, Tween 80 (Roth & Williams, 1979) and will delay equilibration between cannabinoid molecules in free solution and those present in the tissue. The low solubility of these drugs in water would be expected to delay their onset of action by limiting their rate of diffusion across the aqueous phase from solubilizing agent to tissue. The finding that the rate of onset of action of (−)-DMH was less than that of (−)-$\Delta^9$-THC is consistent with these ideas as (−)-DMH is known to be somewhat more lipophilic than (−)-$\Delta^9$-THC (Thomas *et al.*, 1990). Interestingly, (−)-DMH has also been reported to have a slower onset of action than (−)-$\Delta^9$-THC *in vivo* (Järbe *et al.*, 1989).

(−)-DMH and (−)-$\Delta^9$-THC were each about 10 times more potent as inhibitors of the twitch response in the mouse vas deferens than in the myenteric plexus preparation, (−)-DMH being the more potent of the two cannabinoids in both preparations. (+)-DMH was less potent than (−)-$\Delta^9$-THC, the order of potency of the three cannabinoids studied correlating with their psychotropic activity (Little *et al.*, 1989). The relative potency of these cannabinoids was approximately the same in the myenteric plexus preparation as in the mouse isolated vas deferens, supporting the notion that their mode of action in each tissue is the same. A fuller comparison of the structural requirements for inhibition of the twitch response with those for psychotropic activity is now required, as such experiments would help to establish whether the myenteric plexus preparation and the mouse vas deferens could be used as models for studying the central pharmacology of cannabinoids or whether they are more suitable as models for investigating the peripheral pharmacology of these drugs. The observations made in this investigation, that (−)-$\Delta^9$-THC and (−)-DMH are highly potent as inhibitors of the twitch response of the guinea-pig myenteric plexus preparation and the mouse vas deferens and that DMH shows considerable stereoselectivity, suggest that inhibition of the twitch response by cannabinoids in both tissues are mediated by cannabinoid receptors. If such receptors are indeed present, it should be possible to demonstrate that, like certain areas of the brain (Herkenham *et al.*, 1990; 1991), the guinea-pig small intestine and the mouse vas deferens contain specific high affinity cannabinoid binding sites.

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# Studies on the cardiac actions of flosequinan *in vitro*

[1]R.W. Gristwood, J. Beleta, J. Bou, I. Cardelús, A.G. Fernández, J. Llenas & P. Berga

Division of Biological Sciences, Laboratorios Almirall, Cardener 68-74, Barcelona 08024, Spain

**1** We have investigated the *in vitro* cardiac actions of flosequinan and of its major metabolite in man, BTS 53554.

**2** Positive inotropic activity was seen with flosequinan in guinea-pig isolated ventricles, the threshold concentration for effect being less than $1 \times 10^{-5}$ M. BTS 53554 was approximately half as potent as the parent compound.

**3** In guinea-pig working whole hearts flosequinan increased left ventricular $dp/dt_{max.}$, indicating a positive inotropic action. This effect was accompanied by increases in heart rate, cardiac output and stroke volume.

**4** The virtual complete inhibition of inotropic responses to flosequinan and BTS 53554 by carbachol suggests that these responses are adenosine 3':5'-cyclic monophosphate (cyclic AMP)-mediated.

**5** Flosequinan was shown to increase calcium inward current in guinea-pig ventricle, an action consistent with a cyclic AMP involvement in the response.

**6** The inotropic activity of flosequinan was not potentiated by the selective phosphodiesterase (PDE) III inhibitor SK&F 94120, a result which indicates that flosequinan does not increase cyclic AMP concentrations via stimulation of adenylate cyclase.

**7** Flosequinan inotropic responses were potentiated by rolipram, a selective PDE IV inhibitor, a result consistent with flosequinan being itself a PDE III inhibitor.

**8** Biochemical studies with purified enzymes confirmed that flosequinan and BTS 53554 are relatively selective inhibitors of PDE III.

**9** A comparison of pharmacological and biochemical data for both flosequinan and BTS 53554 indicates that their PDE III inhibitory potency is sufficient to account for their inotropic activity.

**Keywords:** Flosequinan; BTS 53554; inotropic activity *in vitro*; phosphodiesterase inhibition

## Introduction

Flosequinan (BTS 49465) is an agent currently under development for the treatment of congestive heart failure and hypertension (Cowley *et al.*, 1984; Kessler & Packer, 1987; Sim *et al.*, 1988). Flosequinan was initially described as a vasodilator agent (e.g. Cowley *et al.*, 1984) although recently it has become recognized that the compound has additional positive inotropic properties (e.g. Yates & Hicks, 1988; Falotico *et al.*, 1989; Greenberg & Touhey, 1990) which may have clinical relevance. Clinical studies have shown that flosequinan has an active sulphone metabolite, BTS 53554, which probably contributes to the overall haemodynamic response in man following oral administration (Wynne *et al.*, 1985).

The exact mechanisms of action of flosequinan (or its active metabolite) on the heart and blood vessels are currently unclear. It has been reported that the *in vitro* vasodilator effects of large concentrations of flosequinan are associated with intracellular increases in guanosine 3':5'-cyclic monophosphate (cyclic GMP) concentrations (Allcock *et al.*, 1988) and that it produces weak non selective inhibition of cyclic nucleotide phosphodiesterases (PDEs) in bovine and guinea-pig cardiac tissue, (Frodsham *et al.*, 1989; 1990) and in guinea-pig vascular tissue (Yates, 1991). The positive inotropic responses in dogs *in vivo* persist after β-adrenoceptor blockade (Falotico *et al.*, 1989) implying a mode of action independent of β-receptor stimulation.

The purpose of the present study was to investigate further the effects of flosequinan on cardiac function in guinea-pigs and then to examine effects on PDE isoenzymes. There are currently known to be at least 5 distinct families of PDE isoenzymes: PDEs I to V, these differ in their substrate specificity and affinity as well as in their regulatory properties (see Beavo & Houslay, 1990). Some of the studies were also carried out with the metabolite BTS 53554, two reference PDE III inhibitors, SK&F 94120 and amrinone (Gristwood *et al.*, 1986b) and the non selective PDE inhibitor, 3 isobutyl-1-methylxanthine (IBMX).

Preliminary presentations of some of this work were made to the British Pharmacological Society meetings in London in January and December 1990 (Gristwood *et al.*, 1990; Beleta *et al.*, 1991).

## Methods

### *Inotropic activity in guinea-pig isolated ventricles*

Hearts were removed from male guinea-pigs (weight 500–600 g). Strips were dissected from the right ventricles having the dimensions 1 cm long x 1 mm wide (maximum). These were mounted in 30 ml organ baths containing Krebs Henseleit solution gassed with 5% $CO_2$ in $O_2$ at 37°C. The preparations were placed under 1 g resting tension whilst electrically stimulated to contract at 1 Hz (threshold voltage +20%). Isometric tension was recorded onto a Letica 4000 polygraph by use of isometric force transducers (Letica TRI 010). Preparations were allowed 30 min to stabilize, prior to drug addition by a cumulative concentration procedure. Drug effects were measured as percentage increases in development of tension (force of contraction) over pre-drug basal values. $Inc_{50}$ values (concentrations causing a 50% increase in force of contraction) were calculated by a least squares regression analysis and s.e.mean values calculated from individual response curves.

[1]Author for correspondence.

### *Effects in guinea-pig isolated working hearts*

Animals were killed 20 min after heparin administration (2000 i.u., i.p.) The heart was quickly excised and placed in a beaker containing Krebs solution at 4°C where any remaining pericardial tissue was removed. The heart was then mounted on the working heart apparatus as described by Flynn *et al.* (1978). When working, Krebs solution (equilibrated with 5% $CO_2$ in $O_2$ at 37.0°C) entered the left atrium at a fixed filling pressure of 12.5 $cmH_2O$ and cardiac output was ejected by the left ventricle against a fluid column of height 70 cm. During drug administration by use of a cumulative concentration procedure, the perfusion system was closed, i.e., both coronary and aortic flows were recirculated. Parameters measured were: aortic flow (Skalar 4 mm i.d. electromagnetic flow probe connected to a Skalar MDL 1401 flow meter), coronary flow (Data Logic RC1 drop counter), left intraventricular pressure (LVP) (Druck PDCR 75 pressure transducer), $dp/dt_{max}$. (Lectromed 5270 differentiator) and heart rate (Lectromed 5250 rate meter triggered by the LVP pressure signal). Responses were recorded directly onto a Lectromed MT8P polygraph and expressed as percentage increases over pre-drug values.

### *Mechanism of action studies*

*Effects of carbachol* The effects of carbachol were studied on inotropic responses to flosequinan and other drugs in guinea-pig ventricle strips. For this, inotropic responses to the drugs were allowed to stabilize and then carbachol added over the concentration range $1 \times 10^{-8}$ M to $1 \times 10^{-6}$ M.

*Interaction with isoprenaline* The interaction of flosequinan with isoprenaline was studied in guinea-pig ventricle strips. Pairs of strips were obtained from the same ventricle one of which was initially treated with flosequinan $1 \times 10^{-4}$ M and the other with isoprenaline $3 \times 10^{-9}$ M. Following stabilization of responses the second drug was then added.

*Interaction with other phosphodiesterase isoenzyme selective inhibitors* The interaction of flosequinan with a known PDE III inhibitor, SK&F 94120 at $3 \times 10^{-5}$ M, was studied in guinea-pig ventricle strips. Pairs of strips were obtained from the same ventricle, one of which was treated with SK&F 94120 before addition of flosequinan.

The interaction of drugs with the PDE IV inhibitor, rolipram (Reeves *et al.*, 1987) were also studied. For this, preparations were pretreated with SK&F 94120 $3 \times 10^{-5}$ M, flosequinan $1 \times 10^{-4}$ M, BTS 53554 or vehicle and allowed 10 min to equilibrate before the addition of rolipram $1 \times 10^{-6}$ M.

*Separation of phosphodiesterase isoenzymes* The effects of drugs on purified PDE enzymes were studied. Cyclic nucleotide phosphodiesterases I to IV were obtained from guinea-pig ventricular tissue following the procedure decribed by Reeves *et al.* (1987), except that the chromatographic step was performed with a MONO-Q ion exchange column attached to a Pharmacia FPLC system. A representative elution profile of PDE isoenzyme activities is shown in Figure 1. The isoenzymes were characterized before use in terms of substrate selectivity and affinity and by the effect of calcium ions (10 μM) plus calmodulin (1.2 μM), cyclic GMP and the selective inhibitors rolipram, SK&F 94120 and zaprinast (see Table 1A). Active enzyme fractions were pooled and kept frozen at −20°C in the presence of 1 g $l^{-1}$ bovine serum albumin until used.

Cyclic GMP specific phosphodiesterase (PDE V) was purified from dog platelets. Briefly, blood was freed of red and white cells by differential centrifugation and the platelet rich plasma was washed three times with phosphate buffered saline (10 mM, Na, K phosphate, 140 mM NaCl, EDTA 2 mM, pH 7.4). The pellet was resuspended in 20 mM BisTris pH 6.5 buffer, containing 50 mM sodium acetate, 2 mM benzamidine, 2 mM EDTA, 5 mM β-mercaptoethanol and 50 μM phenylmethylsuphonylfluoride (PMSF), and disrupted by sonication (Branson Sonifier 250, 50% output, 1 min). All procedures were performed at 4°C. The cell lysate was centrifuged at 40000 *g* for 20 min and the supernatant was filtered through a 0.22 μM filter. A volume of 2 ml (corresponding to $10^{10}$ cells) was chromatographed, characterized and stored following the same procedures described for the guinea-pig enzymes. The preparation of PDE V was known not to be contaminated with PDE I because it was not active when cyclic AMP was used as substrate and was not affected by $Ca^{2+}$ calmodulin.

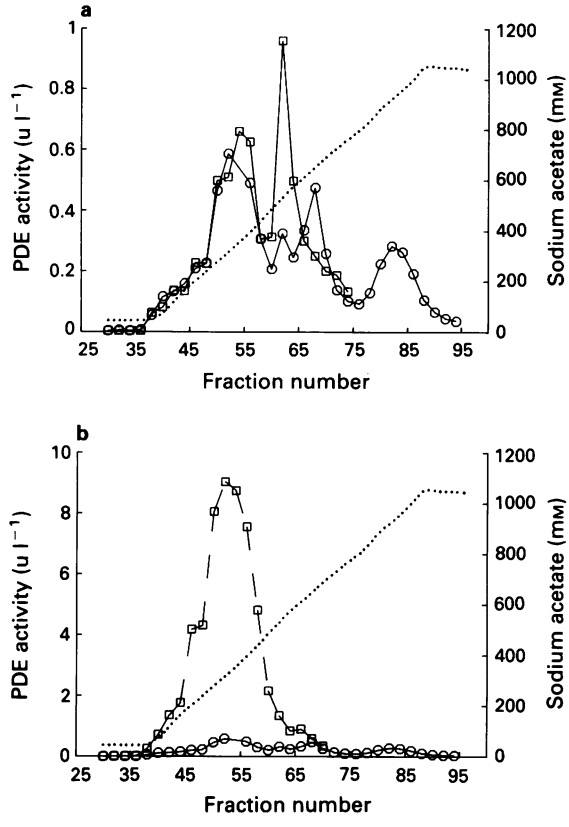


**Figure 1** Elution profiles of cyclic nucleotide phosphodiesterase (PDE) activities from guinea-pig ventricular muscle on a MONO Q ion exchange column. The supernatant from the low speed centrifugation was filtered through 0.22 μM filters and 5 to 10 ml were applied at a flow rate of 1 ml $min^{-1}$ to a 1 ml column. This was washed with 15 ml of homogenization buffer and the isoenzymes eluted with a linear gradient of sodium acetate (50–1000 mM) in the same buffer. Fractions (0.5 ml) were collected and assayed for PDE activity. (a) Substrate was 1 μM cyclic AMP in the absence (○) and in the presence of 1 μM cyclic GMP (□). (b) Substrate was 1 μM cyclic AMP in the absence (○) and in the presence of calcium ions and calmodulin (□). The different isoenzymes were identified in the following fractions:: PDE I, 48 to 56; PDE II, 61 to 63; PDE IV, 68 to 70; PDE III, 84 to 87.

Cyclic nucleotide phosphodiesterases were assayed following the procedure of Thompson & Strada (1984). Inhibition assays at 30°C for 20 min were run in duplicate at a substrate concentration of 0.25 μM. The substrate was cyclic AMP for PDE I to IV and cyclic GMP for PDE V. PDE I was assayed in the presence of calcium ions and calmodulin at the above specified concentrations and PDE II was assayed in the presence of 5 μM unlabelled cyclic GMP. For all purified enzymes the slopes of the inhibition curves obtained with drugs were not significantly different from −1.

**Table 1** (A) Characterization of the isolated phosphodiesterase (PDE) isoenzymes used

| | *Phospodiesterase isoenzyme* | | | | |
|---|---|---|---|---|---|
| | I | II | III | IV | V |
| $K_m$ cyclic AMP (μM) | 1.6 | 86** | 0.3 | 2.3 | >1000 |
| $K_m$ cyclic GMP (μM) | 1.8 | 23*** | n.d. | >1000 | 2.7 |
| $Ca^{2+}$/calmodulin | 15 ± 2.5* | n.e. | n.e. | n.e | n.e. |
| Cyclic GMP | n.d. | 15 ± 3* | 7.0 ± 0.17 | <3.7 (27) | n.d. |
| Zaprinast | 5.1 ± 0.1 | 4.2 ± 0.3 | <3.7 | 4.1 ± 0.05 | 6.8 ± 0.05 |
| SK&F 94120 | <3.7 (26) | <3.7 (33) | 5.1 ± 0.1 | <3.7 (2.5) | <3.7 (30) |
| Amrinone | <3.7 (13) | <3.7 (43) | 4.7 ± 0.2 | <3.7 (26) | n.d. |
| Rolipram | <3.7 (19) | <3.7 (18) | 3.9 ± 0.13 | 6.4 ± 0.06 | 3.7 ± 0.02 |
| IBMX | 5.2 ± 0.04 | 4.3 ± 0.15 | 5.3 ± 0.02 | 5.0 ± 0.03 | 5.3 ± 0.01 |
| (B) Inhibition of the same isoenzymes by flosequinan and BTS 53554 | | | | | |
| Flosequinan | <3.7 (21) | <3.7 (26) | 4.2 ± 0.03 | <3.7 (16) | <3.7 (38) |
| BTS 53554 | <3.7 (14) | <3.7 (30) | 3.9 ± 0.02 | <3.7 (16) | <3.7 (33) |

For each drug 5–7 concentrations were tested in duplicate for at least 2 different enzyme preparations. Values are $-\log_{10}$ $IC_{50}$ ± s.e.mean except where stated. Numbers in parentheses indicate percentage inhibition at the highest drug concentration tested (200 μM).
n.d. not determined.
n.e. no effect.
* indicates fold activitation.
** indicates $S_{0.5}$ because of non hyperbolic kinetics.
*** $K_m$value for cyclic AMP in the presence of 5 μM cyclic GMP.

*Effects on slow inward current* Intracellular action potentials along with force of contraction were recorded from guinea-pig papillary muscles by methods previously described (Gristwood *et al.*, 1987).

Preparations were electrically stimulated to contract (0.5 Hz using large supramaximal voltages) in depolarizing Krebs solution at 37°C (see drugs and solutions). Following a 15 min period of equilibration under these conditions flosequinan was administered to the perfusion medium and its effect evaluated at the time of maximal response (10 min later).

### *Drugs, reagents and solutions*

The following drugs were used, SK&F 94120 (5-(4-acetamidophenyl)-pyrazin-2(H1)-one acetamidophenyl; a gift from SK&F Ltd., Welwyn, U.K.), rolipram (a gift from Schering A.G., Germany), zaprinast (a gift from May and Baker, U.K.), amrinone, obtained from Resfar, Italy, isoprenaline sulphate obtained from Boehringer Ingelheim, W. Germany and propranolol hydrochloride obtained from Chemo Iberica, Spain. Flosequinan and the sulphone metabolite BTS 53554 were synthesized in the Department of Chemical Synthesis, Laboratorios Almirall, S.A., Spain.

[8-$^3$H]-adenosine 3':5'-cyclic monophosphate and [8-$^3$H]-guanosine 3':5'-cyclic monophosphate were from Amersham International (Bucks, U.K.). Benzamidine, cyclic AMP, cyclic GMP, calmodulin, IBMX, carbachol and PMSF were obtained from Sigma-Aldrich Química S.A. (Madrid, Spain).

For the pharmacological studies, drugs were prepared as stock solutions of $10^{-2}$ M. Initial solvents used were: for SK&F 94120 1% NaOH 1 N in water, for flosequinan 10% polyethylene glycol (PEG) 300 in water and for BTS 53554 50% PEG 300 in water. All other drugs were prepared in physiological saline. Dilutions were made with Krebs solution. For the biochemical studies drugs were dissolved in dilute HCl or dimethylsulphoxide (DMSO). Drug vehicles at concentrations employed did not affect enzyme activities.

The composition of the normal Krebs solution used was as follows (in mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, $MgCl_2$ 1.2, $CaCl_2$ 2.55, $NaH_2PO_4$ 1 and glucose 11.0. For the depolarizing solution used for electrophysiological experiments the potassium ion concentration was elevated from 4.7 to 22 mM with an equimolar reduction in sodium ion concentration. Propranolol $3 \times 10^{-7}$ M was included in this solution to inhibit effects attributed to endogenous catecholamines that may have been released by the large stimulus intensity.

### *Statistics*

Values are given as mean ± s.e. of mean.

Where relevant, statistical analyses were carried out by Student's *t* test for paired or unpaired data as appropriate.

## Results

### *Inotropic effects on guinea-pig ventricles*

Guinea-pig ventricles consistently responded to flosequinan with increases in force of contraction as shown in Figure 2. Responses commenced within 30 s of drug addition and had plateaued by 5 min. Responses were sustained. The threshold concentration for effect was less than $1 \times 10^{-5}$ M and the $Inc_{50}$ value was $2.9 \times 10^{-5}$ M ($\pm 1.2 \times 10^{-5}$ M). SK&F 94120 was approximately 7 fold more potent than flosequinan ($Inc_{50}$, $4 \times 10^{-6}$ M ± $2.4 \times 10^{-6}$ M), whilst BTS 53554 was about half as potent as flosequinan ($Inc_{50}$, $6.0 \times 10^{-5}$ M ± $0.8 \times 10^{-5}$ M), as shown in Figure 2. The differences in potencies between all 3 drugs were significant ($P<0.05$).

Propranolol $3 \times 10^{-7}$ M had no effect on the potency of flosequinan ($Inc_{50}$ value with propranolol $2.1 \times 10^{-5}$ M ± $0.8 \times 10^{-5}$ M, $n = 3$).

### *Effects on guinea-pig isolated working hearts*

Immediately prior to administration of flosequinan, values of $dp/dt_{max.}$, heart rate, cardiac output and coronary flow were: 2100 ± 200 mmHg $s^{-1}$, 226 ± 5 beats $min^{-1}$, 76 ± 10 ml $min^{-1}$ and 18 ± 2 ml $min^{-1}$ ($n = 4$) respectively. Flosequinan pro-

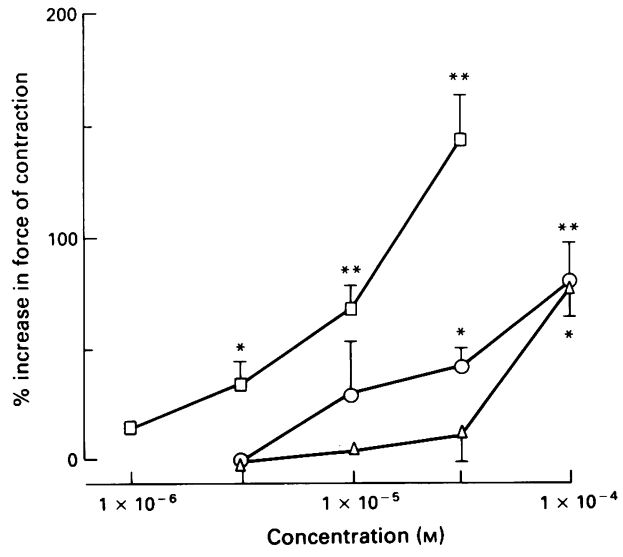


**Figure 2** Effects of flosequinan (○), BTS 53554 (△) and SK&F 94120 (□), on the force of contraction of guinea-pig ventricle strips. Basal control values of force of contraction were 82 ± 7 mg, 76 ± 12 mg and 95 ± 8 mg respectively. Points are means of $n = 7$; vertical bars show s.e.mean. Significant responses are indicated $*P<0.05$; $**P<0.001$.

duced significant concentration related increases in $dp/dt_{max.}$, indicating an enhancement of left ventricular contractility (Figure 3). Heart rate and cardiac output were also significantly increased by flosequinan. Coronary flow was little affected at concentrations up to $1 \times 10^{-4}$ M and a small decrease of this parameter occurred at $1 \times 10^{-3}$ M.

Although not shown in Figure 3, flosequinan also consistantly caused increases in stroke volume. Thus the mean pre-drug value of stroke volume was 0.34 ± 0.04 ml and after administration of the compound at $1 \times 10^{-4}$ M was 0.39 ± 0.04 ml (difference significant, $P<0.05$) indicating a mean increase of 15%.

### *Effects of carbachol on inotropic responses in guinea-pig ventricles*

Carbachol caused concentration-dependent inhibition of positive inotropic responses to flosequinan and BTS 53554 (both at $1 \times 10^{-4}$ M) and SK&F 94120 ($3 \times 10^{-5}$ M), as shown in Figure 4. Carbachol, $1 \times 10^{-6}$ M, produced a complete reversal of positive inotropic activity to all 3 drugs.

### *Interaction with SK&F 94120 in guinea-pig ventricles*

Flosequinan, $1 \times 10^{-4}$ M, in untreated preparations caused an 80% ± 15 increase in force of contraction. In the paired preparations SK&F 94120 at $3 \times 10^{-5}$ M caused a mean 143 ± 21% increase force of contraction and the subsequent addition of flosequinan, $1 \times 10^{-4}$ M, did not produce a further increase (increase from control remained at 140 ± 18%, $n = 5$). In contrast, responses to isoprenaline, $1 \times 10^{-8}$ M were markedly larger in the presence of SK&F 94120, increases in the force of the contraction being 82 ± 15% with isoprenaline alone and 220 ± 30% in combination with SK&F 94120, $3 \times 10^{-5}$ M ($n = 5$).

### *Interaction with rolipram in guinea-pig ventricles*

Rolipram in untreated preparations produced no response, but in preparations treated with flosequinan, BTS 53554 or SK&F 94120 resulted in further inotropic responses as shown in Figure 5, indicating a synergistic interaction with all three drugs. The magnitude of this subsequent rolipram response was greatest in SK&F 94120-treated preparations, intermediate in flosequinan-treated preparations and smallest in the BTS 53554-treated preparations.

### *Interaction with isoprenaline in guinea-pig ventricles*

Flosequinan $1 \times 10^{-4}$ M or isoprenaline $3 \times 10^{-9}$ M alone produced increases in force of contraction of 85 ± 29% and 67 ± 17% ($n = 4$) respectively. In combination the 2 drugs caused an increase of 381 ± 58% ($n = 8$) indicating a synergistic interaction.

### *Studies with isolated cyclic nucleotide phosphodiesterase enzymes*

The effects of flosequinan and its metabolite (BTS 53554), on the activity of the five isolated PDE's are shown in Table 1B. It can be seen that both compounds, like SK&F 94120 and amrinone, inhibited PDE type III, although the compounds differed in their potencies against this enzyme with a potency order of SK&F 94120 > amrinone > flosequinan > BTS 53554.

None of the four compounds achieved 50% inhibition of the other PDE isoenzymes at the highest concentration tested. In contrast the non-selective PDE inhibitor IBMX inhibited all isoenzyme types (Table 1A).

### *Effects of flosequinan on slow response action potentials in guinea-pig ventricles*

Flosequinan ($1 \times 10^{-4}$ M), 10 min after administration, produced a stable increase in force of contraction that was accompanied by increases in $dV/dt_{max.}$ and the amplitude and duration of the slow response action potential. These electrophysiological effects of flosequinan were consistently observed in 5 preparations, as shown in Table 2.

## Discussion

The results from the present study have confirmed previous findings that flosequinan has positive inotropic activity *in vitro* (Falotico *et al.*, 1989; Greenberg & Touhey, 1990). Thus, increases in ventricular contractility were observed in

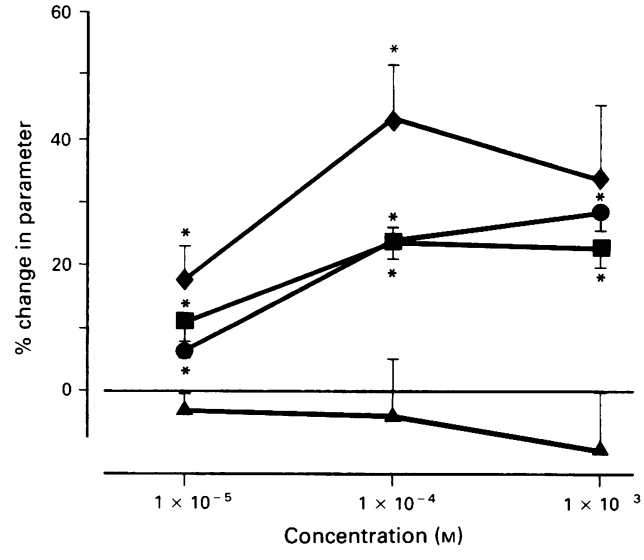


**Figure 3** Effects of flosequinan on guinea-pig isolated working hearts. Parameters shown are left ventricular $dp/dt_{max}$ (●), heart rate (■), cardiac output (◆) and coronary flow (▲). Points show mean values with s.e.mean indicated by vertical bars. $*P<0.05$; $**P<0.005$.

**Table 2** Effects of flosequinan on slow response action potentials in guinea-pig ventricle

| *Condition* | $dV/dt_{max.}$ ($V\ s^{-1}$) | *Action potential amplitude* (mV) | *Action potential duration* (ms) |
|---|---|---|---|
| Control | 7.3 ± 0.8 | 81.9 ± 3.7 | 67.2 ± 8.4 |
| Flosequinan $1 \times 10^{-4}$ M | 11.5 ± 1.1* | 88.6 ± 3.7* | 101.8 ± 9.0* |

*$P<0.05$: significantly different from control values; ($n = 5$).

guinea-pig ventricles and in guinea-pig working whole hearts. The increased contractility of the working hearts was associated with a tachycardia and an increase in cardiac output. In addition the results showed that flosequinan can cause an increase in cardiac output via a direct action on the heart. The finding that stroke volume was increased indicates that the enhanced contractility played a part in the output increase.

Concerning mechanism of action: reversal of positive inotropic responses by carbachol has been shown to be selective for cyclic AMP-dependent inotropic responses (Endoh, 1980; Gristwood *et al.*, 1987) and, therefore, represents a relatively simple procedure to test for this. That carbachol at

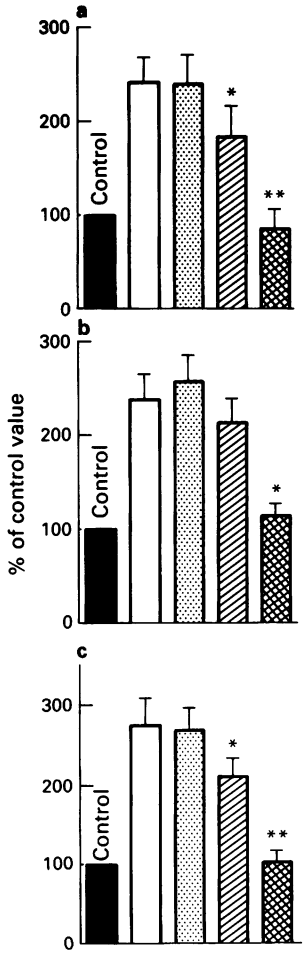


**Figure 4** Effects of carbachol at $1 \times 10^{-8}$ M (▒), $1 \times 10^{-7}$ M (▨) and $1 \times 10^{-6}$ M (▩) on inotropic responses to (a) flosequinan $1 \times 10^{-4}$ M, (b) BTS 53554 $1 \times 10^{-4}$ M and (c) SK&F 94120 $3 \times 10^{-5}$ M. Basal control values of force of contraction prior to drug addition were (a) 83 ± 8 mg, (b) 50 ± 14 mg and (c) 49 ± 10 mg. Values shown are means with s.e.mean shown by vertical bars, $n = 6-7$. *$P<0.05$; **$P<0.005$.

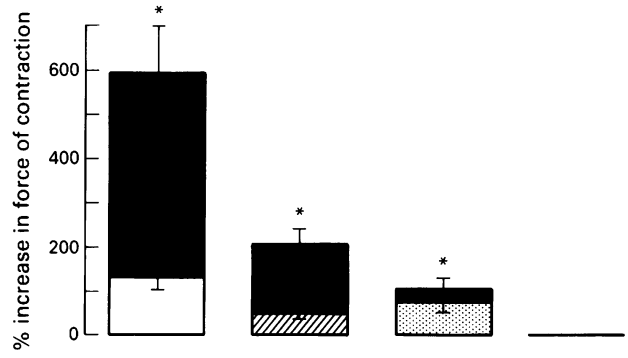


**Figure 5** Responses to rolipram $1 \times 10^{-6}$ M (■) in guinea-pig ventricles pretreated with SK&F 94120 $3 \times 10^{-5}$ M (□), flosequinan $1 \times 10^{-4}$ M (▨) and BTS 53554 $1 \times 10^{-4}$ M (▒), and also in non pretreated preparations (no response). Basal control values of force of contraction were 72 ± 28 mg, 86 ± 14 mg, 110 ± 5 mg and 70 ± 10 mg respectively. Values are means with s.e.mean shown by vertical bars, $n = 5-8$. *Response to rolipram significant: $P<0.05$.

$1 \times 10^{-6}$ M virtually abolished the inotropic activity of effective concentrations of flosequinan, BTS 53554 and SK&F 94120 is consistent with a major cyclic AMP involvement in their responses. The lack of effect of propranolol on the flosequinan $Inc_{50}$ indicated that these responses were not mediated via β-adrenoceptors.

Selective PDE III inhibitors like SK&F 94120 or amrinone have been previously shown to potentiate the positive inotropic effects of adenylate cyclase stimulants, such as isoprenaline (Gristwood *et al.*, 1987) or histamine (Honerjager *et al.*, 1981) in guinea-pig ventricle. Thus, the lack of synergy between effective inotropic concentrations of flosequinan and SK&F 94120 provided further evidence that flosequinan is not stimulating adenylate cyclase activity via β-adrenoceptors or other cyclase coupled receptors, at these concentrations.

In contrast to the lack of potentiation of flosequinan response by the PDE III inhibitor SK&F 94120, our studies have clearly shown that the inotropic effects of flosequinan are potentiated by the PDE IV inhibitor, rolipram. It was previously reported (Gristwood & Owen, 1986; Gristwood *et al.*, 1986a) that in guinea-pig and cat ventricles there is a specific synergistic interaction on force of contraction between the selective PDE III inhibitor SK&F 94120 and rolipram. Subsequently it was reported in rabbit heart muscle that the simultaneous inhibition of PDE IV potentiated the inotropic activity of PDE III inhibitors, whereas in rat heart neither selective inhibitors of PDE III or IV alone produced an inotropic response but a response was observed when these were administered in combination (see Nicholson *et al.*, 1991). Thus, one explanation for the present results with flosequinan, as well as BTS 53554, is that they are, like SK&F 94120, functional inhibitors of PDE III. Further indirect evidence for PDE inhibitory activity was provided by the finding that flosequinan potentiated inotropic responses to isoprenaline.

The studies with isolated PDE isoenzymes confirmed that flosequinan and BTS 53554, like SK&F 94120, can inhibit cardiac PDE III activity. Furthermore, our data indicate that flosequinan, and to a lesser extent BTS 53554, are relatively selective inhibitors of PDE III. The selectivity profile for flosequinan was similar to that of amrinone, a well known selective PDE III inhibitor currently used for the therapy of congestive heart failure. The oral dose range of amrinone tested clinically (100–400 mg) is similar to that currently used for flosequinan (100–150 mg) and it is interesting that these 2 compounds have very similar PDE III inhibitor potencies as well as oral potencies in man. The absolute selectivity for flosequinan and BTS 53554 cannot be calculated accurately from our data. However, extrapolation of inhibitory data observed (to yield theoretical $IC_{50}$ values) with the other cardiac enzymes studied suggest that flose-

quinan has a selectivity of about 20 fold for PDE III whilst BTS 53554 has a selectivity of about 4 fold.

The order of inotropic potency and PDE III inhibitory potency of SK&F 94120 > flosequinan > BTS 53554, was the same and if one considers the ratio of $Inc_{50}$ to $IC_{50}$ values it is found that the values obtained for flosequinan (1.8) and BTS 53554 (2.0) are very similar to that for SK&F 94120 (1.9). It is this similarity that leads us to conclude that the PDE III inhibitory potencies of flosequinan and BTS 53554 are sufficient to account for their inotropic activity in guinea-pig ventricle.

Our electrophysiological studies in guinea-pig heart provided further evidence for this. Thus, in partially depolarized preparations in which the action potential configuration is due predominantly to $I_{si}$, flosequinan at $1 \times 10^{-4}$ M caused large increases in all measured parameters, consistent with an enhancement of $I_{si}$. This activity which occurred in parallel with the positive inotropic activity is consistent with a cyclic AMP mediated increase in $I_{si}$ (see Honerjager *et al.*, 1981).

An important question is whether, at plasma concentrations achieved clinically, flosequinan acts as a positive inotropic agent. Based on our results, we would argue that the inotropic activity of flosequinan is clinically relevant. Thus, peak blood levels of both flosequinan and subsequently its metabolite at hypotensive doses in man are around $1 \times 10^{-5}$ M (Yates & Hicks, 1988). At this concentration we saw clear inotropic activity with flosequinan both in guinea-pig ventricles and whole hearts as well as significant inhibition of guinea-pig PDE III isoenzyme (20% inhibition). Our argument is strengthened by the finding that an important feature of PDE III inhibitors is their ability to interact synergistically with β-adrenoceptor stimulation in human isolated heart (see Gristwood *et al.*, 1987). Thus, it is probable that *in vivo*, an interaction of flosequinan and/or BTS 53554 with the sympathetic drive to the myocardium would be important. Other workers have also argued, based on studies in dogs, that the positive inotropic effect significantly contributes to the overall haemodynamic response of flosequinan (Falotico *et al.*, 1989; Greenberg & Touhey, 1990).

In conclusion our studies have confirmed that flosequinan has positive inotropic activity in guinea-pig ventricle. The mechanism of inotropic action appears to be related to PDE III inhibition resulting in increased intracellular concentrations of cyclic AMP and an enhancement of calcium influx into the ventricle. The inotropic potency of flosequinan suggests that an inotropic effect should be expected in man at doses used clinically. The positive inotropic activity was shown to contribute to the increase in cardiac output seen in the guinea-pig whole heart *in vitro*. Such an effect in man would probably play an important role in the haemodynamic improvements seen with flosequinan in CHF patients (Cowley, 1991).

After submission of this paper we became aware of an abstract by Frodsham *et al.* (1991) describing weak and non-selective inhibition by flosequinan and BTS 53554 of guinea-pig ventricular PDE isoenzymes separated by Mono Q FPLC. Although we cannot explain the differences between our study and that of Frodsham *et al.*, it is obvious that a major difference is that of the substrate concentrations used (1 μM, for Frodsham *et al.*, 1991). A concentration of 0.25 μM cyclic AMP, used in the present study, is believed to reflect the basal concentration of substrate within the cell (Reeves *et al.*, 1987) and allows a more precise measurement of $IC_{50}$ values for drugs with relatively low potency and limited solubility.

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# Cardiovascular actions of a new selective postjunctional α-adrenoceptor antagonist, SK&F 104856, in normotensive and hypertensive dogs

J. Paul Hieble, Andrew J. Nichols, Todd A. Fredrickson, P. Dennis DePalma, Robert R. Ruffolo, Jr. & [1]David P. Brooks

Department of Pharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, PA, U.S.A.

**1** SK&F 104856 (2-vinyl-7-chloro-3,4,5,6-tetrahydro-4-methylthieno[4,3,2ef][3]benzazepine) is a novel postjunctional $\alpha_1$- and $\alpha_2$-adrenoceptor antagonist.

**2** SK&F 104856 as well as prazosin and SK&F 86466 reduced blood pressure in the anaesthetized normotensive dog.

**3** SK&F 86466 and rauwolscine but not SK&F 104856 or prazosin, produced a marked increase in myocardial contractility which corresponds with their ability to block prejunctional $\alpha_2$-adrenoceptors.

**4** Intravenous or oral administration of SK&F 104856 resulted in dose-dependent antihypertensive responses in 1-kidney, 1-clip (1-K, 1-C) Goldblatt hypertensive dogs with baseline blood pressure of approximately 140 mmHg. At 0.1 and 1 mg $kg^{-1}$, i.v., mean arterial blood pressure fell by $11 \pm 5$ and $23 \pm 5$ mmHg, respectively. At 3 and 10 mg $kg^{-1}$, p.o., blood pressure fell by $9 \pm 3$ and $22 \pm 5$ mmHg, respectively. At 10 mg $kg^{-1}$, p.o., the antihypertensive effect of SK&F 104856 was still evident at 4 h.

**5** The data indicate that SK&F 104856 shows selectivity *in vivo* for postjunctional versus prejunctional α-adrenoceptors and is a potent and long-acting antihypertensive agent in 1-K, 1-C Goldblatt hypertensive dogs.

**Keywords:** Hypertension; α-adrenoceptors; normotensive and hypertensive dogs; SK&F 104856

## Introduction

SK&F 104856 (2-vinyl-7-chloro-3,4,5,6-tetrahydro-4- methylthieno[4,3,2ef][3]benzazapine) is a novel α-adrenoceptor antagonist having pharmacological properties distinct from both non-selective antagonists such as phentolamine and selective $\alpha_1$-adrenoceptor antagonists such as prazosin. SK&F 104856 produces competitive blockage of both $\alpha_1$-adrenoceptors and $\alpha_2$-adrenoceptors located on isolated blood vessels and will inhibit the binding of both [$^3$H]-rauwolscine and [$^3$H]-prazosin to rat cortical homogenates (Hieble *et al.*, 1991). However, in several field-stimulated preparations, SK&F 104856 neither enhances neurotransmitter release nor inhibits the neuroinhibitory action of an exogenous $\alpha_2$-adrenoceptor agonist (Hieble *et al.*, 1991). Hence, SK&F 104856 appears to have the ability to block vascular α-adrenoceptors, both $\alpha_1$ and $\alpha_2$ , without interfering with the neuronal $\alpha_2$-adrenoceptor responsible for feedback control of neurotransmitter release.

There is evidence for a contribution of both $\alpha_1$- and $\alpha_2$-adrenoceptor activation to the maintenance of elevated blood pressure in hypertension. In normotensive rats, selective $\alpha_1$-adrenoceptor blockade with prazosin, but not selective $\alpha_2$-adrenoceptor blockade with rauwolscine, would lower blood pressure. However, in either DOCA-salt hypertensive or spontaneously hypertensive rats, rauwolscine will reduce blood pressure when given alone, and produce an additional antihypertensive effect following a maximally effective dose of prazosin (McCafferty *et al.*, 1982). Other investigators have observed an additional blood pressure reduction by rauwolscine in prazosin-treated spontaneously hypertensive rats (Sawyer *et al.*, 1985). Functional vasoconstrictor responses mediated by vascular $\alpha_2$-adrenoceptors have been demonstrated in both normotensive and hypertensive human subjects (Bolli *et al.*, 1984; Jie *et al.*, 1986; Brown, 1989), and may be activated to a greater extent in the hypertensive state (Bolli *et al.*, 1984).

Hence, the ability of an agent, such as SK&F 104856, to block both $\alpha_1$-adrenoceptors and $\alpha_2$-adrenoceptors on vascular smooth muscle while, like prazosin, preserving prejunctional $\alpha_2$-adrenoceptor-mediated control of transmitter release, may be advantageous in the treatment of conditions associated with elevated vascular resistance. In the present study, we characterize the *in vivo* effects of SK&F 104856 in the dog, evaluating antihypertensive efficacy in the conscious 1-kidney, 1-clip Goldblatt preparation, and using myocardial contractility in the anaesthetized normotensive animal as an index of interaction with prejunctional $\alpha_2$-adrenoceptors.

## Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee and were in accordance with National Institutes of Health guidelines for the care and use of animals.

### *Haemodynamic studies in the anaesthetized dog*

*Myocardial contractility* Adult mongrel dogs of either sex (12–20kg) were anaesthetized with sodium pentobarbitone (50 mg $kg^{-1}$, i.p.) and prepared as described previously (Hieble *et al.*, 1987). Briefly, a tracheotomy was performed and the animal was connected to a large animal respirator, delivering a tidal volume of 15–20 ml $kg^{-1}$ at 16 cycles $min^{-1}$. The right femoral vein and artery were isolated and cannulated. A left thoracotomy was performed at the fourth intercostal space. The pericardial sac was opened, and a wide bore catheter was inserted into the left ventricle near the apex to measure left ventricular pressure. The left ventricular pressure signal was used to obtain $dP/dt$. After allowing at least 30 min post surgery to allow stabilization of haemodynamic parameters, α-adrenoceptor antagonists were

[1] Author for correspondence

infused for 10 min. Blood pressure, heart rate and $dP/dt$ were monitored continuously during infusion and for 30 min post infusion.

*Vascular α-adrenoceptor responsiveness* Dogs were anaesthetized as above and the right femoral artery and vein were cannulated for the measurement of blood pressure and the administration of fluids and supplemental anaesthesia, respectively. The left femoral vein was cannulated for the intravenous infusion of SK&F 104856. In order to measure the interaction of SK&F 104856 with vascular $\alpha_1$-adrenoceptors, methoxamine (3 doses, 5 min intervals between doses) was administered before and during infusion of SK&F 104856. Following initiation of antagonist infusion, a 30 min stabilization period was allowed before repeating the methoxamine challenge. In experiments to evaluate blockade of vascular postjunctional $\alpha_2$-adrenoceptors, the left femoral artery was isolated and femoral arterial blood flow was measured with a Carolina Medical Electronics flow probe. A needle catheter was inserted into the femoral artery distal to the flow probe for the intraarterial administration of the $\alpha_1$-adrenoceptor agonist, B-HT 933 (2-amino-6-ethyl-5,6,7,8-tetrahydro-4H-oxazolo[4,5-d]azepine). Following a 30 min stabilization period, increasing doses of B-HT 933 were injected into the femoral artery, and the increase in femoral vascular resistance was measured as the change in mean arterial pressure/the change in femoral arterial blood flow. When femoral vascular resistance had returned to control levels, SK&F 104856 was infused at 100 $\mu g\,kg^{-1}\,min^{-1}$, i.v. and the dose-response curve to B-HT 933 was repeated.

### *Conscious hypertensive dogs*

*Surgical procedure* Animals were appropriately premedicated; anaesthesia was induced with sodium biotol and maintained with isofluorane. With use of standard aseptic techniques and via a flank incision, a right uninephrectomy was performed. Approximately 6 weeks later and via a midline incision, a Goldblatt clamp (Braintree Scientific) was placed on the left renal artery. The clamp was constricted to reduce blood flow (measured acutely during the surgery with a flow meter) by approximately 50%. Dogs were not studied for at least 4 weeks following clamping of the renal artery.

By use of the same incision site and creation of a subcutaneous pocket, the access portion of a Vascular-Access-Port (VAP; Norfolk Medical Products, Inc.) was sutured (000-silk) to the underlying muscle in the area of the paralumbar fossa. The catheter end of the VAP was tunnelled to the area of the left femoral triangle. A second incision was made and the catheter inserted in the femoral artery to a length of approximately 12 cm and secured with 0-silk. The patency of the VAP was maintained by flushing weekly with saline and locking with a heparinized glucose solution (50% glucose-500u $ml^{-1}$ of heparin). Animals were routinely given a broad-spectrum antibiotic for 5 days after surgery.

*Experimental protocol* Dogs were fasted for 18 h with free access to water before study. The dogs were placed in a sling and the glucose/heparin lock of the VAP was evacuated and the VAP connected to a Gould P23 XL pressure transducer via an infusion set. MABP and heart rate (determined from the arterial pulse) were recorded on a Gilson duograph. When i.v. administration of drugs was performed, a catheter was placed in the saphenous vein.

At least 60 min was allowed for MABP and heart rate to stabilize and then a mean of 4 values were recorded at 10 min intervals before drug administration. This was considered the control value. SK&F 104856 was administered either intravenously at 0.1 or 1 $mg\,kg^{-1}$, i.v. or p.o. at 3 or 10 $mg\,kg^{-1}$ (in a gelatin capsule). Blood pressure and heart rate were monitored over the next 4 h. Four animals were used in each group and a period of at least 2 weeks was allowed between each experiment.

### *Data analyses*

All data are reported as means ± s.e. Statistical analysis was performed by analysis of variance for repeated measures.

## Results

Intravenous infusion of either prazosin (10 $\mu g\,kg^{-1}\,min^{-1}$), SK&F 86466 (100 $\mu g\,kg^{-1}\,min^{-1}$) or SK&F 104856 (100 or 200 $\mu g\,kg^{-1}\,min^{-1}$) reduced blood pressure in the anaesthetized, normotensive dog. The blood pressure reduction produced by SK&F 104856 appeared to be dose-related. In contrast, rauwolscine (10 $\mu g\,kg^{-1}\,min^{-1}$) produced a slight increase in blood pressure. All drugs produced a small increase in heart rate (<15% increase, data not shown). However, SK&F 86466 and rauwolscine, but not prazosin or SK&F 104856, produced a marked increase in myocardial contractility, as measured by ventricular $dP/dt$ (Figure 1).

Infusion of SK&F 104856 at 100 $\mu g\,kg^{-1}\,min^{-1}$ was shown to block both the pressor response to $\alpha_1$-adrenoceptor activation by methoxamine and the ability of the selective $\alpha_2$-adrenoceptor agonist, B-HT 933, to increase femoral vascular resistance (Table 1), demonstrating *in vivo* blockade of both vascular α-adrenoceptor subtypes at this dose. Increases in hind limb vascular resistance were used as an index of $\alpha_2$-adrenoceptor activation, rather than systemic pressor responses, since the pressor dose-response curve to selective $\alpha_2$-adrenoceptor agonists in the anaesthetized dog is shallow and has a low maximum response.

The ability of SK&F 104856 to lower blood pressure in conscious dogs in which hypertension had been induced by renal artery clamping was studied. Baseline blood pressure in normotensive animals prior to placing the Goldblatt clamp on the renal artery was between 100–110 mmHg. Four weeks following placement of the clip, MABP averaged approximately 140 mmHg. Intravenous administration of SK&F 104856 resulted in a rapid, dose-dependent decrease in mean

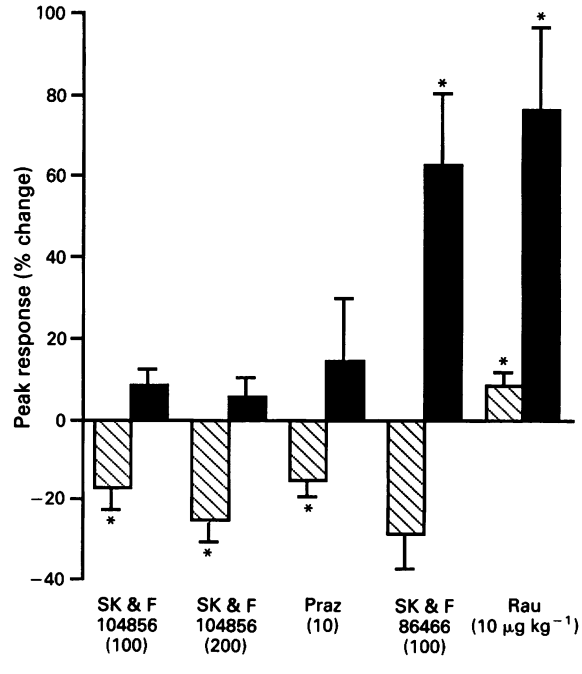


**Figure 1** Comparison of the effects of various α-adrenoceptor antagonists on mean arterial blood pressure (MAP, hatched columns) and myocardial contractility ($dP/dt$, solid columns) in the anaesthetized dog. Praz, prazosin; Raw, rauwolscine $*P<0.05$ ($n=4$).

**Table 1** Blockade of postjunctional $\alpha_1$-adrenoceptors and $\alpha_2$-adrenoceptors by SK&F 104856 in the anaesthetized dog

| *Agonist dose* ($\mu g\ kg^{-1}$) | $\alpha_1$[a] *Control* | *SK&F 104856*[c] | $\alpha_2$[b] *Control* | *SK&F 104856*[c] |
|---|---|---|---|---|
| 1 | | | 0.2 ± 0.1 | 0 |
| 3 | 5.8 ± 2.8 | 0 | 0.9 ± 0.2 | 0.1 ± 0.1 |
| 10 | 7.8 ± 4.8 | 0 | 1.5 ± 0.2 | 0.2 ± 0.1 |
| 30 | 39.3 ± 11.1 | 6.7 ± 4.4 | 3.7 ± 0.2 | 0.6 ± 0.2 |

[a]Blockade of methoxamine-induced increases in diastolic blood pressure; agonist administered intravenously. Responses in mmHg.
[b]Blockade of B-HT 933-induced increases in vascular resistance in femoral arterial bed; agonist administered intra-arterially. Responses in $mmHg\ ml^{-1}\ min^{-1}$.
[c]SK&F 104856 administered intravenously at $0.1\ mg\ kg^{-1}\ min^{-1}$ during repeat challenge.

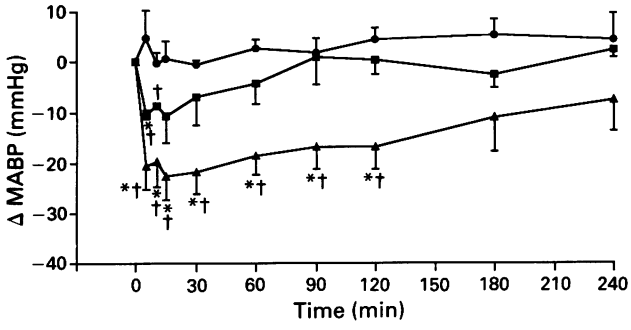


**Figure 2** Time course of the effect of i.v. administration of SK&F 104856 on mean arterial blood pressure (MABP) in hypertensive dogs. Control MABP in dogs treated with vehicle (●), $0.1\ mg\ kg^{-1}$ SK&F 104856 (■) or $1\ mg\ kg^{-1}$ SK&F 104856 (▲) were 143 ± 11, 140 ± 10 and 138 ± 6 mmHg, respectively. *$P<0.05$ versus time 0; †$P<0.05$ versus vehicle.

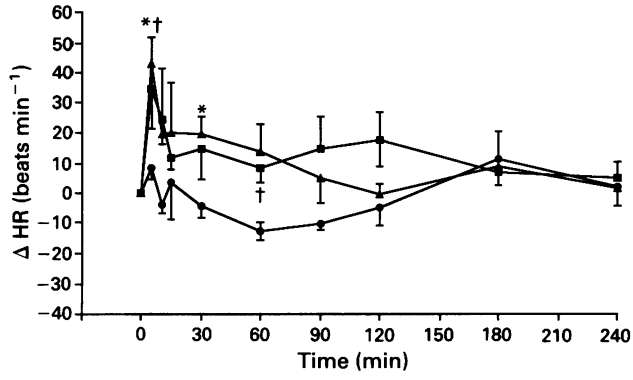


**Figure 3** Time course of the effect of i.v. administration of SK&F 104856 on heart rate (HR) in hypertensive dogs. Control HR in dogs treated with vehicle (●), $0.1\ mg\ kg^{-1}$ SK&F 104856 (■) or $1\ mg\ kg^{-1}$ SK&F 104856 (▲) were 80 ± 3, 75 ± 4 and 81 ± 7 beats $min^{-1}$, respectively. *$P<0.05$ versus time 0; †$P<0.05$ versus vehicle.

arterial blood pressure (Figure 2). At $0.1\ mg\ kg^{-1}$, i.v. blood pressure decreased by approximately 10 mmHg, but the response was short-lived. At $1\ mg\ kg^{-1}$, i.v. blood pressure fell by 23 ± 5mmHg, and the antihypertensive response was maintained for at least 2 h (Figure 2). There was a significant reflex tachycardia associated with the initial drop in blood pressure, with both doses of SK&F 104856 increasing heart rate by approximately 40 beats $min^{-1}$ (Figure 3); however, this response was not maintained. Thus, heart rate had returned to control levels at a time when MABP was still significantly reduced (Figures 2 and 3).

Oral administration of SK&F 104856 at $3\ mg\ kg^{-1}$ had relatively little effect on mean arterial blood pressure. However, at $10\ mg\ kg^{-1}$, blood pressure was reduced by 22 ± 5 mmHg, and this was maintained for the duration of the experiment (Figure 4). Oral administration of SK&F 104856 resulted in a slow onset of the antihypertensive action, with blood pressure reaching a minimum at 90 min. This was different from that observed after intravenous administration when blood pressure fell immediately (Figure 2). There was a small but significant increase in heart rate associated with oral administration of the higher dose of SK&F 104856 (Figure 5). This increase in heart rate paralleled, for the most part, the reduction in blood pressure.

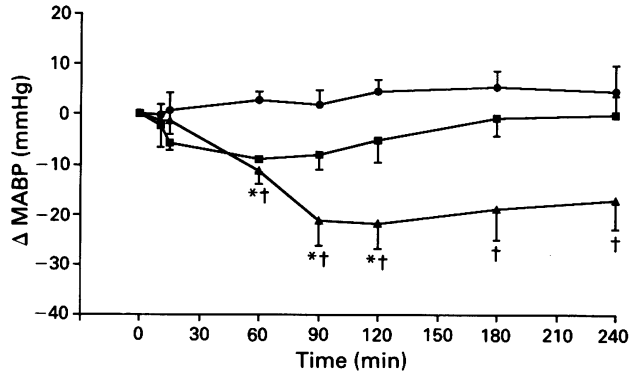


**Figure 4** Time course of the effect of oral administration of SK&F 104856 on mean arterial blood pressure in hypertensive dogs. Control MABP in dogs treated with vehicle (●), $3\ mg\ kg^{-1}$ SK&F 104856 (■) or $10\ mg\ kg^{-1}$ SK&F 104856 (▲) were 143 ± 11, 142 ± 9 and 136 ± 5 mmHg, respectively. *$P<0.05$ versus time 0; †$P<0.05$ versus vehicle.

## Discussion

SK&F 104856 can be considered to be structurally derived from the 3-benzazepine α-adrenoceptor antagonists, SK&F 86466 (Hieble *et al.*, 1986) and SK&F 104078 (Hieble *et al.*, 1988b). As observed with SK&F 104078, but not SK&F 86466, SK&F 104856 can discriminate between $\alpha_2$-adrenoceptor populations. Based on the current observations, the *in vitro* selectivity for vascular $\alpha_2$-adrenoceptors of canine and human saphenous vein, *vis-a-vis* neuronal $\alpha_2$-adrenoceptors of human saphenous vein or guinea-pig atrium, can be extended to an *in vivo* preparation.

Rauwolscine and SK&F 86466, compounds of diverse chemical structure showing potent *in vitro* antagonist activity at prejunctional $\alpha_2$-adrenoceptors of the guinea-pig atrium, have been shown to increase ventricular $dP/dt$, as assessed by studies in the anaesthetized dog. The doses required to produce maximal cardiac stimulation appear to correspond with *in vitro* $\alpha_2$-adrenoceptor antagonist potency at the prejunctional receptor, with rauwolscine being 3–10 times more potent than SK&F 86466. However, in contrast, the maximal degree of inotropic stimulation produced at a given infusion

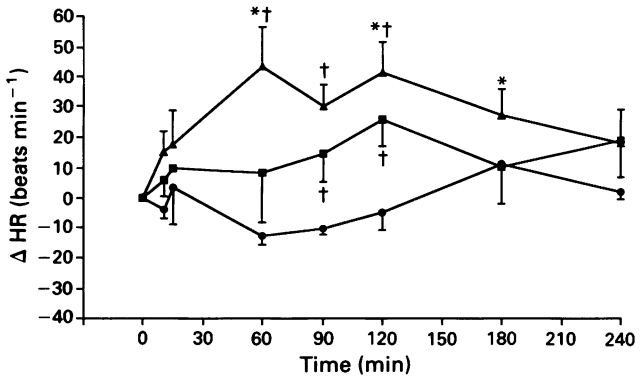


**Figure 5** Time course of the effect of oral administration of SK&F 104856 on heart rate in hypertensive dogs. Control HR in dogs treated with vehicle (●), 3 mg kg$^{-1}$ SK&F 104856 (■) or 10 mg kg$^{-1}$ SK&F 104856 (▲) were 80 ± 3, 84 ± 11 and 75 ± 5 beats min$^{-1}$, respectively. *$P<0.05$ versus time 0; †$P<0.05$ versus vehicle.

rate of rauwolscine and SK&F 86466 is approximately equal (Table 2). Prazosin and SK&F 104856, which have a very low *in vitro* affinity ($K_b>1$ μM) for prejunctional $\alpha_2$-adrenoceptors, produce no significant effect on contractility in the anaesthetized dog (Table 2). Our results are compatible with those observed in a similar canine preparation with two other antagonists, yohimbine and WY26392 (Paciorek & Shepperson, 1985). It is likely that the increase in $dP/dt$ results from an enhancement in neurotransmitter release from cardiac sympathetic terminals, producing an activation of ventricular β-adrenoceptors. This is supported by the ability of propranolol to produce complete blockade of the inotropic effect of rauwolscine in this model (Hieble *et al.*, 1988a). It is interesting to note that the effects of the $\alpha_2$-adrenoceptor antagonists on $dP/dt$, both in this study and in that described by Paciorek & Shepperson (1985), are much more dramatic than their effects on heart rate, perhaps reflecting vagally mediated compensatory mechanisms to control heart rate.

Studies in conscious hypertensive dogs show that the α-adrenoceptor blockade produced by SK&F 104856 results in a decrease in blood pressure. SK&F 104078, which shows an *in vitro* α-adrenoceptor antagonist profile similar to SK&F 104856, is not active orally, due to degradation by stomach acid (Hieble *et al.*, 1990). In contrast, SK&F 104856 shows antihypertensive activity by both oral and intravenous routes (Figures 2 and 4). Assuming we can extrapolate the data from the anaesthetized to the conscious dog, which we believe is reasonable, the inability of SK&F 104856 to elevate myocardial contractility in the anaesthetized dog indicates that its antihypertensive effect in the conscious dog might not be accompanied by an increase in plasma catecholamine levels. Plasma catecholamines were not measured in the present study since it is difficult to obtain consistent data from a conscious dog; however, studies in anaesthetized spontaneously hypertensive rats show that SK&F 104856 does not produce a statistically significant elevation of plasma noradrenaline at an effective antihypertensive dose (Sauermelch *et al.*, 1991). The effects of SK&F 104856 in this model were similar to prazosin, and in contrast to the 3–4 fold elevation in plasma noradrenaline produced by an equieffective antihypertensive dose of SK&F 86466.

Although SK&F 104856 has a haemodynamic profile in the anaesthetized instrumented dog that is similar to prazosin, the potent antagonist activity of SK&F 104856 at vascular $\alpha_2$-adrenoceptors, both *in vitro* and *in vivo*, is likely to contribute to its haemodynamic effects. Studies in hypertensive rats suggest a contribution of both $\alpha_1$-adrenoceptors and $\alpha_2$-adrenoceptors to the maintenance of vascular tone (McCafferty *et al.*, 1982; Sawyer *et al.*, 1985), but such a contribution has not yet been established in canine models of hypertension. Nevertheless, $\alpha_2$-adrenoceptors are known to play an important role in specific vascular beds.

Studies in rats (Willette *et al.*, 1991), cats (Koss *et al.*, 1991) and man (Brown, 1989) have shown an important role of the vascular $\alpha_2$-adrenoceptor in the control of cutaneous blood flow. In the rat, SK&F 104856, but not prazosin, can increase flow in the cutaneous vascular bed (Sauermelch *et al.*, 1991). Since the vascular $\alpha_2$-adrenoceptor makes an important contribution to coronary vascular resistance in the dog (Kopia *et al.*, 1986; Hieble *et al.*, 1988a), the addition of vascular $\alpha_2$-adrenoceptor blockade may be advantageous in the treatment of hypertension with concomitant coronary artery disease.

An agent such as SK&F 104856, blocking both $\alpha_1$-adrenoceptors and $\alpha_2$-adrenoceptors on the vasculature, may have advantages over a selective $\alpha_1$-adrenoceptor antagonist such as prazosin in the treatment of congestive heart failure. The development of tolerance to the beneficial haemodynamic effects of prazosin in heart failure patients has been postulated to result from an increased contribution of an $\alpha_2$-adrenoceptor-mediated vasoconstrictor effect of the high levels of plasma catecholamines associated with heart failure (Smyth *et al.*, 1986). Furthermore, elevated pulmonary vascular tone is a clinical feature of heart failure, and studies in both dogs (Shebuski *et al.*, 1986) and cats (Hyman & Kadowitz, 1985) have shown an enhanced sensitivity to $\alpha_2$-adrenoceptor-induced increases in pulmonary vascular tone under conditions where active tone was elevated. The high density of postjunctional $\alpha_2$-adrenoceptor in the venous system also supports utility of a mixed antagonist such as

**Table 2** Correlation of haemodynamic effects with prejunctional $\alpha_2$-adrenoceptor blockade *in vitro*

| *Antagonist* | *Postjunctional*[a] $\alpha_2K_B$(nM) | *Prejunctional*[b] $\alpha_2K_B$(nM)[a] | *Dose*[c] (mg kg$^{-1}$) | dP/dt[d] |
|---|---|---|---|---|
| Rauwolscine | 1* | 4.5* | 0.10 | 57 ± 18†† |
| SK&F 86466 | 42* | 17* | 0.80 | 63 ± 18† |
| Prazosin | >500* | >1000* | 0.1 | 15 ± 15† |
| SK&F 104856 | 29** | >3000** | 2.0 | 6 ± 5 |

*Daly *et al.* (1988)
**Hieble *et al.* (1991)
† Valocik & Blumberg (1983)
†† Hieble *et al.* (1988a)
[a]Blockade of B-HT 920-induced contraction in canine saphenous vein.
[b]Blockade of B-HT 920-induced inhibition of neurotransmission in guinea-pig atrium.
[c]Dose required to produce maximum effect on $dP/dT$. Drugs infused intravenously at a rate of 0.01 mg kg$^{-1}$min$^{-1}$ (rauwolscine and prazosin) or 0.1 mg kg$^{-1}$min$^{-1}$ (SK&F 86466 and SK&F 104856) over a 10 min interval.
[d]Maximum increase in left ventricular $dP/dT$ achieved.

SK&F 104856 in congestive heart failure, since cardiac preload would be expected to be reduced more effectively as a result of the addition of vascular $\alpha_2$-adrenoceptor blockade.

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# Comparative effects of BRL 38227, nitrendipine and isoprenaline on carbachol- and histamine-stimulated phosphoinositide metabolism in airway smooth muscle

[1]R.A. John Challiss, Neela Patel & *Jonathan R.S. Arch

Department of Pharmacology & Therapeutics, University of Leicester, Leicester LE1 9HN and *SmithKline Beecham Pharmaceuticals, Great Burgh, Epsom, Surrey KT18 5XQ

**1** The ability of BRL 38227 and nitrendipine to affect muscarinic agonist and histamine-stimulated [$^3$H]-inositol phosphate accumulation in slices of bovine tracheal smooth muscle has been studied and compared with the established inhibitory effects of isoprenaline on this pathway.

**2** Pre-addition of BRL 38227 (5 μM), nitrendipine (1 μM) or isoprenaline (10 μM) significantly inhibited the subsequent inositol phosphate response to histamine at all concentrations studied (10– 1000 μM). BRL 38227 and nitrendipine also significantly inhibited the [$^3$H]-inositol phosphate response to low (1 μM), but not high (100 μM) concentrations of carbachol. Isoprenaline had no effect at any concentration of carbachol studied.

**3** Nitrendipine ($IC_{50}$ = 95 nM) and BRL 38227 ($IC_{50}$ = 322 nM) caused concentration-related inhibitions of the inositol phosphate response to histamine (100 μM). Similar maximal inhibitions were caused by each agent (55– 58%). Inhibitory effect of BRL 38227 was reduced in potency ($IC_{50}$ = 5.5 μM), but not magnitude, in the presence of glibenclamide (0.5 μM).

**4** Time-course studies comparing the effects of BRL 38227 addition 15 min before, and 10 min after histamine challenge showed that for pre-addition a distinct (<2 min) lag occurred following histamine addition before the inhibitory effect of BRL 38227 was manifest. In contrast, when BRL 38227 was added 10 min after histamine, an inhibitory effect was immediately apparent.

**5** Further evidence for an initial, 'protected' phase of inositol phosphate accumulation was provided by the finding that BRL 38227 pre-addition had no effect on the early (0–300 s) time-course of inositol 1,4,5-trisphosphate mass accumulation.

**6** The inhibitory effect of BRL 38227, but not that of nitrendipine or isoprenaline, on histamine-stimulated [$^3$H]-inositol phosphate accumulation was completely prevented in the presence of an elevated extracellular $K^+$ (65 mM) concentration.

**7** The results demonstrate that membrane hyperpolarization, and/or blockade of voltage-operated $Ca^{2+}$-channels can regulate agonist-stimulated phosphoinositide metabolism in airway smooth muscle. The possible contribution of this regulatory mechanism to the relaxant properties of these agents is discussed.

**Keywords:** BRL 38227; phosphoinositides; airway smooth muscle; histamine; nitrendipine; isoprenaline

## Introduction

It is now generally accepted that spasmogenic agonists such as carbachol and histamine exert their contractile action in airway smooth muscle through activation of the phosphoinositide pathway (Baron *et al.*, 1984; Takuwa *et al.*, 1986; Meurs *et al.*, 1988; Coburn & Baron, 1990). Receptor-mediated activation of phosphoinositidase C (PIC) increases the rate of phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) hydrolysis and formation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and *sn*-1,2-diacylglycerol (DAG) (Takuwa *et al.*, 1986; Chilvers *et al.*, 1989b; 1991; Katsuyama *et al.*, 1990). The rapid, but transient, increase in $Ins(1,4,5)P_3$ accumulation has been implicated in the mobilisation of $Ca^{2+}$ from non-mitochondrial, intracellular stores (Hashimoto *et al.*, 1985) and the initiation of contraction, whilst the sustained activation of PIC (Chilvers *et al.*, 1991) and maintained DAG accumulation (Takuwa *et al.*, 1986) may have a role in the sustained (tonic) phase of contraction (Rasmussen *et al.*, 1990).

β-Adrenoceptor agonists exert a spasmolytic or anti-spasmogenic effect in airway smooth muscle which can be mimicked by other agents which increase cellular cyclic AMP concentration (Andersson *et al.*, 1978; Polsen *et al.*, 1982; Rasmussen *et al.*, 1990). The extent of the relaxant effect caused by the β-adrenoceptor agonist is dependent on the nature of the spasmogenic stimulus. Thus, airway smooth muscle contraction induced by muscarinic cholinoceptor agonists is relatively resistant to relaxation by isoprenaline compared to contractions induced by other spasmogens (Torphy, 1984; Jenne *et al.*, 1987). This spasmogen-specific effect is also seen at the level of second messenger generation. β-Adrenoceptor stimulation causes a marked inhibition of histamine-stimulated phosphoinositide hydrolysis in canine (Madison & Brown, 1988) and bovine (Hall & Hill, 1988; Hall *et al.*, 1989) tracheal smooth muscle, whereas these effects are either small or absent when muscarinic agonists are employed to stimulate phosphoinositide turnover (Madison & Brown, 1988; Hall & Hill, 1988; Hall *et al.*, 1989; 1990). The relative resistance of muscarinic cholinoceptor-mediated response to β-adrenoceptor agonists may relate to the large muscarinic receptor reserve seen in tracheal smooth muscle (Gunst *et al.*, 1989), although a recent report of differential sensitivities of the β-adrenoceptor-mediated inhibition of phosphoinositide turnover to two muscarinic partial agonists argues against this explanation (Offer *et al.*, 1991).

A distinct class of smooth muscle relaxant drugs has recently been described which act by increasing the open probability of membrane $K^+$-channels (Hamilton & Weston,

[1] Author for correspondence.

1989). The prototypes of this class of compound, cromakalim, and its more active enantiomer BRL 38227 (lemakalim), have been shown to exert a bronchodilator action *in vivo* (Arch *et al.*, 1988; Bowring *et al.*, 1991) and to cause relaxation of airway smooth muscle *in vitro* (Allen *et al.*, 1986; Arch *et al.*, 1988; Black *et al.*, 1990; Raeburn & Brown, 1991), and these effects appear to be independent of changes in cellular cyclic nucleotide concentration (Gillespie & Sheng, 1988; Berry *et al.*, 1991).

In the present investigation, we have studied the effects of BRL 38227 on muscarinic cholinoceptor- and histamine-stimulated phosphoinositide turnover and compared the effects of this agent with those elicited by the voltage-operated $Ca^{2+}$-channel antagonist, nitrendipine and the β-adrenoceptor agonist, isoprenaline.

## Methods

### *Incubation methods*

Bovine trachealis muscle was obtained and prepared as described previously (Chilvers *et al.*, 1989a). Tissue was maintained at 4°C in a modified Krebs-Henseleit buffer (KHB; composition in mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, $CaCl_2$ 1.3, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, HEPES 5, glucose 10) during the careful removal of epithelium and connective tissue. The smooth muscle was cross-chopped (300 μm × 300 μm) with a McIlwain tissue-chopper. The resulting slices were washed and incubated in 100 ml KHB at 37°C for 60 min. At 15 min intervals, the slices were allowed to sediment and the buffer replaced by fresh medium. Gravity-packed slices (2 ml) were then transferred to fresh KHB containing 1 μM carbachol and 1 μCi $ml^{-1}$ [$^3$H]-inositol (cleaned of polar contaminants immediately before use by passing it through a 0.25 ml bed-volume Dowex 1-X8 (100–200 mesh, $Cl^-$ form) column). Samples were gassed with $O_2/CO_2$ (19:1) at regular intervals during the 60 min radiolabelling period.

At the end of the labelling period, slices were washed thoroughly with 6 × 20 ml KHB for 15–20 min. Gravity-packed slices (75 μl) were then transferred to 400 μl KHB in plastic insert vials containing 5 mM LiCl and 1 μCi $ml^{-1}$ [$^3$H]-inositol. Incubation vials were gassed with $O_2/CO_2$, capped and incubated for 30 min. At this point appropriate additions of agonists and antagonists were made as detailed in the Results section. In general, incubations were continued at 37°C for 30 min after muscarinic cholinoceptor agonist or histamine addition, unless otherwise stated.

All incubations were terminated by addition of 500 μl of ice-cold 1 M trichloroacetic acid (TCA) and samples left to extract on ice for 20 min.

### *Measurement of [$^3$H]-inositol phosphates and [$^3$H]-inositol phospholipids*

Samples were centrifuged (20 min, 4000 *g*, 4°C) and 900 μl of the supernatant neutralized by washing with 4 × 3 vol water-saturated diethylether. A 750 μl aliquot of the extracted supernatant was brought to pH 7 by addition of 150 μl 60 mM $NaHCO_3$, and the entire sample washed onto a 0.5 ml bed-volume Dowex 1-X8 (100–200 mesh, $Cl^-$ form) column with 20 ml $H_2O$. The column was washed with 10 ml 0.025 M ammonium formate and then total [$^3$H]-$InsP_x$ were eluted in 10 ml 1 M HCl. Radioactivity in a 2 ml sample of the latter eluate was determined by scintillation counting.

In some cases the original tissue pellet was washed in 0.9% NaCl, dissolved in 1 M NaOH and the protein concentration determined. Alternatively, the pellet was washed sequentially with 1 ml 5% TCA containing 1 mM EDTA, and 1 ml $H_2O$ before phospholipid extraction as described by Downes & Wusteman (1983) and determination of total [$^3$H]-$PtdIns(P_x)$ in the chloroform-phase.

### *Measurement of Ins(1,4,5)$P_3$ concentration*

Smooth muscle slices for experiments involving mass determinations of $InsP_3$ were conducted as described above, except that the [$^3$H]-inositol-labelling step was omitted. Samples were stimulated with 100 μM histamine or 100 μM carbachol, following a 15 min pre-incubation in the absence or presence of 5 μM BRL 38227, and incubations terminated 5–300 s after spasmogen addition with 500 μl 1 M TCA. Following diethylether extraction, a 200 μl sample was taken and 50 μl 60 mM $NaHCO_3$ and 50 μl 30 mM EDTA added. $InsP_3$ concentration was determined as described previously (Challiss *et al.*, 1988). The pellet protein content was determined for each sample and results expressed as pmol $InsP_3$ $mg^{-1}$ of protein.

### *Materials*

[$^3$H]-*myo*-inositol (12–20 Ci $mmol^{-1}$) was purchased from, and [$^3$H]-inositol 1,4,5-trisphosphate (17–20 Ci $mmol^{-1}$) was generously donated by DuPont (U.K.) Ltd. (Stevenage, Herts). Histamine, carbachol, oxotremorine, pilocarpine, isoprenaline, nitrendipine and glibenclamide were purchased from Sigma Chemical Co. Ltd. (Poole, Dorset). Dowex 1-X8 (100–200 mesh, $Cl^-$ form) was purchased from BioRad Laboratories Ltd. (Watford, Herts.). BRL 38227 and the less active (+)-enantiomer BRL 38226 were synthesized as described by Ashwood *et al.* (1986).

### *Data analysis*

All values are presented as means ± s.e.mean for *n* separate experiments. Statistical comparisons of values obtained in the absence or presence of BRL 38227, nitrendipine or isoprenaline were performed by use of Student's *t* test for unpaired observations. Concentration-response curves were drawn by inspection and $IC_{50}$ values determined with a computer-assisted curve-fitting programme (ALLFIT; DeLean *et al.*, 1978).

## Results

Optimal conditions for [$^3$H]-inositol labelling of the agonist-sensitive inositol phospholipid pool in bovine tracheal smooth muscle slices have been described previously (Chilvers *et al.*, 1989a): the presence of a low concentration of carbachol (or histamine) during the labelling period accelerates inositol phospholipid labelling and allows steady-state labelling to be approached within a 1 h incubation period (Chilvers *et al.*, 1989a; 1990).

The stimulations of total $InsP_x$ accumulation by maximally effective concentrations of histamine (1 mM), and the muscarinic cholinoceptor agonists carbachol (100 μM), oxotremorine (100 μM) and pilocarpine (100 μM), in the presence of 5 mM LiCl, are shown in Figure 1a. As reported by a number of other groups (Hall & Hill, 1988; Madison & Brown, 1988; Chilvers *et al.*, 1989a; Van Amsterdam *et al.*, 1989; Offer *et al.*, 1991), carbachol elicited the largest [$^3$H]-$InsP_x$ accumulation, which in this experiment was a 37 fold increase over the basal accumulation. Histamine, and the partial muscarinic agonists oxotremorine and pilocarpine elicited responses of 54%, 80% and 22% respectively, relative to that evoked by carbachol (Figure 1a).

The effects of BRL 38227 (5 μM), isoprenaline (10 μM) and nitrendipine (1 μM), each added 15 min before agonist challenge, are also shown in Figure 1a. None of these agents had any effect on basal [$^3$H]-$InsP_x$ accumulation, but all caused significant 30–60% inhibitions of the histamine-stimulated response. In contrast, none of these agents had any effect on [$^3$H]-$InsP_x$ accumulation elicited by 100 μM carbachol. In agreement with the data of Offer *et al.* (1991), isoprenaline caused a small (24%), but significant inhibition

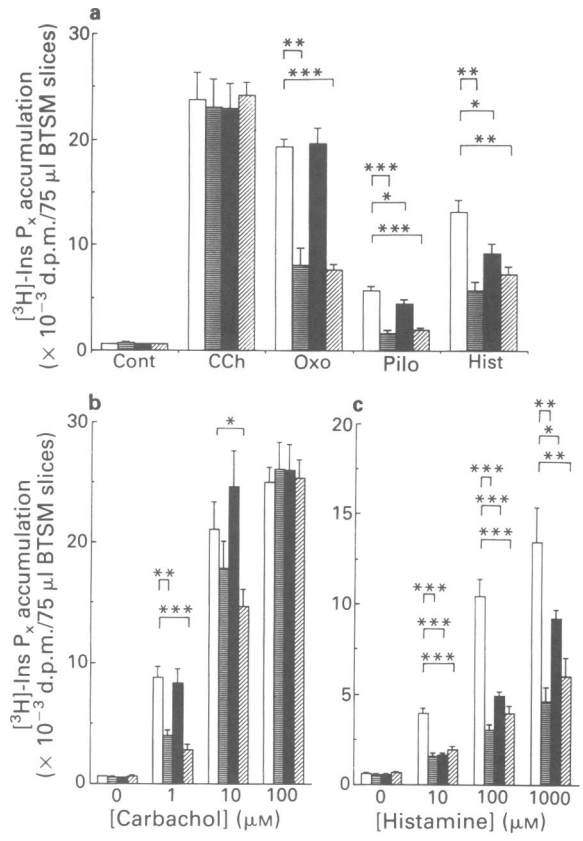


**Figure 1** Effects of BRL 38227, isoprenaline and nitrendipine on muscarinic cholinoceptor – and histamine-stimulated inositol phosphate accumulations in bovine tracheal smooth muscle. [$^3$H]-inositol pre-labelled bovine tracheal smooth muscle (BTSM) slices were pre-incubated for 15 min in the absence ( □ ) or presence of 5 µM BRL 38227 ( ▤ ), 10 µM isoprenaline ( ■ ) or 1 µM nitrendipine ( ▨ ) followed by addition of 100 µM carbachol (CCh), 100 µM oxotremorine (Oxo), 100 µM pilocarpine (Pilo) or 1 mM histamine (Hist) for 30 min. Panels shown are: comparison of [$^3$H]-InsP$_x$ accumulations in the absence (Cont) or presence of various agonists (**a**); effects of BRL 38227, isoprenaline or nitrendipine on [$^3$H]-InsP$_x$ accumulations evoked by 1, 10 or 100 µM carbachol (**b**) or 10, 100 or 1000 µM histamine (**c**). Values represent means for 5 (**a**), 3 (**b**) and 3 (**c**) separate experiments each performed in triplicate; s.e.mean shown by vertical bars. Statistical significance is indicated as $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ for each agonist in the absence compared to the presence of BRL 38227, isoprenaline or nitrendipine.

of the pilocarpine-stimulated [$^3$H]-InsP$_x$ accumulation, but was without effect on the oxotremorine-stimulated response (Figure 1a). However, both BRL 38227 and nitrendipine markedly inhibited (>60%) both pilocarpine- and oxotremorine-stimulated [$^3$H]-InsP$_x$ accumulations.

The inhibitory effects of BRL 38227, isoprenaline and nitrendipine on [$^3$H]-InsP$_x$ accumulations elicited by submaximally effective concentrations of carbachol and histamine are shown in Figure 1. The histamine-stimulated response was significantly inhibited by each of the agents at all of the concentrations of histamine studied. At a low concentration of carbachol (1 µM), which evoked a [$^3$H]-InsP$_x$ accumulation which was 34% of that caused by 100 µM carbachol, BRL 38227 and nitrendipine, but no isoprenaline, caused respectively, significant 59% and 73% inhibitions of the control response. At 10 µM carbachol, only nitrendipine caused a small (31%), but significant inhibitory effect (Figure 1b).

The concentration-response relationships for the inhibitory effects of BRL 38227 and nitrendipine on histamine-stimulated [$^3$H]-InsP$_x$ accumulation are shown in Figure 2. Both agents caused similar maximal inhibitions (55–58%) of the histamine-stimulated response, with half-maximal inhibitions being observed at 95 ± 27 nM for nitrendipine and 322 ± 33 nM for BRL 38227 (Figure 2). The inhibitory effect of BRL 38227 occurred over a narrow concentration-range, giving rise to a slope factor greater than unity (1.91 ± 0.05). In the presence of 0.5 µM glibenclamide, a sulphonylurea that blocks $K^+$-channel opening by BRL 38227 (Buckingham *et al.*, 1989; Murray *et al.*, 1989a), the curve for the inhibitory effect of BRL 38227 was shifted to the right, in an apparently parallel fashion, such that 5.5 ± 0.7 µM BRL 38227 was required to produce a half-maximal inhibition of this response. From the shift in the BRL 38227 inhibition curve caused by glibenclamide addition, a $K_i$ value of 31.3 ± 1.1 nM for glibenclamide antagonism of the BRL 38227 effect can be calculated. The (+)-enantiomer of cromakalim, BRL 38226, was approximately 100 fold weaker as an inhibitor than BRL 38227 (Figure 2).

The time-course of [$^3$H]-InsP$_x$ accumulation following addition of 100 µM histamine is shown in Figure 3. The effects of 5 µM BRL 38227, added either 15 min before, or 10 min after histamine addition are also shown. These orders of addition are analogous to the investigation of the anti-spasmogenic and spasmolytic actions of an agent in functional studies. Prior addition of BRL 38227 did not affect the initial increase in histamine-stimulated [$^3$H]-InsP$_x$ accumulation; however, a significant inhibitory effect was observed 5 min after histamine addition, and BRL 38227 caused a 63% inhibition of the rate of [$^3$H]-InsP$_x$ accumulation between 10 and 30 min after histamine addition (Figure 3). In contrast, BRL 38227 addition 10 min after histamine challenge appeared to cause an immediate inhibition of the rate of [$^3$H]-InsP$_x$ accumulation (71%, measured between 10 and 30 min after histamine addition). The changes in histamine-stimulated [$^3$H]-InsP$_x$ accumulation in the presence of BRL 38227 are unlikely to be related to differences in inositol phospholipid labelling, as it can be seen from Figure

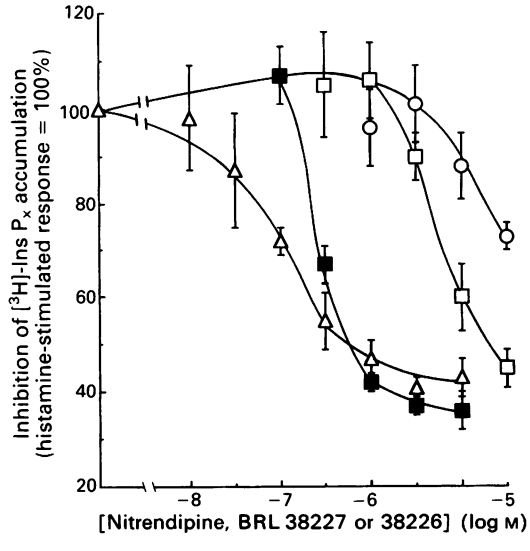


**Figure 2** Effects of increasing concentrations of nitrendipine, BRL 38227 and BRL 38226 on histamine-stimulated inositol phosphate accumulation in bovine tracheal smooth muscle. [$^3$H]-inositol pre-labelled bovine tracheal smooth muscle (BTSM) slices were incubated in the presence of various concentrations of nitrendipine (△), BRL 38227 (■), BRL 38227 plus 0.5 µM glibenclamide (□) or BRL 38226 (○) for 15 min before addition of 100 µM histamine for 30 min. Values are means for 3 separate experiments performed in triplicate and are expressed as a percentage of the histamine-stimulated (-basal) [$^3$H]-InsP$_x$ accumulation in the absence of other additions; vertical bars show s.e.mean. The group mean histamine stimulation for the 6 experiments required to produce these data was 10541 ± 509 d.p.m./75 µl BTSM slices.

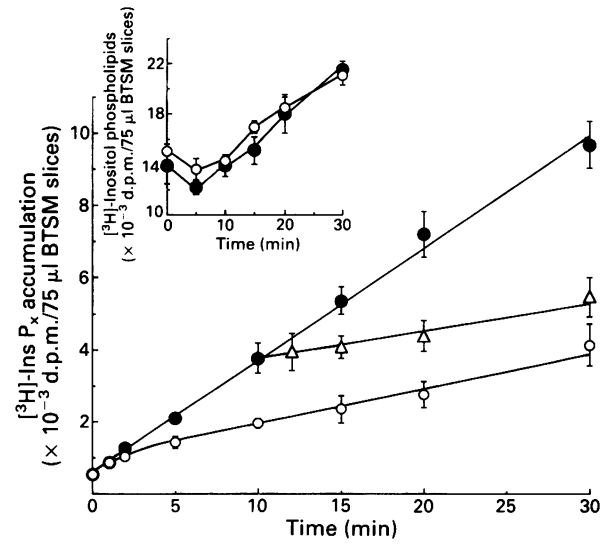


**Figure 3** Effect of BRL 38227 on the time-course of histamine-stimulated inositol phosphate accumulation and changes in inositol phospholipid labelling (inset) in bovine tracheal smooth muscle (BTSM). [$^3$H]-inositol pre-labelled BTSM slices were incubated in the absence (●) or presence (○) of 5 µM BRL 38227 for 15 min before addition of 100 µM histamine at zero-time. Incubations were continued for the times indicated. In some incubations, 5 µM BRL 38227 was added 10 min after histamine addition (△). [$^3$H]-InsP$_x$ accumulation and [$^3$H]-inositol phospholipid labelling (inset) was assessed. Values are means for four separate experiments performed in duplicate or triplicate; s.e.means shown by vertical bars.

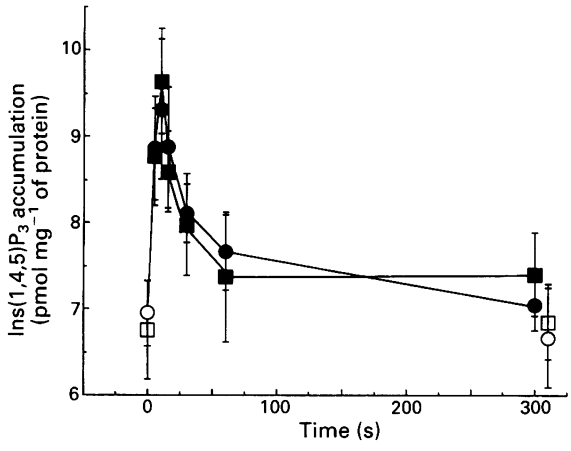


**Figure 4** Effect of BRL 38227 on the initial time-course of Ins(1,4,5)P$_3$ mass accumulation in bovine tracheal smooth muscle (BTSM). BTSM slices were incubated in the absence (○,●) or presence (□,■) of 5 µM BRL 38227 for 15 min before addition of 100 µM histamine (●,■) at zero-time. Values are means for 4 experiments performed in triplicate; vertical bars show s.e.mean.

3 (inset) that levels of [$^3$H]-inositol phospholipids are similar throughout the time-course of histamine stimulation.

In the light of the fact that there appears to be a significant lag-phase before an inhibitory effect of BRL 38227 pre-addition was observed on histamine stimulated [$^3$H]-InsP$_x$ accumulation, it was considered important to investigate whether an inhibitory effect of BRL 38227 could be observed at the level of Ins(1,4,5)P$_3$ generation. To study this, mass accumulations of Ins(1,4,5)P$_3$ were determined 5–300 s after histamine challenge (Figure 4). As observed previously (Chilvers *et al.*, 1989b), histamine caused a small (43 ± 4%), but significant increase in Ins(1,4,5)P$_3$ concentration which was maximal at 10 s after histamine addition; in the same experiments, 100 µM carbachol caused a 115 ± 9% increase in Ins(1,4,5)P$_3$ accumulation, 5 s after agonist addition. Addition of 5 µM BRL 38227 15 min before histamine had no effect on the time-course of Ins(1,4,5)P$_3$ accumulation (Figure 4).

To check that BRL 38227 was eliciting its effects by $K^+$-channel activation, the effect of increasing extracellular potassium concentration ($[K^+]_e$) on the inhibitory effect of BRL 38227 on histamine-stimulated [$^3$H]-InsP$_x$ accumulation was investigated (Figure 5). Increasing $[K^+]_e$ to 65 mM caused a small (30–50%) increase in basal [$^3$H]-InsP$_x$ accumulation and a small (18–25%) inhibition of the histamine-stimulated [$^3$H]-InsP$_x$ accumulation. The inhibitory effect of prior addition of 5 µM BRL 38227 was decreased as $[K^+]_e$ increased, such that it had no inhibitory effect on histamine-stimulated [$^3$H]-InsP$_x$ accumulation when $[K^+]_e$ was elevated to 65 mM (Figure 5). The dependency on $[K^+]_e$ of the effects of prior addition of isoprenaline or nitrendipine on histamine-stimulated [$^3$H]-InsP$_x$ accumulation are compared with those of BRL 38227 in Figure 6. The decrease in the magnitude of the control response to histamine in the presence of 65 mM $K^+$ (Figure 6a) was paralleled by a small decrease in [$^3$H]-inositol phospholipid labelling (Figure 6b). The inhibitory effect of isoprenaline on histamine-stimulated [$^3$H]-InsP$_x$ accumulation was decreased at $[K^+]_e$ (% inhibition at 5 mM: 53.0 ± 3.1; at 65 mM: 26.8 ± 6.3); whereas there was a small increase in the inhibitory effect of nitrendipine at high $[K^+]_e$ (% inhibition at 5 mM: 41.7 ± 2.3; at 65 mM: 55.4 ± 4.6).

## Discussion

In this study we have measured [$^3$H]-InsP$_x$ accumulations in [$^3$H]-inositol pre-labelled, lithium-treated bovine tracheal smooth muscle (BTSM) slices as an index of agonist-stimulated rates of phosphoinositide hydrolysis. Using this method, we have demonstrated that the $K^+$-channel opener BRL 38227 and the $Ca^{2+}$-channel blocker nitrendipine cause inhibition of both muscarinic agonist- and histamine-stimulated [$^3$H]-InsP$_x$ accumulation in BTSM.

The inhibitory actions of these agents differ in a number of respects from the inhibitory effects of β-adrenoceptor

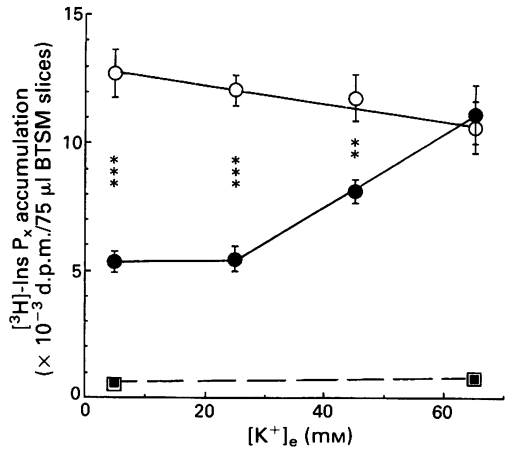


**Figure 5** Effect of extracellular $K^+$ concentration on the inhibitory action of BRL 38227 on histamine-stimulated inositol phosphate accumulation in bovine tracheal smooth muscle (BTSM). [$^3$H]-inositol pre-labelled BTSM slices were incubated in the absence (□,○) or presence (■,●) of 5 µM BRL 38227 for 15 min in Krebs-Henseleit buffer containing the indicated extracellular concentrations of KCl, before addition of 100 µM histamine (○,●) for 30 min. Values are means for three experiments performed in triplicate; vertical bars show s.e.mean. Statistical significance is indicated as: $^{**}P < 0.01$; $^{***}P < 0.001$ for the inhibitory effect of BRL 38227 on histamine-stimulated [$^3$H]-InsP$_x$ accumulations.

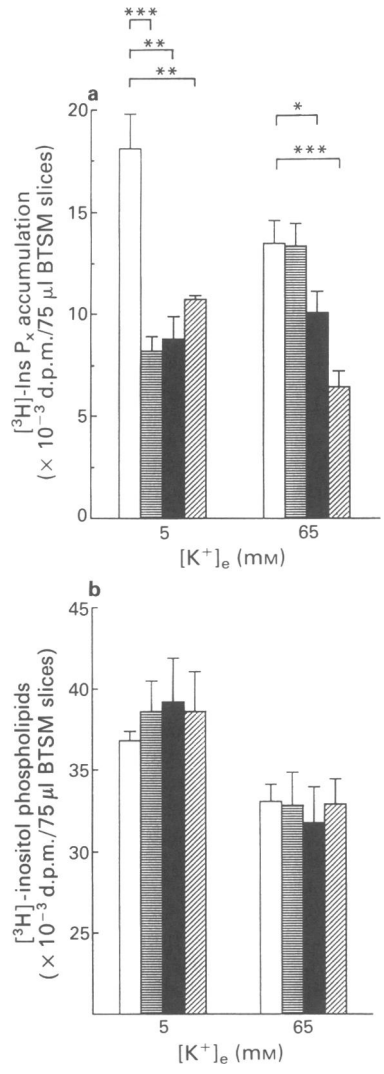


**Figure 6** Dependence on extracellular $K^+$ concentration of the inhibitory effects of BRL 38227, isoprenaline and nitrendipine on histamine-stimulated inositol phosphate accumulations in bovine tracheal smooth muscle (BTSM). [$^3$H]-inositol pre-labelled BTSM slices were pre-incubated for 15 min in the absence (□) or presence of 5 µM BRL 38227 (▤), 10 µM isoprenaline (■) or 1 µM nitrendipine (▨) in Krebs-Henseleit buffer containing 5 or 65 mM KCl, followed by addition of 100 µM histamine for 30 min. Panels shown are: effects on [$^3$H]-$InsP_x$ accumulation (**a**); Effects on [$^3$H]-inositol phospholipid labelling (**b**). Values are means for three separate experiments performed in triplicate; s.e.means shown by vertical bars. Statistical significance as indicated as: $*P < 0.05$; $**P < 0.01$ or $***P < 0.001$ for each $[K^+]_e$ in the absence, compared to the presence of BRL 38227, nitrendipine or isoprenaline. In the absence of histamine, 65 mM $K^+$ caused a small increase in basal [$^3$H]-$InsP_x$ accumulation (5 mM $K^+$ 588 ± 41; 65 mM $K^+$ 750 ± 32 d.p.m./75 µl BTSM slices; $P < 0.05$) with no effect on inositol phospholipid labelling (5 mM $K^+$ 24937 ± 474; 65 mM $K^+$ 25654 ± 883 d.p.m./75 µl BTSM slices).

agonists. In common with previous reports, isoprenaline was ineffective in inhibiting carbachol-stimulated [$^3$H]-$InsP_x$ accumulations at all concentrations of carbachol studied (Madison & Brown, 1988; Hall *et al.*, 1990; Offer *et al.*, 1991). β-Adrenoceptor agonists also have the, as yet unexplained, action of differentially affecting the [$^3$H]-$InsP_x$ response to the partial muscarinic agonists, oxotremorine and pilocarpine (Offer *et al.*, 1991). In contrast, BRL 38227 and nitrendipine each inhibit both pilocarpine and oxotremorine-stimulated responses and exert a significant inhibitory action on [$^3$H]-$InsP_x$ accumulations stimulated by low concentrations of carbachol. Therefore, the actions of BRL 38227 and nitrendipine, but not those of the β-adrenoceptor agonist, on [$^3$H]-$InsP_x$ accumulation can be accommodated by current receptor theory, which would predict that because of the large muscarinic receptor reserve demonstrated in this tissue (Meurs *et al.*, 1988; Gunst *et al.*, 1989), these agents should cause a rightward shift of the carbachol concentration-response curve and a suppression of the maximal effects of partial muscarinic agonists.

Particular attention has been given in this study to the mechanism whereby BRL 38227 exerts its observed inhibitory effects on histamine-stimulated phosphoinositide metabolism in this tissue. Previous studies have shown that the effects of $K^+$-channel openers are independent of changes in cyclic nucleotide concentrations (Gillespie & Sheng, 1989; Berry *et al.*, 1991), and can be antagonized by the sulphonylurea glibenclamide (Murray *et al.*, 1989a; Black *et al.*, 1990; Raeburn & Brown, 1991). In BTSM slices, maximally effective concentrations of BRL 38227 caused a 60–65% suppression of histamine-stimulated [$^3$H]-$InsP_x$ accumulation, with a half-maximal inhibition of this response being observed at a BRL 38227 concentration of 0.3 µM. Glibenclamide (0.5 µM) caused a parallel rightward shift of the BRL 38227 inhibition curve. Whilst this is consistent with a competitive antagonism, functional studies in tracheal preparations with a range of glibenclamide concentrations strongly suggest that the effects of this agent may be more complex (Murray *et al.*, 1989a; Berry *et al.*, 1991). Furthermore, $K^+$-channel openers, including cromakalim, have been shown not to displace specific [$^3$H]-glibenclamide binding from muscle membrane preparations (Gopalakrishnan *et al.*, 1991).

The time-course studies presented here demonstrate that pre-addition of BRL 38227 has no effect on the initial rates of $Ins(1,4,5)P_3$ mass or [$^3$H]-$InsP_x$ formation, and therefore strongly suggest that BRL 38227 has no effect on the initial rate of agonist-stimulated PIC activity; however, this agent does affect the sustained phase of [$^3$H]-$InsP_x$ accumulation seen with prolonged agonist exposure. During this period, $Ca^{2+}$ influx is known to be an important determinant of the contractile response (Coburn & Baron, 1990). The failure to affect the initial rate of phosphoinositide hydrolysis is consistent with the poor anti-spasmogenic activity of $K^+$-channel openers observed in some tissues (Allen *et al.*, 1986; Cook *et al.*, 1988; Bray *et al.*, 1991; Raeburn & Brown, 1991). In contrast to the present findings, Ito *et al.* (1991) have recently reported that BRL 38227 does inhibit $Ins(1,4,5)P_3$ accumulation (at least when experiments are performed under '$Ca^{2+}$-free' conditions) in rabbit mesenteric artery strips 10 s after noradrenaline challenge. This finding is consistent with the effective anti-spasmogenic activity of cromakalim observed in this tissue (Clapham & Wilson, 1987), and suggests that the ability of $K^+$-channel openers to affect the generation of $Ins(1,4,5)P_3$ and the consequent initiation phase of contraction in smooth muscle may be source and/or species dependent.

The inhibition of histamine-stimulated [$^3$H]-$InsP_x$ accumulations by BRL 38227 seems to be dependent on the ability of this agent to modify membrane potential. Decreases in membrane potential, caused by increasing $[K^+]_e$, resulted in a decreased ability of BRL 38227 to inhibit histamine-stimulated [$^3$H]-$InsP_x$, such that at 65 mM $[K^+]_e$ (which the Nernst equation predicts would shift the $K^+$ equilibrium potential in the depolarizing direction by approximately 60 mV) BRL 38227 had no inhibitory effect. These data strongly suggest that the inhibitory effects of BRL 38227 on [$^3$H]-$InsP_x$ accumulation are a direct consequence of membrane hyperpolarization, caused by the action of this agent in increasing the open probability of a sulphonylurea-sensitive class of $K^+$-channel present in BTSM. Cellular hyperpolarization will, in turn, decrease the open probability of voltage-operated $Ca^{2+}$-channels and so reduce $Ca^{2+}$-influx.

In many tissues a $Ca^{2+}$-activated component of the phosphoinositide response can be discerned (Eberhard & Holz, 1988). Thus, in membranes prepared from airway smooth

muscle, changes in calcium concentration within the physiological range have been shown to stimulate PIC activity (Murray *et al.*, 1989b). Therefore, it is possible to put forward the simple hypothesis that agents which directly block $Ca^{2+}$-entry (nitrendipine) or decrease $Ca^{2+}$-channel open probability by membrane hyperpolarization (BRL 38227), can decrease $Ca^{2+}$-stimulated PIC activity and decrease the rate of agonist-stimulated [$^3$H]-Ins$P_x$ accumulation. Furthermore, the time-dependence of the observed inhibition can be explained by an immediate activation of PIC with an initial increase in Ins(1,4,5)$P_3$ and consequent mobilization of intracellular $Ca^{2+}$ stores; thus the $Ca^{2+}$-entry component for maintaining $Ca^{2+}$-stimulated PIC activity will only become significant during the period subsequent to the initial transient increase in Ins(1,4,5)$P_3$ concentration.

There are, however, a number of aspects of the results which are not entirely consistent with the contention that the effects of nitrendipine or BRL 38227 are a consequence of direct or indirect blockade of a pathway of $Ca^{2+}$-entry in this tissue. It has been known for sometime that β-adrenoceptor agonists also cause hyperpolarization of tracheal smooth muscle (Allen *et al.*,, 1985; Honda *et al.*, 1986) through a covalent modification of the $Ca^{2+}$-dependent $K^+$-channel by adenosine 3':5'-cyclic monophosphate (cyclic-AMP)-dependent protein kinase (Kume *et al.*, 1989). In common with the observed effects of $K^+$-channel openers, the β-adrenoceptor agonist-induced hyperpolarization approaches the $K^+$ equilibrium potential (Allen *et al.*, 1985; Honda *et al.*, 1986). However, the β-adrenoceptor effect on transmembrane potential is not required for the relaxant response (Allen *et al.*, 1985) and the failure of elevated $[K^+]_e$ to prevent completely the inhibition of histamine-stimulated [$^3$H]-Ins$P_x$ accumulation by isoprenaline also suggests a difference in the consequences of hyperpolarization caused by isoprenaline and BRL 38227. Furthermore, although extensive evidence exists demonstrating the presence of voltage-operated $Ca^{2+}$-channels in tracheal smooth muscle (Coburn & Baron, 1989; Worley & Kotlikoff, 1990), the importance of such channels as mediators of the maintained $Ca^{2+}$-influx response to spasmogens is still uncertain (Murray & Kotlikoff, 1991).

A further possible inconsistency is that, in agreement with the results of others (Takuwa *et al.*, 1986), we have shown that depolarizing concentrations of $K^+$ cause only a very small increase in [$^3$H]-Ins$P_x$ accumulation, and a slight reduction in histamine-stimulated [$^3$H]-Ins$P_x$ accumulation. This has led some workers to conclude that PIC activity in airway smooth muscle is insensitive to physiological changes in $[Ca^{2+}]_i$. However, recent work by Murray and Kotlikoff (1991), using fura-2 to monitor changes in $[Ca^{2+}]_i$ in human cultured airway smooth muscle cells, has demonstrated that elevating $[K^+]_e$ causes only a modest increase in $[Ca^{2+}]$, whilst $[Ca^{2+}]_i$ actually decreases following histamine challenge in the presence of elevated $[K^+]_e$. If similar changes in $[Ca^{2+}]_i$ occur in bovine tracheal smooth muscle slices in the presence of depolarizing and/or spasmogenic stimuli then this may provide an explanation for the observed changes in [$^3$H]-Ins$P_x$, and in no way argues against the presence of a $Ca^{2+}$-activated PIC activity in this tissue.

The relationship between the actions of BRL 38227, nitrendipine and isoprenaline on spasmogen-stimulated phosphoinositide hydrolysis and the smooth muscle relaxant properties of these agents must also be considered. Interpretation of the functional consequences of inhibition of the sustained phase of agonist-stimulated [$^3$H]-Ins$P_x$ accumulation, which can be rapidly affected by addition of BRL 38227 subsequent to histamine challenge, is limited by our knowledge of the role of phosphoinositide hydrolysis during the period which corresponds to the tonic phase of contraction. The inhibition of phosphoinositide hydrolysis will clearly reduce the rate of DAG formation. Whether this results in a change in DAG concentration and consequently protein kinase C activity is, however, unknown. Indeed, it is not known whether phosphoinositide-derived DAG constitutes an important source of this second messenger in BTSM.

A clear discrepancy between the effects on phosphoinositide metabolism and relaxant effects is evident from comparisons of the actions of BRL 38227 and nitrendipine. It is now recognized that $K^+$-channel openers have a wider spectrum of activity than $Ca^{2+}$-channel blockers both *in vitro* and *in vivo*. For example, cromakalim or BRL 38227 are more effective than nifedipine in protecting guinea-pigs from bronchospasm induced by histamine or 5-hydroxytryptamine (Arch *et al.*, 1988; Bowring *et al.*, 1991). This has led to the proposal that the actions of $K^+$-channel openers are not simply due to effects on voltage-operated channel activity through their hyperpolarizing action (Arch *et al.*, 198; Cook *et al.*, 1988; Bowring *et al.*, 1991, Bray *et al.*, 1991; Raeburn & Brown, 1991). However, the data presented here, which demonstrate a clear effect of both BRL 38227 and nitrendipine at the level of the histamine-and muscarinic agonist-stimulated phosphoinositide response of bovine tracheal smooth muscle, provide no evidence to support this contention.

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# Changes in adenosine sensitivity in the hippocampus of rats with streptozotocin-induced diabetes

P.D Morrison, M.W.B. Mackinnon, J.T. Bartrup, P.G. Skett & [1]T.W. Stone

Department of Pharmacology, University of Glasgow, Glasgow, G12 8QQ

**1** Hippocampal slices have been used to assess the sensitivity of the CNS to adenosine and γ-aminobutyric acid (GABA) in diabetes. The effects of adenosine, 2-chloroadenosine, GABA, muscimol and baclofen were studied on orthodromic synaptic potentials recorded in the CA1 region of slices taken from normal rats or animals made diabetic by the injection of streptozotocin.

**2** In diabetic animals the sensitivity to adenosine was increased 4 fold compared with normal rats. The potency of 2-chloroadenosine was unchanged.

**3** The nucleoside transport inhibitor, hydroxynitrobenzylthioinosine (HNBTI), increased the potency of adenosine in slices from normal rats but not in slices from diabetic rats.

**4** No change was observed in the potency of GABA or muscimol, although a small but significant decrease was detected in the $EC_{50}$ value for baclofen.

**5** Treatment of diabetic animals with insulin restored the potency of adenosine to control levels.

**6** It is concluded that the diabetic state is accompanied by substantial changes of adenosine sensitivity due to the loss of nucleoside uptake processes. Secondary neurochemical changes following from this in human diabetic patients may contribute to the reported behavioural changes.

**Keywords:** Adenosine; purines; diabetes; hydroxynitrobenzylthioinosine; hippocampus

## Introduction

Diabetes mellitus is often accompanied, especially in younger patients, by alterations of behaviour. These include lessened locomotor activity (Rowland *et al.*, 1985; Chu *et al.*, 1986) decreased reaction times and marked affective changes including depression and alterations in emotionality and personality (Burch, 1949; Sterns, 1953; Ryan *et al.*, 1984). Many of these symptoms are directly attributable to the diabetic state and can be reversed by insulin therapy. Behavioural changes have also been noted in animals made diabetic by streptozotocin administration (Rowland *et al.*, 1985; Hilakivi-Clarke *et al.*, 1990).

In general the neurochemical bases for these phenomena remain largely unexplored. In the peripheral nervous system modifications in neurotransmitter function in the diabetic state have been well documented. These include changes of acetylcholine receptor number or affinity and sensitivity to cholinomimetics of several tissues (Latifpour *et al.*, 1989; Carrier & Aronstam, 1987; Aronstam & Carrier, 1989). There are also marked alterations of peripheral catecholamine receptor function or sensitivity in vascular and non-vascular smooth muscle, including changes of α-adrenoceptor (Wald *et al.*, 1988; Drukarch *et al.*, 1989) and β-adrenoceptor mediated responses (Ingebretsen *et al.*, 1983; Berlin *et al.*, 1986; Ramanadhan & Tenner, 1987; Kashiwagi *et al.*, 1989).

Less is known about possible changes of transmitter function in the central nervous system although fragmentary evidence exists for both a hypersensitivity of central cholinoceptors (Page *et al.*, 1987; Arends *et al.*, 1988; Coiro *et al.*, 1989) which would be compatible with the enhanced cholinoceptor function reported peripherally, and changes of monoamine (Lozovsky *et al.*, 1981; Trulson & Himmel, 1983; Rowland *et al.*, 1985; Bitar *et al.*, 1986), opioid (Tang, 1989) and substance P (Kamei *et al.*, 1990) systems.

Another neuroactive compound with changed sensitivity in diabetes is adenosine. In streptozotocin diabetic lambs, Downing (1985) has demonstrated a marked reduction in the coronary dilator activity of adenosine, which can be largely reversed by insulin. Similarly adipocytes from diabetic animals have a reduced sensitivity to adenosine (Solomon *et al.*, 1987; Green & Johnson, 1991). Adenosine is now well recognised as an ubiquitous neuromodulator (Stone, 1991; Stone & Simmonds, 1991) which is released from many tissue during normal cellular activity (White & Hoehn, 1991) and which acts both presynaptically, to depress neurotransmitter release, and postsynaptically to modify cellular activity in a direction which tends to oppose or preclude the development of hypoxic stress (Stone *et al.*, 1990). Any change in the function of adenosine receptors centrally could, therefore, have a variety of secondary effects on other transmitter systems and on behaviour. The present study was designed to compare neuronal effects of adenosine in the normal and streptozotocin diabetic rat. The highest density of the presynaptic $A_1$ receptors for adenosine in the brain occurs in the hippocampal formation, especially on the terminals of those neurones projecting to the CA1 pyramidal cell layer (Goodman & Snyder, 1982; Reddington & Lee, 1991), and this site was consequently chosen as the focus of the present experiments. For comparison, the effects of γ-aminobutyric acid acid (GABA) and the respective $GABA_A$ and $GABA_B$ receptor agonists muscimol and baclofen were also studied.

## Methods

### *Induction of diabetes*

Male Wistar rats (150–250 g) were used throughout this study. Diabetes was induced by a single dose of streptozotocin (60 mg $kg^{-1}$) injected into the tail artery under nitrous oxide/halothane anaesthesia. Animals were maintained for at least 5 days prior to experimentation. At the time of death, blood glucose and ketone bodies were estimated with Keto-Diabur test sticks. Normal rats had blood glucose levels of approximately 2.5 g $l^{-1}$. Rats treated with streptozotocin were classified as diabetic if the blood glucose level exceeded 15 g $l^{-1}$. All animals were given free access to food and water throughout the treatments.

[1] Author for correspondence.

In one group of animals the diabetic state was allowed to develop for 5 days and then a course of insulin replacement was instituted. This consisted of a single daily injection of insulin zinc suspension (10 i.u. per rat) administered s.c. These injections were made for at least 4 days before experimentation and animals were killed approximately 24 h after the final injection of insulin. At death the blood glucose levels in these animals were generally in the range of 2.5–5 g $l^{-1}$.

### *Preparation of brain slices*

Male Wistar rats were anaesthetized with urethane 1.5 g $kg^{-1}$, i.p. and then killed by cervical dislocation. The brain was removed into cold artificial cerebrospinal fluid (ACSF) of the following composition (mM): NaCl 115, KCl 2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $KH_2PO_4$ 2.2, $NaHCO_3$ 25, D-glucose 10 (gassed with 5% $CO_2$ in $O_2$; pH 7.4). The hippocampus was removed and cut into slices 500 μm with a McIlwain chopper. These were then preincubated at room temperature in ACSF under an atmosphere of 5% $CO_2$ in $O_2$ for at least 1 h before use.

Individual slices were then transferred to a 0.5 ml recording chamber and superfused with ACSF at 30°C at a rate of approximately 4 ml $min^{-1}$. The slices were fully submerged in the superfusion medium. For stimulation, the tip of a concentric bipolar electrode was located in the stratum radiatum near the CA1/CA2 border to activate the Schaffer collaterals and commissural fibres projecting to the pyramidal cell layer in the CA1 region. The stimulation parameters normally employed were 0.1 ms duration, 200–500 μA intensity at a frequency of 0.1 Hz.

Recording was effected by single glass microelectrodes containing 1 M sodium chloride (resistances 1–4 megohms), the tips of which were positioned in the CA1 stratum pyramidale so as to record the evoked potentials. These were amplified and displayed on digital storage oscilloscopes from which they were plotted immediately onto a chart recorder. The amplitude of the evoked population spike was adjusted to be submaximal, normally approximately 75% of maximum. The full amplitude (peak positivity to negativity) of the population spike was measured and used to study the effects of agents applied into the superfusion medium.

### *Statistics*

Wherever possible, comparisons between groups were made by a two sample *t* test, but where the number of samples was substantially different or the variance was large, a Mann-Whitney test was employed. Results were considered to be significantly different if $P<0.05$.

### *Compounds*

All compounds were purchased from Sigma Chemical Company. Hydroxynitrobenzylthioinosine (HNBTI) was dissolved in 10% ethanol before dilution to the required concentration. The amount of ethanol in the final superfusate was found to have no effect on the slices. All other compounds were dissolved directly in ACSF.

## Results

### *Adenosine and 2-chloroadenosine*

Adenosine had a concentration-related inhibitory effect on the evoked population spikes in both normal and diabetic rats (Figure 1). In control animals the $EC_{50}$ concentration, measured from individual concentration-response curves, was calculated as 27.7 ± 5.4 μM ($n = 8$ animals). In diabetic animals the concentration-response relationship was displaced to the left, the $EC_{50}$ now being 6.6 ± 1.0 μM ($n = 9$). This reduction in the $EC_{50}$ value was highly significant ($P<0.005$) (Figure 2).

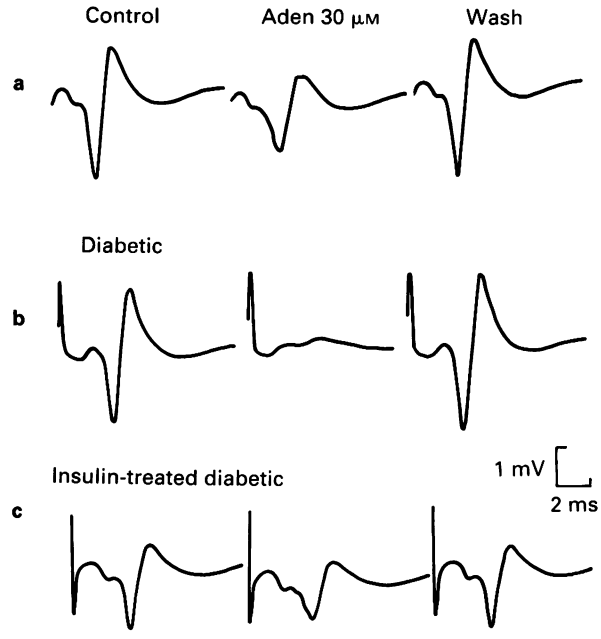


**Figure 1** Records of the orthodromic CA1 population spike potential in slices taken from (a) control animals, (b) streptozotocin diabetic animals and (c) insulin-treated diabetic animals. Each sequence of traces shows responses respectively before, during and following washout of, superfusion with adenosine (Aden) 30 μM. Calibrations 1 mV and 2 ms.

In control rats the inclusion of the nucleoside transport inhibitor HNBTI (10 μM) in the superfusion medium enhanced the effectiveness of adenosine (Figure 3) and shifted the concentration-response curves significantly to the left (Figure 4a), yielding an $EC_{50}$ value of 11.1 ± 2.1 μM ($n = 5$) ($P<0.05$). In contrast, the presence of HNBTI had no significant effect on th $EC_{50}$ value of adenosine tested on slices from diabetic animals; the difference between the $EC_{50}$ concentration of adenosine in slices from diabetic rats without (6.58 ± 1.0 μM, $n = 9$) or with (6.25 ± 1.05 μM, $n = 5$) superfusion of HNBTI (10 μM) was not significant (Figure 4b).

2-Chloroadenosine also exhibited a concentration-dependent inhibition of the CA1 population spike, with an $EC_{50}$ of 0.68 ± 0.07 μM ($n = 6$) in normal animals and 0.69 ± 0.07 μM ($n = 6$) in those treated with streptozotocin. These values are not significantly different.

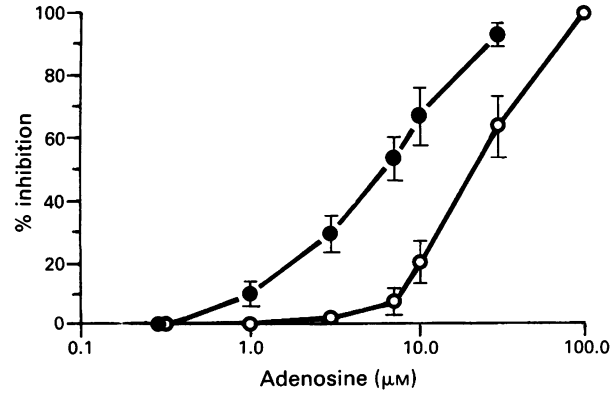


**Figure 2** Concentration-response curves for the inhibitory effect of adenosine upon the CA1 evoked population spike in slices from control animals (○; $n = 8$) and animals with streptozotocin-induced diabetes (●; $n = 9$). Symbols indicate the mean with s.e.mean shown by vertical bars.

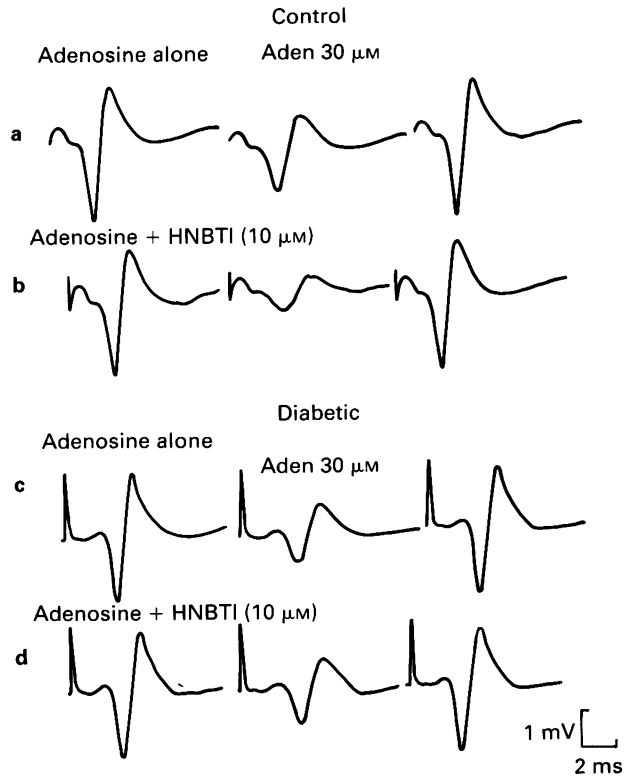


**Figure 3** Records of the orthodromic CA1 population spike in slices taken from control (a and b) or diabetic rats (c and d). Each horizontal sequence shows the response respectively before, during and after superfusion with adenosine (Aden), 30 μM either of naive slices (a and c) or in the presence of the nucleoside transport inhibitor HNBTI, 10 μM (b and d). Calibrations 1 mV and 2 ms.

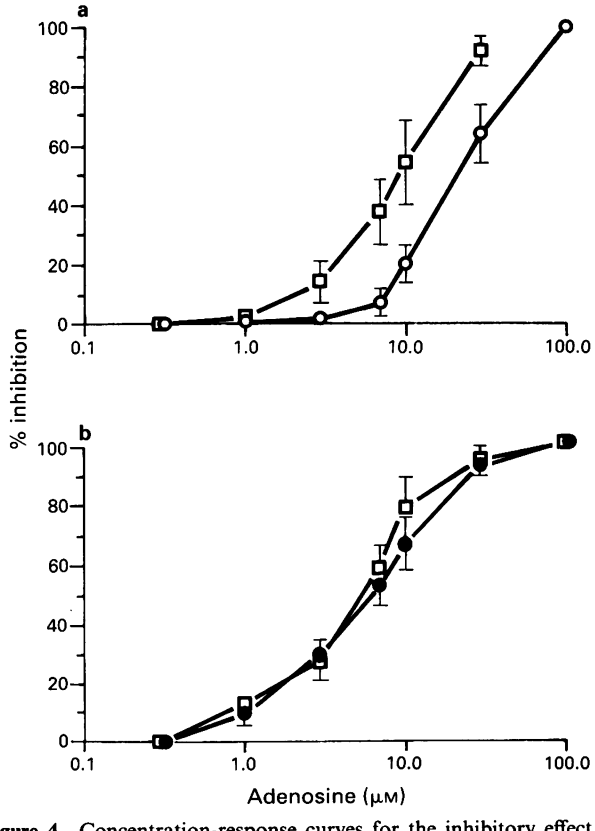


**Figure 4** Concentration-response curves for the inhibitory effect of adenosine upon the CA1 evoked population spike in slices taken from (a) normal animals and (b) diabetic animals. Each graph summarises data from control (naive) slices (circles) and for data obtained in the presence of hydroxynitrobenzylthioinosine (HNBTI) 10 μM (squares). Symbols show the mean with s.e.mean indicated by vertical bars. Statistics are given in the text.

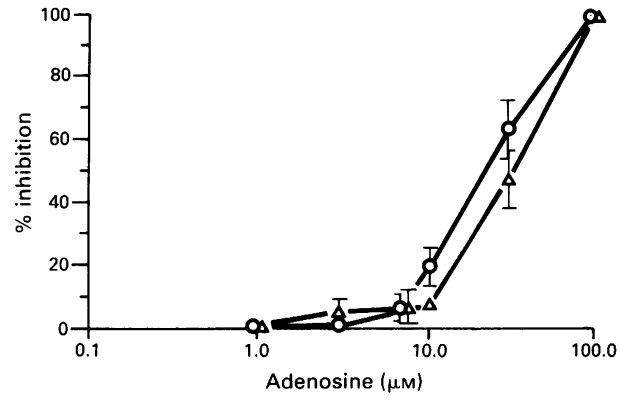


**Figure 5** Concentration-response curves for the inhibitory effect of adenosine on the CA1 evoked population spike in slices taken from normal animals (○; $n = 8$) and from insulin-treated diabetic animals (△; $n = 6$). Symbols show the mean with s.e.mean shown by vertical bars. Statistics are given in the text.

### *Insulin replacement*

In diabetic rats treated with insulin from diabetic day 5, the leftward shift of the adenosine concentration-response curve was no longer observed. The $EC_{50}$ for adenosine in the insulin-treated diabetics was 34.5 ± 4.4 μM ($n = 6$), a value not significantly different from that in normal animals (Figure 5).

### *GABA analogues*

GABA itself, muscimol and (±)-baclofen applied in the superfusion medium all induced a concentration-dependent reduction in the amplitude of orthodromic potentials in slices taken from control animals. The $EC_{50}$ values are summarized in Table 1.

In slices taken from diabetic rats the concentration-response curves were consistently displaced to the left (Figure 6a), the $EC_{50}$ concentrations being decreased in all cases, but only significantly so in the case of baclofen (Table 1). Following treatment of a group of animals with insulin, the curve for (±)-baclofen was no longer shifted and the $EC_{50}$ values for control and diabetic animals were no longer significantly different (Figure 6b).

## Discussion

The present results reveal a striking and highly significant increase in the sensitivity of hippocampal slices to the presynaptic inhibitory action of adenosine. The data contrast with those from studies on coronary vessels (Downing, 1985) and adipocytes (Green & Johnson, 1991) in which a decreased sensitivity to this purine was observed. In the latter case, however, the mechanism of the change was attributed to a decrease in the coupling of the $A_1$ receptor to adenylate cyclase via the associated G-protein (Gi). The presynaptic $A_1$ receptor in the hippocampus, however, is not coupled to

**Table 1** $EC_{50}$ values (μM) for the inhibitory effects of γ-aminobutyric acid (GABA) analogues upon CA1 orthodromic hippocampal potentials

| *Agonist* | *Control* | *Diabetic* |
|---|---|---|
| GABA | 710 ± 80 (5)[a] | 640 ± 140 (5) |
| Muscimol | 3.7 ± 0.6 (7) | 2.3 ± 0.5 (4) |
| Baclofen | 2.3 ± 0.2 (10) | 1.2 ± 0.3 (10)* |

[a]Mean ± s.e.mean ($n$ slices)
*$P < 0.005$ (two-sample $t$ test).

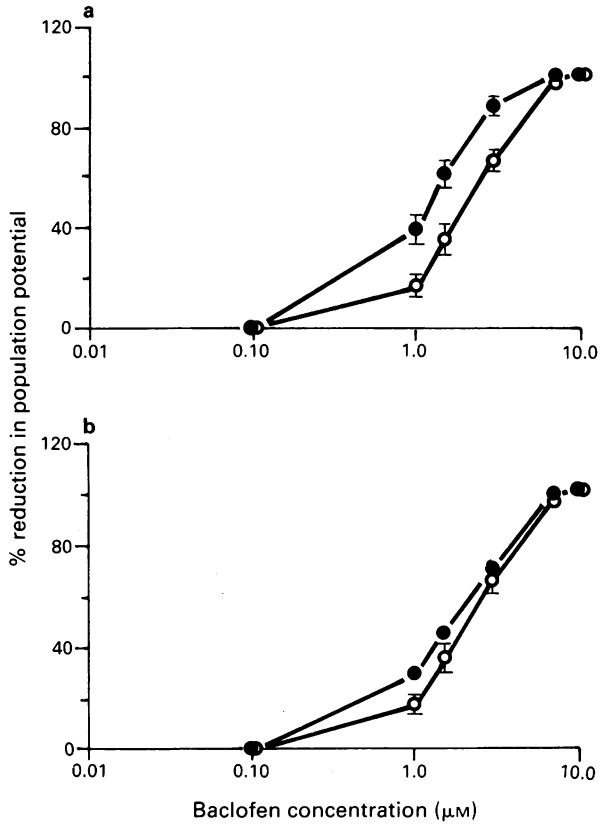


**Figure 6** Concentration-response curves for the inhibitory effect of (±)-baclofen on the CA1 population spike in slices taken from (a) normal (○; $n = 10$) and diabetic animals (●; $n = 7$); (b) normal animals (○; $n = 10$) and insulin-treated diabetic animals (●; $n = 7$). Symbols show the mean with s.e.mean indicated by vertical bars. Statistics are given in Table 1.

cyclase (Fredholm *et al.*, 1986). The present work indicates a different but highly specific mechanism for the change of purine sensitivity in the hippocampus, firstly because no change in the sensitivity of adenosine receptors could be demonstrated with the directly acting agonist 2-chloradenosine. Secondly, the nucleoside transport inhibitor HNBTI proved able to enhance the activity of adenosine only in slices taken from normal animals. These findings suggest that the diabetic state has induced, not a change of receptor properties, but a loss, down-regulation or inactivation of the transport system. This phenomenon does not appear to have been encountered, or studied, elsewhere, since most reported diabetic alterations of transmitter sensitivity have been attributed to changes either of receptor binding site number or affinity or of second messenger function (Cros *et al.*, 1986; Aronstam & Carrier, 1989). It remains unclear whether any change of transport function could have been associated, directly or indirectly, with these phenomena.

The shift of the adenosine concentration-response curve induced either by diabetes or HNBTI was relatively modest, but the hippocampus is an area of brain which has, on the one hand, a particularly high density of adenosine $A_1$ receptors (Reddington & Lee, 1991) but on the other hand possesses a relatively low density of transport sites (Geiger & Nagy, 1984). It is quite conceivable, therefore, that if the loss of uptake activity occurs elsewhere in the CNS a much greater increase of adenosine sensitivity may result than can be demonstrated in the hippocampus. The failure of HNBTI to shift the adenosine concentration-response curve to the same extent as was noted in diabetic animals may reflect the difference between a complete absence of uptake in the latter, and the ability of pharmacological agents to achieve only partial inhibition. It is now well established that there are at least two pharmacologically distinguishable forms of the neuronal transporter, demonstrable using HNBTI and dipyridamole as binding ligands (Hammond & Clanachan, 1984; Marangos & Deckert, 1987; Geiger & Fyde, 1991).

A critical part of this study is the demonstration that insulin replacement therapy beginning at diabetic day 5 fully reverses the change of adenosine sensitivity. This important result indicates that the observed changes are directly attributable to the diabetic state and are not the result of tissue damage occurring as a consequence of the hyperglycaemic and/or ketotic condition. This result also indicates that the alteration of adenosine sensitivity is not due to the presence or toxic actions of streptozotocin itself.

The purine nucleoside transporter is believed to function as a transmembrane shuttle, mediating both the efflux as well as the uptake of purines. However, this is not the only route by which adenosine reaches the extracellular space; it is also produced by the metabolism of extracellular nucleotides, including ATP, 5′-adenosine monophosphate (5′ AMP) and adenosine 3′:5′-cyclic monophosphate (cyclic AMP), (Stone *et al.*, 1990; Meghji, 1991) the efflux of which does not involve the nucleoside transporter. The net effect of a loss of transport activity would therefore be an elevation of extracellular adenosine concentration. The presynaptic inhibitory properties of adenosine (Stone & Simmonds, 1991) on diverse neuronal pathways including those releasing glutamate (Corradetti *et al.*, 1984; Fastbom & Fredholm, 1985), acetylcholine (Spignoli *et al.*, 1984; Jackisch *et al.*, 1984) and dopamine (Michaelis *et al.*, 1979) may thus be substantially enhanced, leading indirectly to some of the neurotransmitter, hormonal and behavioural abnormalities listed in the Introduction.

It is also possible that the changes of adenosine uptake are themselves a reflection of a more general disturbance of neuronal membrane function in the diabetic state. It was to examine this particular question that studies were also conducted on sensitivity to the inhibitory amino acid, GABA, and its analogues muscimol and baclofen. Only in the case of baclofen was the concentration-response relationship displaced to the left in streptozotocin-treated animals, and this shift was considerably smaller than that obtained for adenosine. It is therefore clear that there was no loss of GABA transport activity (which could be demonstrated as a greatly increased potency of GABA in the presence of nipecotic acid, results not shown) in the diabetic rats to compare with the marked loss of purine transport. The absence of any change in muscimol sensitivity would also indicate that $GABA_A$ receptor function was unimpaired. Together with the absence of 2-chloroadenosine sensitivity these data on GABA-related mechanisms strengthen the view that the loss of purine transport is not a reflection of a generalized membrane disorder, but indicates a rather specific abnormality.

The small change in baclofen sensitivity is of interest since Martin *et al.* (1988) demonstrated a decrease of $GABA_B$, but not $GABA_A$ receptor binding in streptozotocin-treated rats. This change is in the wrong direction to permit a simple correlation with the present results, but one interpretation of the results from these two studies may be that a post-receptor change of $GABA_B$ receptor function can be induced in the CNS by the diabetic state.

In conclusion, induction of the diabetic state in rats induces a selective loss of the adenosine transporter, with no change either in the transport mechanism for GABA or in receptor sensitivity to 2-chloroadenosine, GABA or muscimol. It is proposed that the loss of the nucleoside transporter may be indirectly responsible for some of the neurotransmitter and hormonal abnormalities in the diabetic central nervous system of man and animals.

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# Effect of metformin on glucose metabolism in the splanchnic bed

[1]C.J. Bailey, Carol Wilcock & Caroline Day

Department of Pharmaceutical Sciences, Aston University, Birmingham B4 7ET

**1** Use of the antihyperglycaemic agent, metformin, is often associated with a small rise in circulating lactate. This study investigates the source of the lactate and examines the effect of metformin on glucose metabolism by the intestine and liver of rats.

**2** Changes in plasma glucose and lactate were measured in the inferior vena cava (IVC), hepatic portal vein (HPV), hepatic vein (HV) and aorta (A) after intrajejunal administration of metformin (50 and 250 mg $kg^{-1}$) without and with glucose (2 g $kg^{-1}$).

**3** Metformin 250 mg $kg^{-1}$ reduced the hyperglycaemic response to a glucose challenge, associated with a greater reduction of glucose concentrations in the HPV (average decrease of 33% at 60 and 120 min) than at other sites.

**4** Both doses of metformin increased lactate concentrations in the glucose-loaded state: the highest concentration (2.5 fold increase) was recorded in the HPV 60 min after administration of 250 mg $kg^{-1}$ metformin, with a high lactate concentration persisting in the HV at 120 min. Metformin 250 mg $kg^{-1}$ also increased lactate concentrations in the basal state, with highest concentrations (2 fold increase) in the HPV.

**5** Two hours after intrajejunal administration of metformin, 50 mg $kg^{-1}$, rings of tissue from the small intestine showed an average 22% decrease in glucose oxidation ([$^{14}$C]-glucose conversion to $^{14}CO_2$) and a 10% increase in lactate production. Since glucose metabolism in the gut is predominantly anaerobic, metformin caused an overall 9.5% increase of intestinal glucose utilization.

**6** Metformin, $10^{-6}$ and $10^{-4}$ mol $l^{-1}$, did not significantly alter glucose oxidation or lactate production by isolated hepatocytes, but a very high concentration of metformin ($10^{-2}$ mol $l^{-1}$) increased lactate production by 60%.

**7** The results support the view that metformin increased intestinal glucose utilization and lactate production by the intestine. Under basal conditions there was net extraction of lactate by the liver but not after an enteral glucose load.

**Keywords:** Metformin; glucose metabolism; lactate; splanchnic bed; liver; intestine

## Introduction

Metformin (dimethylbiguanide) is an antihyperglycaemic agent used for the treatment of non-insulin-dependent diabetes mellitus (NIDDM) (Bailey, 1988; Bailey & Nattrass, 1988). The glucose-lowering action is associated with increased peripheral glucose disposal, particularly by skeletal muscle, and decreased hepatic glucose production (Bailey & Puah, 1986; Prager *et al.*, 1986; Nosadini *et al.*, 1987; Wollen & Bailey, 1988). There is also evidence that metformin decreases the rate of intestinal glucose absorption (Lorch, 1971; Wilcock & Bailey, 1990a). The antihyperglycaemic action of metformin requires the presence of insulin but the drug does not stimulate insulin secretion (Bailey, 1988; Bailey & Nattrass, 1988).

Concern about the use of metformin has focused on its propensity to raise circulating lactate concentrations, mainly after meals, although the magnitude of this effect is generally small (<2 mmol $l^{-1}$) (Campbell *et al.*, 1987; Jackson *et al.*, 1987; Bailey, 1988; Bailey & Nattrass, 1988).

Metformin does not cause lactic acidosis if appropriately prescribed, i.e. if patients with renal and hepatic insufficiency are excluded (Bailey, 1988; Bailey & Nattrass, 1988; Campbell, 1990). Extra lactate production during metformin therapy was presumed to arise from peripheral tissues such as skeletal muscle, but recent studies failed to confirm that notion (Bailey & Puah, 1986; Jackson *et al.*, 1987).

The present study investigates the possibility that increased lactate production by metformin arises from the splanchnic bed. *In vivo* and *in vitro* experiments in rats have been undertaken to examine the effect of metformin on glucose metabolism and lactate production by the intestine and liver.

[1] Author for correspondence.

## Methods

### *Animals*

Adult male Wistar rats weighing about 200 g were maintained in an air-conditioned room at 22 ± 2°C with 12 h light (08 h 00 min–20 h 00 min), and supplied a standard pellet diet (Rat breeding diet, Heygate and Sons, Northampton) and tap water.

### *Blood sampling studies*

Rats were anaesthetized with sodium pentobarbitone (60 mg $kg^{-1}$ i.p.) and maintained under anaesthesia with further doses of 15 mg $kg^{-1}$ $h^{-1}$. Rectal temperature was held at 34–36°C. The abdomen was opened and blood samples (50 µl) were taken through fine heparinised needles inserted at 4 sites: aorta (A) immediately anterior to the branching of the iliac arteries; inferior vena cava (IVC) adjacent to the right ilio-lumbar vein; hepatic portal vein (HPV) immediately before branching into the liver; and hepatic vein (HV) as close to the liver as possible. Test substances were administered by injection into the second loop of the jejunum and massaged distally along the intestine. Groups of 12 h-fasted

rats received either saline (0.9% NaCl, 5 ml $kg^{-1}$) or metformin hydrochloride (50 and 250 mg $kg^{-1}$ 5 $ml^{-1}$). Groups of 4 h fasted rats received either glucose (2 g $kg^{-1}$ 5 $ml^{-1}$) or glucose with metformin hydrochloride (doses as above). Blood samples were taken from all sites immediately before and at 60 and 120 min after administration of test substances. Plasma glucose (Stevens, 1971) and lactate (Noll, 1974) were determined.

### *Intestinal rings*

Fed rats were anaesthetized and treated by intrajejunal administration of either saline or metformin hydrochloride (50 mg $kg^{-1}$) as above. After 2 h, rings of tissue (about 30 mg) were prepared from the proximal, middle and distal regions of the jejunum and ileum. The rings were washed in incubation buffer and incubated for 2 h at 37°C in 3 ml of pregassed (95% $O_2$:5% $CO_2$) Krebs Ringer bicarbonate (KRB) buffer, pH 7.4, containing bovine serum albumin 20 mg $ml^{-1}$, glucose 10 mmol $l^{-1}$, D-[U-$^{14}$C]-glucose 0.5 $\mu$Ci $ml^{-1}$ and insulin $10^{-8}$ mol $l^{-1}$. Production of $^{14}CO_2$ and lactate was determined (Bailey & Puah, 1986).

### *Hepatocytes*

Hepatocytes were isolated from anaesthetized fed rats by a modification of the collagenase method (Berry & Friend, 1969). The following four buffers were infused at 5 ml $min^{-1}$ into the HPV without recirculation: calcium-free KRB supplemented with EGTA (0.5 mmol $l^{-1}$) and sodium heparin (2 units $ml^{-1}$) for 5 min; calcium-free KRB for 5 min; KRB supplemented with collagenase (0.5 mg $min^{-1}$) for 10 min; and KRB supplemented with bovine serum albumin (20 mg $ml^{-1}$) and glucose (10 mmol $l^{-1}$) for 5 min. Buffers were pH 7.4, saturated with 95% $O_2$:5% $CO_2$ and infused at 37°C. The liver was removed and cells were separated by disruption with dissecting needles. The suspension was filtered through muslin, washed and preincubated for 15 min at 37°C in pregassed (95% $O_2$:5% $CO_2$) buffer as used for the last infusion step of the hepatocyte isolation. Cell viability assessed by 0.1% trypan blue exclusion was accepted at >90%, and the number of viable cells was determined. Test incubations were performed with a suspension of $7 \times 10^6$ viable cells $ml^{-1}$ in a final volume of 1.5 ml. Test buffer was the same as for preincubation with the addition of 1.0 $\mu$Ci $ml^{-1}$ D-[U-$^{14}$C]-glucose, without and with insulin ($10^{-8}$ mol $l^{-1}$) and metformin ($10^{-6}$–$10^{-2}$ mol $l^{-1}$). Production of $^{14}CO_2$ and lactate was measured as above after incubation for 2 h at 37°C.

### *Chemicals*

Crystalline bovine insulin (24.3 iu $mg^{-1}$), bovine serum albumin (fraction V, RIA grade), EGTA and collagenase (type IV, from *Clostridium histolyticum*) were from Sigma Chemical Company, Poole, Dorset; D-[U-$^{14}$C]-glucose (specific activity 270 mCi $mmol^{-1}$) was from Amersham International, Amersham, UK; and pure metformin hydrochloride (batch 2452) was from Lipha Pharmaceuticals, West Drayton.

### *Statistical analysis*

Data are presented as mean ± s.e.mean. Data were evaluated for the effect of metformin by one-way analysis of variance and differences between individual groups were compared by Student's *t* test with Bonferroni's correction for multiple comparison. Differences were considered to be significant if $P < 0.05$.

## Results

### *Blood sampling studies*

Intrajejunal administration of saline (5 ml $kg^{-1}$), or metformin (50 and 250 mg $kg^{-1}$) to 12 h-fasted anaesthetized rats did not significantly alter plasma glucose concentrations at each of the four sites sampled (IVC, HPV, HV and A) over

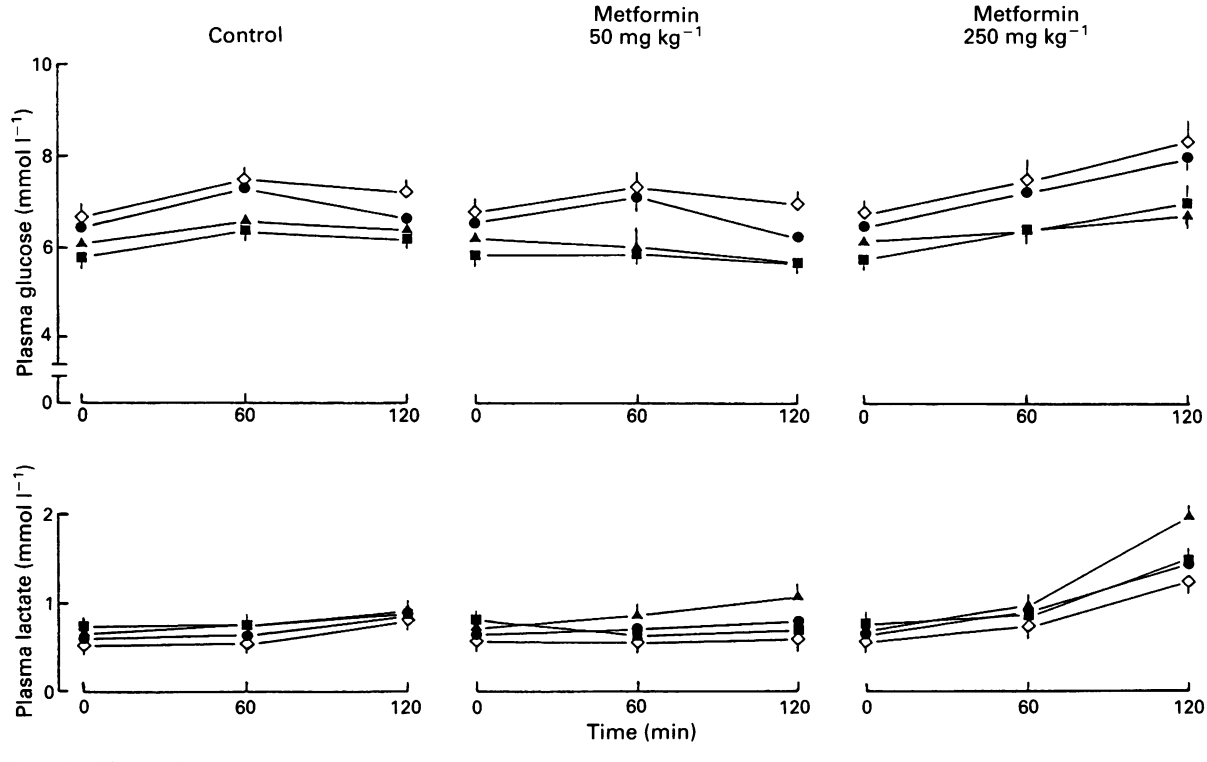


**Figure 1** Plasma glucose and lactate concentrations of 12 h-fasted anaesthetized rats after intrajejunal administration of saline, and 50 and 250 mg $kg^{-1}$ metformin. (■) Inferior vena cava; (▲) hepatic portal vein; (◇) hepatic vein; (●) aorta. Values are mean with s.e.mean shown by vertical lines, $n = 6$.

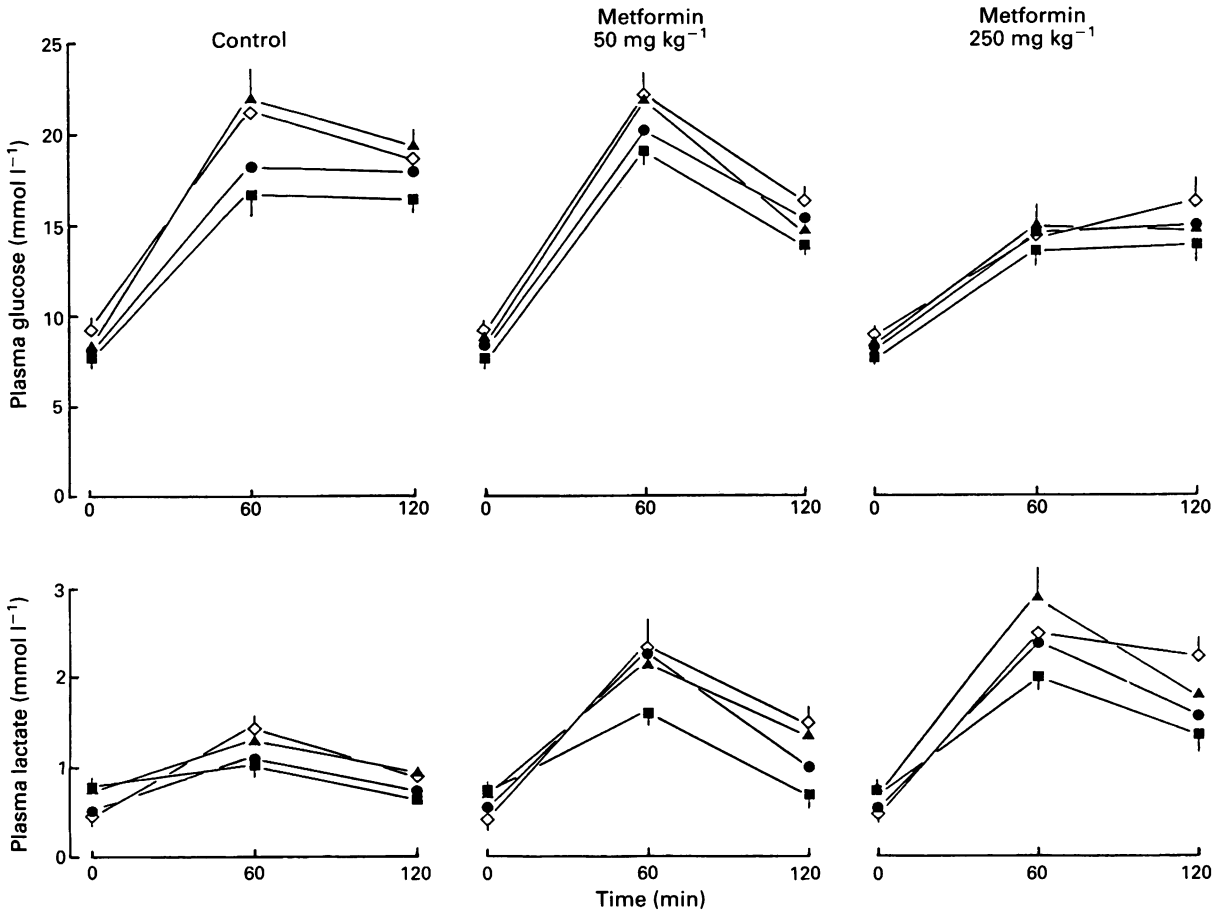


**Figure 2** Plasma glucose and lactate concentrations of 4 h-fasted anaesthetized rats after intrajejunal administration of glucose (2 g kg$^{-1}$) without and with 50 and 250 mg kg$^{-1}$ metformin. (■) Inferior vena cava; (▲) hepatic portal vein; (◇) hepatic vein; (●) aorta. Values are mean with s.e.mean shown by vertical lines, $n = 10$ for controls and $n = 5$ for the metformin-treated groups.

the 120 min duration of the study (Figure 1). Plasma lactate concentrations at each of the four sites were not significantly altered by administration of saline or metformin, 50 mg kg$^{-1}$. However, 250 mg kg$^{-1}$ metformin increased plasma lactate concentrations (ANOVA, $P<0.05$), with the greatest increase occurring in the HPV (by 104%, $P<0.01$) at 120 min.

Intrajejunal administration of glucose (2 g kg$^{-1}$) increased plasma glucose concentrations at all four sites sampled (Figure 2). The greatest increase occurred in the HPV and the smallest increase in IVC as expected. Each dose of metformin exerted an antihyperglycaemic effect but the pattern of the glucose response was different. Administration of 50 mg kg$^{-1}$ metformin with the glucose, reduced plasma glucose concentrations at 120 min in HPV (by 38%, $P<0.01$) and IVC (by 17%, $P<0.05$). When 250 mg kg$^{-1}$ metformin was administered with the glucose, plasma glucose concentrations were reduced at 60 min at all four sites (IVC by 21%, HPV by 37%, HV by 36% and A by 17%, all $P<0.05$), and at 120 min in HPV (by 29%, $P<0.05$). Plasma lactate concentrations were raised at all four sites at 60 min after intrajejunal glucose administration (IVC by 46%, HPV by 77%, HV by 270% and A by 121%, all $P<0.05$ compared with time zero). In glucose-loaded rats, metformin increased lactate concentrations in a dose-dependent manner. Administration of 50 mg kg$^{-1}$ metformin with glucose produced greater increases in plasma lactate than glucose alone at all four sites at 60 min (IVC by 48%, HPV by 52%, HV by 58% and A by 113% greater than glucose only, all $P<0.05$). Administration of 250 mg kg$^{-1}$ metformin with the glucose increased plasma lactate at all four sites at 60 and 120 min: at 60 min IVC by 99%, HPV by 123%, HV by 62% and A by 118% greater than glucose only; at 120 min IVC by 103%, HPV by 89%, HV by 160% and A by 99% greater than glucose only. All values were significantly ($P<0.05$) greater than glucose alone or glucose with 50 mg kg$^{-1}$ metformin. It is noteworthy that 250 mg kg$^{-1}$ metformin caused a protracted increase in plasma lactate in the HV of glucose-loaded rats.

### *Intestinal rings*

Two hours after intrajejunal administration of 50 mg kg$^{-1}$ metformin, rings of proximal, middle and distal regions of the jejunum and ileum were prepared and incubated *in vitro* with 10 mmol l$^{-1}$ U-[$^{14}$C]-glucose. Glucose oxidation assessed by production of $^{14}CO_2$, was decreased (by 19–38%) in the mid-jejunum through to the mid-ileum (Figure 3). Lactate production was increased (by 21–25%) in the mid-jejunum through to the proximal ileum. Since intestinal glucose metabolism was mainly anaerobic, metformin increased glucose utilization by 23% in the mid-jejunum to proximal ileum, and by 9.5% for the overall jejunum and ileum.

### *Hepatocytes*

Glucose oxidation and lactate production were assessed using hepatocytes of fed rats incubated with metformin ($10^{-6}$, $10^{-4}$ and $10^{-2}$ mol l$^{-1}$). Metformin $10^{-6}$ and $10^{-4}$ mol l$^{-1}$ did not significantly alter glucose oxidation or lactate production, either in the absence or presence of $10^{-8}$ mol l$^{-1}$ insulin, although $10^{-4}$ mol l$^{-1}$ metformin increased mean values for lactate production (Figure 4). Metformin, $10^{-2}$ mol l$^{-1}$, decreased glucose oxidation (about 30%) and increased lactate production (about 60%) in the absence and presence of $10^{-8}$ mol l$^{-1}$ insulin. Insulin ($10^{-8}$ mol l$^{-1}$) alone did not significantly alter glucose oxidation or lactate production.

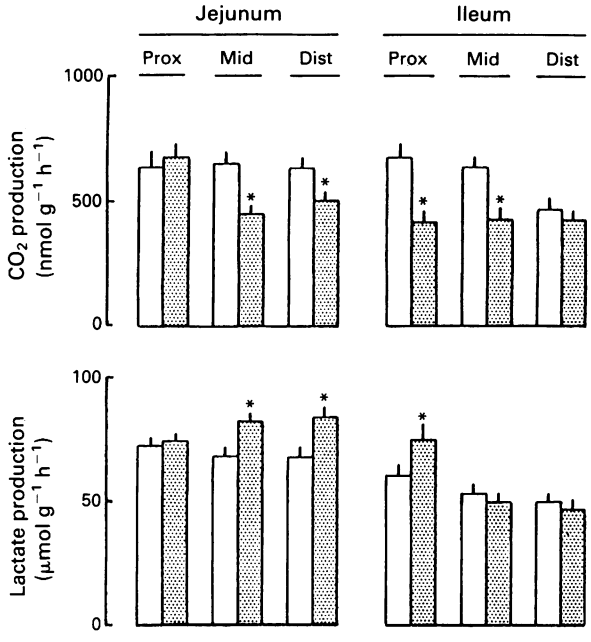


**Figure 3** Glucose oxidation to $CO_2$ and lactate production by intestinal rings prepared from rats 2 h after intrajejunal administration of 50 mg $kg^{-1}$ metformin. Open columns indicate controls; stippled columns indicate metformin-treated. Values are mean with s.e.mean shown by vertical lines, $n = 6$. $^*P < 0.05$ versus control (Student's $t$ test).

## Discussion

Clinically, metformin is prescribed in doses of 500–1000 mg twice or thrice daily with meals. Thus 50 mg $kg^{-1}$ metformin given to a rat is equivalent on a weight related basis to the maximum clinical daily dose (3 g 60 $kg^{-1}$) delivered as a single bolus. In man, consumption of 1 g metformin produces peak peripheral plasma concentrations of $1-5 \times 10^{-5}$ mol $l^{-1}$ at 1–2 h (Pentikainen *et al.*, 1979; Tucker *et al.*, 1981). In rats, an oral bolus of 50 mg $kg^{-1}$ metformin produced similar maximum concentrations of metformin in peripheral plasma after 1 h (Wilcock *et al.*, 1991). Since rats are less sensitive to the antihyperglycaemic effect of metformin than man (Sterne, 1969), the present selection of metformin doses (50 and 250 mg $kg^{-1}$) and concentrations ($10^{-6}$–$10^{-2}$ mol $l^{-1}$) was considered appropriate to investigate therapeutic and supratherapeutic effects.

Unlike sulphonylureas, metformin is not able alone to cause clinical hypoglycaemia, and it has little effect on basal glucose concentrations in the non-diabetic state (Bailey, 1988; Bailey & Nattrass, 1988; Campbell, 1990). However, metformin lowers glucose concentrations in NIDDM patients and in the glucose-loaded non-diabetic state, hence its designation as an 'antihyperglycaemic' agent. Accordingly, metformin lowered the plasma glucose response to an intrajejunal glucose challenge in the present study. Metformin 250 mg $kg^{-1}$ most strongly reduced HPV glucose concentrations consistent with decreased intestinal glucose absorption (Lorch, 1971; Wilcock & Bailey, 1990a). Recent studies have shown that metformin increases glucose utilization by the intestine (Penicaud *et al.*, 1989; Wilcock & Bailey, 1990b), and our preliminary *in vitro* data indicated a concomitant increase in lactate production (Wilcock & Bailey, 1990b). Although the measurement of lactate concentrations at different sites does not provide information on lactate turnover, the present observation that 250 mg $kg^{-1}$ metformin produced the highest lactate concentrations in the HPV, in both the basal and glucose-loaded state, substantiates the theory that metformin can increase intestinal net lactate production independently of intestinal glucose absorption (Wilcock & Bailey, 1990b). However, the effect of metformin on net lactate production was greater during a glucose challenge, although the absolute magnitude of the increase in lactate concentrations was always small (maximum increase 2.5 fold in the HPV).

In the basal state lactate concentrations were consistently higher in the HPV than HV, suggesting net extraction of lactate by the liver, which would buffer the extent of change in peripheral lactate concentrations. During a glucose challenge the concentration of lactate in the HV may exceed that in the HPV, as evident at 120 min after 250 mg $kg^{-1}$ metformin. This is consistent with other evidence that when glucose and lactate concentrations are raised in the HPV, the liver is no longer able to operate as a net lactate extractor (Jackson *et al.*, 1990). This would explain the clinical observation that metformin increases peripheral lactate concentrations mainly during meal absorption (Bailey & Nattrass,

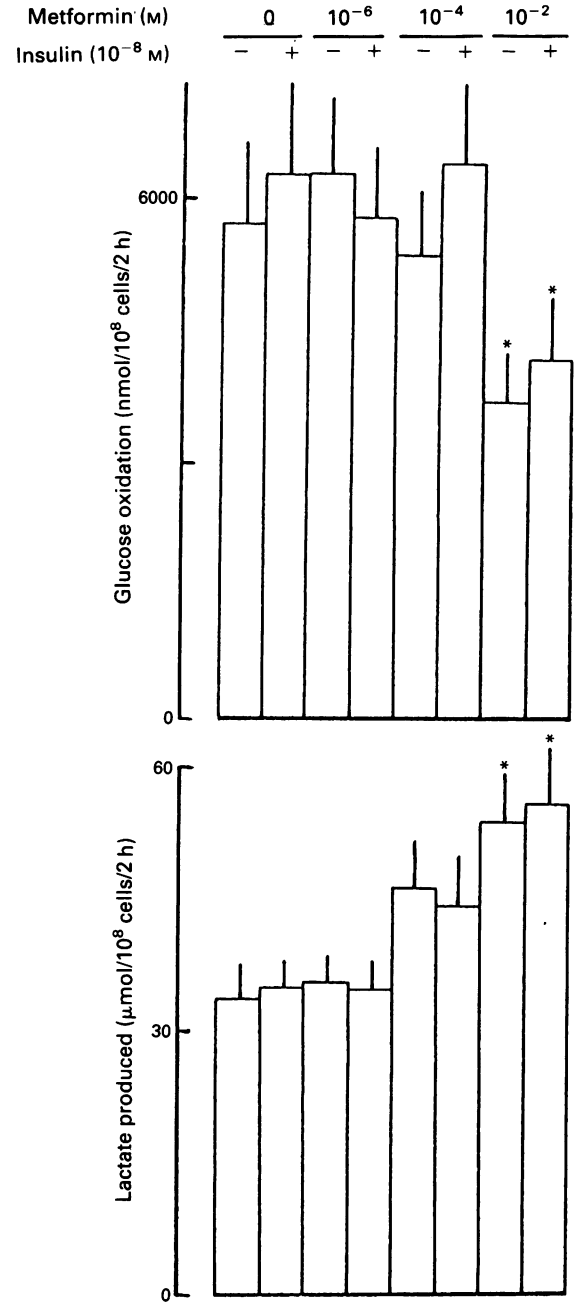


**Figure 4** Glucose oxidation to $CO_2$ and lactate production by rat hepatocytes incubated for 1 h with metformin ($10^{-6}$–$10^{-2}$ mol $l^{-1}$) in the absence and presence of insulin ($10^{-8}$ mol $l^{-1}$). Values are mean with s.e.mean shown by vertical lines, $n = 8$. $^*P < 0.05$ versus control receiving same amount of insulin (Student's $t$ test).

1988; Campbell, 1990), and is consistent with a recent clinical study showing that a net increase in lactate production after metformin does not come from the periphery (Jackson *et al.*, 1987). Indeed *in vitro* studies have discounted muscle, fat, brain and skin as sources of extra lactate production by metformin (Wilcock & Bailey, 1990b). The present study excludes the periphery as the net source of the extra lactate because lactate concentrations were slightly higher in the aorta than IVC.

Although therapeutic concentrations ($10^{-6}$–$10^{-4}$ mol $l^{-1}$) of metformin did not significantly alter glucose oxidation or lactate production by hepatocytes, $10^{-4}$ mol $l^{-1}$ metformin consistently increased mean values for lactate production. Moreover, a very high concentration of metformin ($10^{-2}$ mol $l^{-1}$) reduced glucose oxidation and increased lactate production by hepatocytes, consistent with the measurement of highest glucose and lactate concentrations in the HV at 120 min after treatment of glucose-loaded rats with 250 mg $kg^{-1}$ metformin. The effect of a high metformin concentration in reducing glucose oxidation and increasing lactate production by hepatocytes is comparable with the effect of the drug on the small intestine, but contrasts with the effect of lower (therapeutic) concentrations of metformin that enhance glucose oxidation by peripheral insulin-sensitive tissues, especially in mildly diabetic states (Frayn & Adnitt, 1972; Bailey & Puah, 1986; Wilcock & Bailey, 1990b).

Interestingly, rats appear to be more sensitive than mice to the effects of metformin on glucose metabolism in the intestine and liver; indeed even $10^{-2}$ mol $l^{-1}$ metformin did not significantly alter glucose oxidation by these tissues from mice (Wilcock & Bailey, 1990b). In conclusion, the present study has provided evidence compatible with the view that the main source of extra lactate produced by metformin is the intestine. This is relatively small and may be extracted by the liver unless the liver is presented with high concentrations of both lactate and glucose.

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