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# Expression of nitric oxide synthase in rat glomerular mesangial cells mediated by cyclic AMP

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**1** Treatment of rat mesangial cells with interleukin 1β (IL-1β) or tumour necrosis factor α (TNFα) has been shown to induce a macrophage-type of nitric oxide (NO) synthase. Here we report that adenosine 3′:5′-cyclic monophosphate (cyclic AMP) is another mediator that triggers induction of NO synthase in mesangial cells.

**2** Incubation of mesangial cells with the β-adrenoceptor agonist, salbutamol, forskolin or cholera toxin, which all activate adenylate cyclase and increase intracellular cyclic AMP concentration, increased nitrite formation in a dose-dependent manner. Likewise, the addition of the membrane-permeable cyclic AMP analogue, $N^6$, 0-2′-dibutyryladenosine 3′,5′-phosphate ($Bt_2$cyclic AMP) or the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine enhanced NO synthase activity in a dose-dependent manner.

**3** There was a lag period of about 8 h before a significantly enhanced secretion of nitrite could be detected upon exposure of cells to forskolin and for maximal stimulation, forskolin had to be present during the whole incubation period.

**4** Treatment of mesangial cells with actinomycin D, cycloheximide or dexamethasone completely suppressed forskolin-stimulated NO-synthase activity, thus demonstrating that transcription and protein synthesis are necessary for nitrite formation.

**5** $Bt_2$ cyclic AMP, the most potent inducer of nitrite production, increased NO synthase mRNA levels in mesangial cells in a time- and dose-dependent fashion. Dexamethasone completely inhibited the increase of NO synthase mRNA in response to $Bt_2$ cyclic AMP.

**6** Combination of $Bt_2$ cyclic AMP and IL-1β or TNFα revealed a strong synergy in terms of nitrite formation. Time-course studies indicated that cyclic AMP needed to be increased during the whole period of IL-1β stimulation for maximal nitrite production.

**7** These observations suggest that cyclic AMP controls NO synthase expression in mesangial cells. Furthermore, the signalling cascades triggered by IL-1β and TNFα synergize with the cyclic AMP pathway to stimulate NO synthase activity.

**Keywords:** Nitric oxide synthase; cyclic AMP; interleukin-1β; tumour necrosis factor α; dexamethasone; renal mesangial cells

## Introduction

The generation of nitric oxide (NO) from L-arginine by NO synthase provides a multipurpose messenger molecule responsible for blood vessel relaxation, modulation of synaptic transmission and tumouricidal and bactericidal activities of macrophages (Moncada *et al.*, 1991; Nathan, 1992). Molecular cloning and sequencing analyses revealed the existence of at least three main types of NO synthase isoforms (Nathan, 1992). In 1991, Bredt *et al.* were the first to report the molecular cloning of the brain NO synthase. Lamas *et al.* (1992) reported on the molecular cloning and characterization of a distinct NO synthase isoform from endothelial cells. The brain and endothelial enzymes are 60% identical at the amino acid level. Both enzymes are constitutively expressed and become activated by hormone-stimulated increase in intracellular calcium. A third type of NO synthase has been cloned from macrophages and proved to be approximately 50% identical to the brain and endothelial enzymes, respectively (Xie *et al.*, 1992; Lyons *et al.*, 1992). In contrast to the brain and endothelial enzymes, the macrophage type of NO synthase is not constitutively expressed, but is induced by bacterial endotoxin and γ-interferon, and is calcium insensitive.

Glomerular mesangial cells are a specialized type of vascular smooth muscle cells and take part in the regulation of the glomerular filtration rate (Pfeilschifter, 1989). These cells respond to endothelial-derived NO with increased levels of intracellular guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (Shultz *et al.*, 1990; Marsden *et al.*, 1990). We and others have shown that cytokines, such as interleukin 1 (IL-1) or tumour necrosis factor α (TNFα) induce a macrophage-type of NO synthase in mesangial cells with subsequent elevation of cellular cyclic GMP concentrations (Pfeilschifter & Schwarzenbach, 1990; Marsden & Ballermann, 1990; Pfeilschifter *et al.*, 1992). The excessive formation of NO and cyclic GMP in mesangial cells not only alters contractile responses of the cells (Pfeilschifter *et al.*, 1992), but may also cause tissue injury and thus contribute to the pathogenesis of certain forms of glomerulonephritis (Pfeilschifter *et al.*, 1993). The present paper presents data demonstrating that adenosine 3′:5′-cyclic monophosphate (cyclic AMP) up-regulates NO synthase expression and subsequent nitrite formation in mesangial cells. Moreover, IL-1β and TNFα synergize with cyclic AMP to trigger NO synthase activity, suggesting that two distinct signalling pathways control inducible NO synthase expression in mesangial cells.

## Methods

### *Cell culture*

Rat glomerular mesangial cells were cultured as described previously (Pfeilschifter & Vosbeck, 1991). In a second step, single cells were cloned by limited dilution using 96-microwell plates. Clones with apparent mesangial cell morphology were used for further processing. The cells exhibited the typical stellate morphology. Moreover, there was positive staining for the intermediate filaments desmin and vimentin, which are considered to be specific for myogenic cells (Travo

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et al., 1982), positive staining for Thy 1.1 antigen, negative staining for factor VIII-related antigen and cytokeratin excluded endothelial and epithelial contaminations, respectively. The generation of inositol trisphosphate upon activation of the angiotensin II $AT_1$ receptor was used as a functional criterion for characterizing the cloned cell line. The cells were grown in RPMI 1640 supplemented with 10% foetal calf serum, penicillin (100 u $ml^{-1}$), streptomycin (100 $\mu g\ ml^{-1}$) and bovine insulin at 0.66 u $ml^{-1}$ (Sigma). For the experiments, passages 9–16 were used. Confluent mesangial cells in 24-well plates were incubated with the indicated concentrations of compounds in Dulbecco's modified Eagle's medium (DMEM) without phenol red containing 0.1 mg $ml^{-1}$ of bovine serum albumin for 24 h. Thereafter, the medium was removed for determination of nitrite concentration. Cell protein determination was performed according to the method of Lowry et al. (1951) with bovine serum albumin (Sigma) as standard. For isolation of cellular RNA, cells were cultured in 150 mm diameter dishes.

### *Nitrite analysis*

Nitrite production by rat glomerular mesangial cells was measured as a read-out for NO synthase activity as described previously (Green et al., 1982). Confluent mesangial cells in 24-well plates were washed twice with PBS and incubated in DMEM without phenol red and supplemented with 0.1 mg $ml^{-1}$ of fatty acid-free bovine serum albumin, with or without agents for the indicated time periods. Thereafter, the medium was withdrawn and centrifuged for 10 min at 6000 r.p.m. in an Eppendorf-lab centrifuge. Nitrite was measured by mixing 150 µl of the supernatant with an equal volume of Griess reagent. The absorbance at 550 mm was measured and the nitrite concentration was determined from a calibration curve with sodium nitrite standards.

### *Preparation of cytosol and measurement of L-[$^3$H]-citrulline formation*

Mesangial cells were grown on 100 mm diameter dishes and washed twice with phosphate-buffered saline (PBS). The cells were removed from the culture dishes with a cell scraper and resuspended in HEPES buffer (15 mM, pH 7.4; leupeptin, antipain, pepstatin A (each 10 mg $l^{-1}$), PMSF (172.4 mg $l^{-1}$). After sonification (3 × 10 s; MSE) the homogenate was centrifuged for 1 h at 4°C at 100,000 *g*. Protein content of the cytosol (supernatant) was determined (Bio-Rad assay kit) and assayed for NOS-activity. The conversion of L-arginine to L-citrulline was assayed as reported previously (Förstermann et al., 1992; Goreau et al., 1993) with minor modifications.

Cytosolic samples (200 µl) were incubated for 30 min at 37°C with L-[$^3$H]-arginine (2 µM; 0.1 µCi), dithiothreitol (10 mM), NADPH (0.2 mM), FAD (50 µM), (6**R**)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride (0.2 mM) in a final volume of 220 µl. Some experiments were done in the absence of the cofactors and in the presence of $N^G$-monomethyl-L-arginine (L-NMMA, 400 µM). The reaction was stopped by heating to 90°C for 2 min. After addition of sodium citrate (20 mM; pH 2.2; total volume 230 µl) and centrifugation, 2 ml of a buffer containing EDTA (2 mM), sodium acetate (20 mM; pH 5,5), L-citrulline (0.1 mM) was added. The mixture was loaded on to a strongly acidic cation-exchange column (Bio-Rad AG 50W-X8, $Na^+$-form). The flow-through and the eluate (2 ml of water) were collected and mixed with 8 ml of scintillator (Ultima-Gold, Packard). $^3$H was quantified in a β-counter.

### *Cyclic AMP determination*

Confluent cells in 35 mm diameter dishes were exposed to the different agents for the indicated time periods, washed twice with ice-cold 50 mM Tris/HCl (pH 7.4), 16 mM 2-mercaptoethanol and 8 mM theophylline, then scraped off the dish in 0.5 ml of the above buffer. The reaction was stopped by adding 50 µmol HCl. The cells were disrupted by sonication (10 × 1 s at 20 W) and neutralized with 50 µmol NaOH before aliquots were used to determine cellular cyclic AMP concentrations using a cyclic AMP-binding assay as described by Brown et al. (1972).

### *Statistical analysis*

Statistical analysis was performed by one way analysis of variance (ANOVA). For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons according to Bonferroni.

### *Northern blot analysis*

Confluent mesangial cells were cultured in 150 mm diameter culture dishes. For stimulation, cells were washed twice with PBS and incubated in DMEM without phenol red, supplemented with 0.1 mg $kg^{-1}$ or fatty acid-free bovine serum albumin (Sigma), with or without agents for the indicated time periods. Cells were washed twice with PBS and harvested with a rubber policeman. After centrifugation for 10 min at 1000 r.p.m. in a Heraeus sepatec lab centrifuge the supernatant was removed and the pellet frozen in liquid nitrogen and stored until isolation of RNA. Total cellular RNA was extracted from the cell pellets by the guanidinium thiocyanate/caesiumchloride method (Sambrook et al., 1989). Samples of 20 µg RNA were separated on 0.8% agarose gels containing 0.66 M formaldehyde prior to transfer to gene screen membranes (New England Nuclear). After baking at 80°C for 2 h and prehybridization for 4 h, the filters were hybridized for 16–18 h to a $^{32}$P-labelled Sma I cDNA insert from pMac-NOS (Lyons et al., 1992). To correct for variations in RNA amount, the NOS-probe was stripped with boiling 0.1 × SSPE/1% SDS and the blots were rehybridized to the $^{32}$P-labelled BamHI/SalI cDNA insert from clone pEX 6 coding for β-actin. DNA-Probes (≈2 × $10^6$ c.p.m. $ml^{-1}$) were radioactively labelled with a [$^{32}$P]-dATP by random priming (Boehringer-Mannheim). Hybridization reactions were performed in 50% (v/v) Formamide, 5 × SSPE, 10 × Denhardt's solution, 0.5% (w/v) SDS and 250 $\mu g\ ml^{-1}$ salmon sperm DNA. Filters were washed three times in 2 × SSPE, 0.1% SDS at room temperature for 30 min, and then in 2 × SSPE, 2% SDS at 65°C for 30 min. Filters were exposed for 6–48 h to Kodak X-Omat XAR-film using intensifying screens.

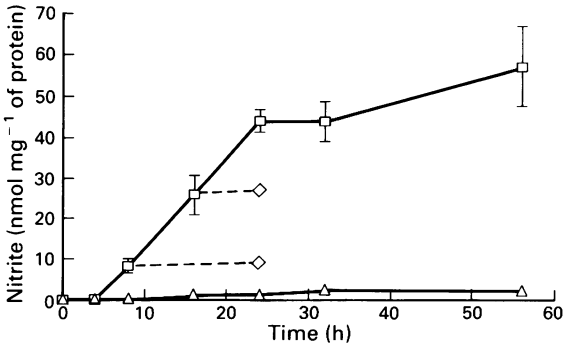


**Figure 1** Time-course of forskolin-stimulated nitrite formation in mesangial cells. Confluent cells were stimulated with forskolin (10 µM, □) or vehicle (△) for the indicated time periods. Thereafter, the medium was removed and used for nitrite determination. Results are means ± s.d. for four experiments. In some experiments forskolin was removed after 8 or 16 h and nitrite production was determined after a total incubation period of 24 h (◇, dashed line), data are indicated as means of two experiments.

### *Chemicals*

Recombinant human IL-1β and salbutamol were generously supplied by Dr Klaus Vosbeck and Dr Irmgard Wiesenberg, Ciba-Geigy Ltd., Basel, Switzerland; recombinant human TNFα was from Boehringer, Mannheim, Germany; forskolin was purchased from Calbiochem, Lucerne, Switzerland; cholera toxin, $Bt_2$ cAMP, dexamethasone, actinomycin D and cycloheximide were from Sigma, Buchs, Switzerland. The cDNA clone pMac-Nos, coding for the inducible macrophage NO synthase was kindly provided by Dr J. Cunningham, Boston, MA, U.S.A.; the cDNA clone pEX6, coding for human β-actin, was a gift from Dr U. Aebi, Basel, Switzerland, nylon membranes (Gene Screen) were purchased from DuPont de Nemours International, Regensdorf, Switzerland; [$^{32}$P]-dATP (specific activity 300 Ci mmol$^{-1}$) was from Amersham, Dübendorf; Switzerland; cell culture media and nutrients were from Gibco BRL, Basel, Switzerland and all other chemicals were either from Merck, Darmstadt, Germany or Fluka, Buchs, Switzerland.

## Results

### *Cyclic AMP mediates nitrite formation in mesangial cells*

Glomerular mesangial cells were incubated in the absence or presence of forskolin and cell culture supernatants were assayed for nitrite production, the stable endproduct of NO formation. Figure 1 demonstrates that after a period of approximately 8 h there was a significant and time-dependent increase in nitrite concentration in the culture supernatant. This response to forskolin was dose-dependent, with a threshold at 0.1 μM forskolin, and increased still further in the concentration-range tested, as shown in Figure 2. For maximal stimulation, forskolin had to be present during the whole incubation period (Figure 1) suggesting that a sustained increase in cyclic AMP is necessary for maximal NO synthase induction. In the presence of 10 μM forskolin, cyclic AMP levels in mesangial cells were increased 14 fold during the first hour and remained at an elevated concentration during at least 24 h of incubation (Figure 3).

The diterpene forskolin is known to activate adenylate cyclase by direct stimulation of the catalytic subunit of the enzyme (Seamon & Daly, 1986). The 1,9-dideoxy derivative of forskolin, which is unable to activate the cyclase (Seamon & Daly, 1986), did not increase nitrite generation (data not shown). This result indicates that it was not a nonspecific effect of forskolin that causes the enhanced nitrite production by mesangial cells. We therefore considered the hypothesis that an activation of adenylate cyclase and an increase of intracellular cyclic AMP may be responsible for the observed stimulation of NO formation by mesangial cells. In the next step we investigated the effects of the β-adrenoceptor agonist, salbutamol and cholera toxin, two compounds that activate adenylate cyclase system by different mechanisms, on nitrite formation. Salbutamol binds to β-adrenoceptor on the cell

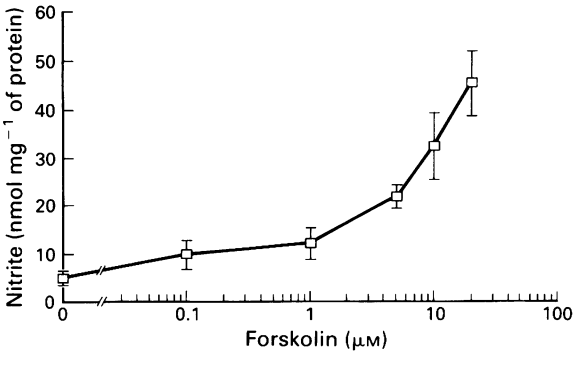


**Figure 2** Dose-response curve of forskolin-stimulated nitrite formation in mesangial cells. Confluent cells were incubated for 24 h with the indicated concentrations of forskolin. Thereafter, the medium was removed and used for nitrite determination. Results were means ± s.d. for four experiments.

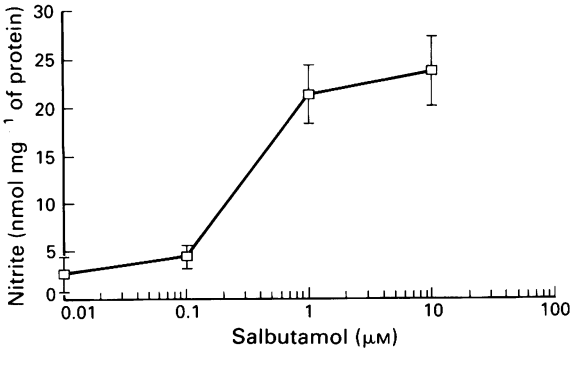


**Figure 4** Dose-response curve of salbutamol-stimulated nitrite formation in mesangial cells. Confluent cells were incubated for 24 h with the indicated concentrations of salbutamol. Thereafter, the medium was removed and used for nitrite determination. Results were means ± s.d. for four experiments.

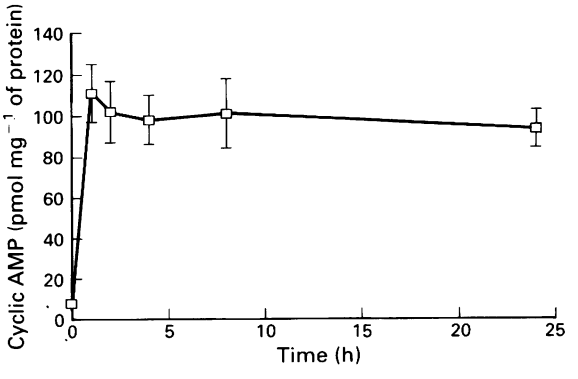


**Figure 3** Time-course of forskolin-stimulated cyclic AMP levels in mesangial cells. Confluent cells were exposed to forskolin (10 μM) for the indicated time periods, then washed twice with ice-cold Tris buffer. Cells were scraped off the dish and disrupted by sonication. Cellular cyclic AMP concentrations were determined as described in the Methods section. Results are means ± s.d. for four experiments.

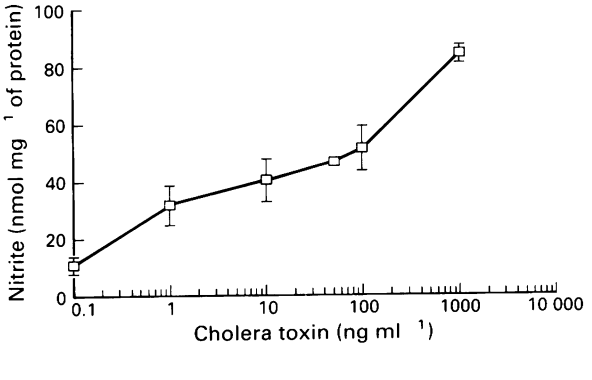


**Figure 5** Dose-response curve of cholera toxin-stimulated nitrite formation in mesangial cells. Confluent cells were incubated for 24 h with the indicated concentrations of cholera toxin. Thereafter, the medium was removed and used for nitrite determination. Results were means ± s.d. for four experiments.

surface of mesangial cells (Pfeilschifter *et al.*, 1991) and activates adenylate cyclase via the stimulatory G-protein, $G_S$. Cholera toxin causes ADP-ribosylation of $G_S$, thus altering this G-protein to a state of permanent activation, leading to the subsequent stimulation of the catalytic moiety of adenylate cyclase. As shown in Figure 4 for salbutamol and in Figure 5 for cholera toxin, both agents enhanced nitrite production in a dose-dependent manner. In a further approach we used the membrane-permeant analogue of cyclic AMP, $Bt_2$ cyclic AMP, to elevate the intracellular concentration of cyclic AMP directly. The data in Figure 6 clearly demonstrate that $Bt_2$ cyclic AMP dose-dependently increased nitrite synthesis by mesangial cells. In addition, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (which increases cyclic AMP by inhibiting the enzyme that degrades it) also enhanced nitrite production and a combination of 3-isobutyl-1-methylxanthine and forskolin resulted in an additive response (Table 1). $N^G$-monomethyl-L-arginine (L-NMMA) was a potent inhibitor of cyclic AMP-stimulated nitrite synthesis and completely abolished forskolin-, cholera toxin- and $Bt_2$ cyclc AMP-induced nitrite production (Table 1). In an alternative approach we have determined NO synthase activity by monitoring the conversion of L-[$^3$H]-arginine to L-[$^3$H]-citrulline. As shown in Table 2, incubation of mesangial cells with $Bt_2$ cyclic AMP for 16 h dose-dependently increased NO synthase activity in the cytosolic fractions of the cells, thus confirming our data obtained by nitrite analysis. Furthermore, L-NMMA prevented $Bt_2$ cyclic AMP-induced L-[$^3$H]-citrulline formation (Table 2), thus ensuring that citrulline production is related to NO synthase activity.

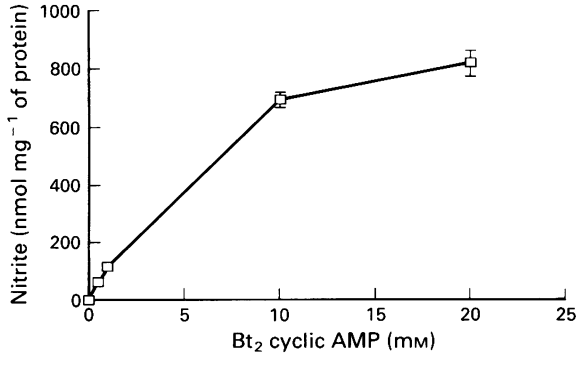


**Figure 6** Dose-response curve of $Bt_2$ cyclic AMP-stimulated nitrite formation in mesangial cells. Confluent cells were incubated for 24 h with the indicated concentrations of $Bt_2$ cyclic AMP. Thereafter, the medium was removed and used for nitrite determination. Results were means ± s.d. for four experiments.

**Table 1** Inhibition of cyclic AMP-induced nitrite production by $N^G$-monomethyl-L-arginine (L-NMMA)

| *Addition* | *Nitrite* (nmol mg$^{-1}$ of protein) |
|---|---|
| Control | 4.5 ± 2.2 |
| L-NMMA | 5.6 ± 3.1 |
| Forskolin | 38.4 ± 3.0* |
| IBMX | 11.0 ± 1.9 |
| Forskolin + IBMX | 51.0 ± 6.2** |
| Forskolin + NMMA | 4.5 ± 2.4 |
| Cholera toxin | 60.0 ± 10.5** |
| Cholera toxin + NMMA | 8.6 ± 2.3 |
| $Bt_2$ cyclic AMP | 206.4 ± 28.5** |
| $Bt_2$ cyclic AMP + NMMA | 12.6 ± 3.4 |

Confluent mesangial cells were incubated for 24 h with vehicle (control), L-NMMA (1 mM), forskolin (10 μM), 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM), cholera toxin (500 ng ml$^{-1}$), $Bt_2$ cyclic AMP (5 mM) or combinations of the cyclic AMP-activators plus L-NMMA or IBMX. Thereafter, the medium was removed and used for nitrite determination. Results are means ± s.d. of four experiments. Significant differences from control: $*P<0.05$; $**P<0.01$ and $***P<0.001$; ANOVA.

### *Actinomycin D, cycloheximide and dexamethasone abolish nitrite synthesis*

Addition of actinomycin D (1 μM) or cycloheximide (10 μM), at the time of stimulation, completely suppressed forskolin (10 μM)-induced formation of nitrite by mesangial cells (Table 3). These results together with the lag period of onset of nitrite production, indicate that RNA synthesis and protein synthesis are necessary for observed nitrite formation. Moreover, dexamethasone, which has been shown to suppress selectively the activity of the inducible NO synthase, but not the constitutitive NO synthase (Radomski *et al.*, 1990; Pfeilschifter & Schwarzenbach, 1990; Pfeilschifter, 1991a), completely prevented nitrite production in response to forskolin stimulation (Table 3), thus suggesting that cyclic AMP triggers the expression of a macrophage-type of NO synthase in mesangial cells. None of the compounds used affected viability of the cells as measured with the MTT test, a sensitive dye exclusion test (Mosmann, 1983).

### *Cyclic AMP increases NO synthase mRNA levels*

Northern blot analysis using a cDNA probe for the inducible mouse macrophage NO synthase revealed a dose-dependent up-regulation of NO synthase steady-state mRNA levels upon stimulation with $Bt_2$ cyclic AMP (Figure 7). The NO synthase mRNA was present as a single band of approximately 4.5 kb. In unstimulated cells there was no detectable NO synthase mRNA. To study the time course of NO synthase mRNA induction following stimulation with $Bt_2$ cyclic AMP, mesangial cells were incubated with 5 mM $Bt_2$ cyclc AMP for 4–24 h. As shown in Figure 8 increases in NO synthase mRNA levels can already be detected after 4 h of $Bt_2$ cyclic AMP stimulation and a maximal level of NO synthase mRNA is seen after 12 h stimulation (Figure 8). The increase of NO synthase mRNA seen after $Bt_2$ cyclic AMP stimulation is comparable to the level observed after stimulation of mesangial cells with IL-1β (Figure 9), a pro-inflammatory cytokine shown to increase potently expression of NO synthase mRNA in mesangial cells (Mühl *et al.*, 1993). Furthermore, addition of dexamethasone completely suppressed the $Bt_2$ cyclic AMP-triggered increase in NO synthase mRNA expression (Figure 9).

**Table 2** Dose-dependence of $Bt_2$ cyclic AMP-stimulated citrulline formation in mesangial cells

| *Addition* | *[$^3$H]-citrulline* (% of total radioactivity) |
|---|---|
| Control | 0.43 ± 0.10 |
| $Bt_2$ cyclic AMP (0.5 mM) | 2.41 ± 0.53 |
| $Bt_2$ cyclic AMP (1 mM) | 5.05 ± 1.72** |
| $Bt_2$ cyclic AMP (5 mM) | 7.75 ± 0.91*** |
| $Bt_2$ cyclic AMP (5 mM) + L-NMMA (0.5 mM) | 0.68 ± 0.17††† |

Confluent cells were incubated for 16 h with the indicated concentrations of $Bt_2$ cyclic AMP. Thereafter cells were homogenized and a cytosolic fraction was used to determine L-[$^3$H]-citrulline formation from L-[$^3$H]-arginine in the absence or presence of L-NMMA as described in the Methods section. Results are means ± s.d. for four experiments. Significant differences from control: $**P<0.01$ and $***P<0.001$, ANOVA. Significant differences from corresponding $Bt_2$ cyclic AMP stimulation in the absence of inhibitor: $†††P<0.001$, ANOVA.

### *Cyclic AMP synergistically interacts with cytokines to stimulate nitrite production*

The proinflammatory cytokines, IL-1β and TNFα, have been reported to induce a macrophage-type of NO synthase in mesangial cells (Pfeilschifter & Schwarzenbach, 1990; Pfeilschifter *et al.*, 1992). We therefore were interested to discover whether the cyclic AMP signalling cascade interacts with the IL-1β and TNFα-triggered signalling pathway to stimulate NO synthase activity in mesangial cells. Figure 10 shows that cytokines and $Bt_2$ cyclic AMP interact in a synergistic fashion to stimulate nitrite generation by mesangial cells. In order to obtain information on the time course of this interaction, cells were incubated with interleukin 1β for 24 h and forskolin was added for different periods during this stimulation. The data in Figures 11 and 12 show that for maximal potentiation of nitrite production, forskolin needs to be present during the whole incubation period, suggesting that a sustained increase in cellular cyclic AMP concentration is necessary for maximal augmentation of NO synthase expression. Furthermore, the data in Figure 12 clearly demonstrate that short-term elevation of cyclic AMP just prior to the end of the stimulation period (20–24 h) is not sufficient to potentiate cytokine-induced NO synthase. These results also argue

**Table 3** Effects of actinomycin D, cycloheximide and dexamethasone on forskolin-stimulated nitrite formation in mesangial cells

| *Addition* | *Nitrite* (nmol $mg^{-1}$ of protein) |
|---|---|
| Control | 4.4 ± 3.0 |
| Forskolin | 35.8 ± 7.4*** |
| Forskolin + actinomycin D | 5.6 ± 1.0††† |
| Forskolin + Cycloheximide | 7.7 ± 5.0††† |
| Forskolin + dexamethasone | 5.9 ± 2.2††† |

Confluent cells were incubated for 24 h with vehicle (control), forskolin alone (10 μM), or in combination with actinomycin D (1 μM), cycloheximide (10 μM) or dexamethasone (10 μM). Thereafter, the medium was removed and used for nitrite determination. Results are means ± s.d. for four experiments. Significant differences from control: ***$P<0.001$, ANOVA. Significant differences from forskolin stimulation (without inhibitors): †††$P<0.001$; ANOVA.

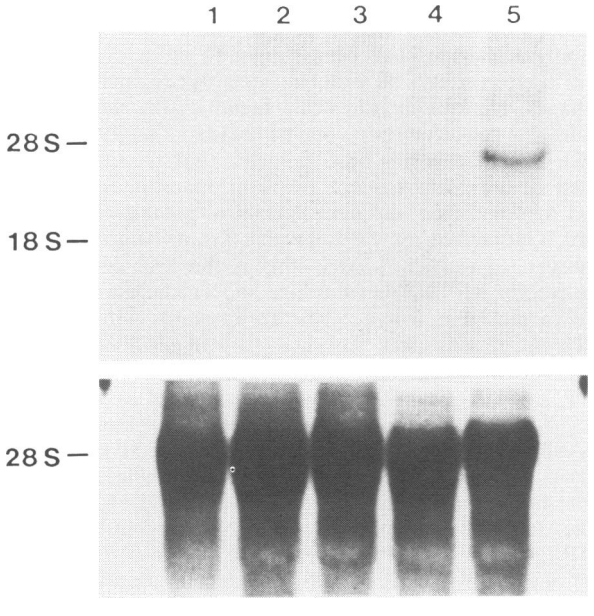


**Figure 7** Dose-dependence of $Bt_2$ cyclic AMP-stimulated NO synthase mRNA accumulation in mesangial cells. Confluent cells were incubated with vehicle (control), or $Bt_2$ cyclic AMP 0.5 mM (2), 1 mM (3), 5 mM (4) and 10 mM (5) for 24 h. Total cellular RNA (20 μg) was successively hybridized to $^{32}$P-labelled NO synthase and 28S ribosomal RNA probes as described in the Methods section.

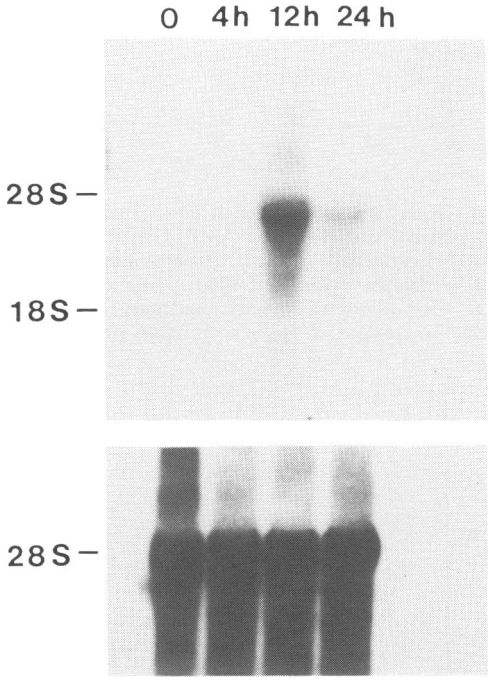


**Figure 8** Time-course of induction of NO synthase mRNA in mesangial cells following stimulation with $Bt_2$ cyclic AMP. Confluent cells were incubated with $Bt_2$ cyclic AMP (5 mM) for the indicated time periods. Total cellular RNA (20 μg) was successively hybridized to $^{32}$P-labelled NO synthase and 28S ribosomal RNA probes as described in the Methods section.

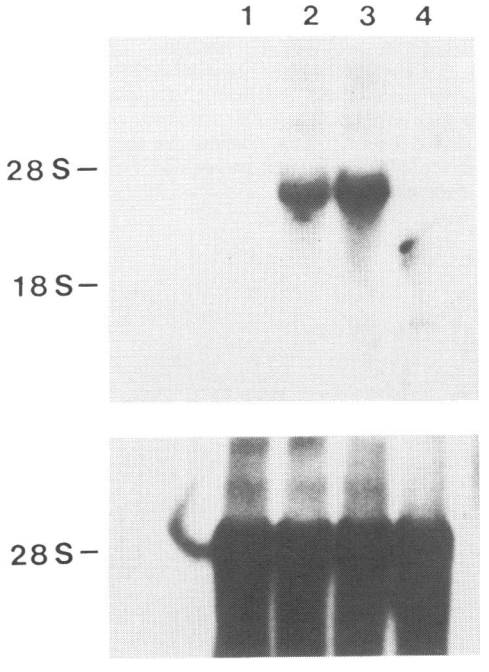


**Figure 9** Comparison of $Bt_2$ cyclic AMP- and IL-1β stimulated NO synthase mRNA accumulation and inhibition by dexamethasone. Confluent mesangial cells were incubated with vehicle (control) (1), IL-1β (1 nM) (2), $Bt_2$ cyclic AMP (5 mM) (3), or $Bt_2$ cyclic AMP (5 mM) plus dexamethasone (1 μM) (4) for 12 h. Total cellular RNA (20 μg) was successively hybridized to $^{32}$P-labelled NO synthase and 28S ribosomal RNA probes as described in the Methods section.

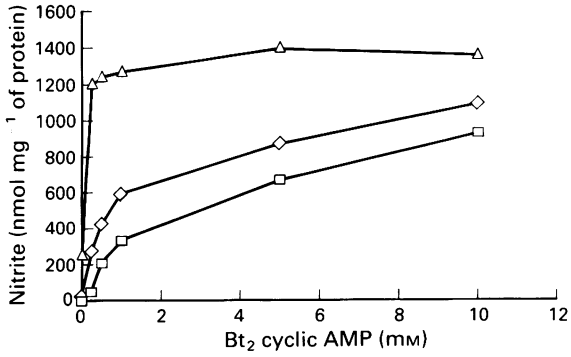


**Figure 10** Synergistic stimulation of nitrite formation in mesangial cells by cytokines and $Bt_2$ cyclic AMP. Confluent cells were stimulated for 24 h with the indicated concentrations of $Bt_2$ cyclic AMP alone (□) or together with IL-1β (500 pM, △) or TNFα (1 nM, ◇). Thereafter, the medium was removed and used for nitrite determination. Results are means of four experiments; s.d. ranged from 5 to 35%.

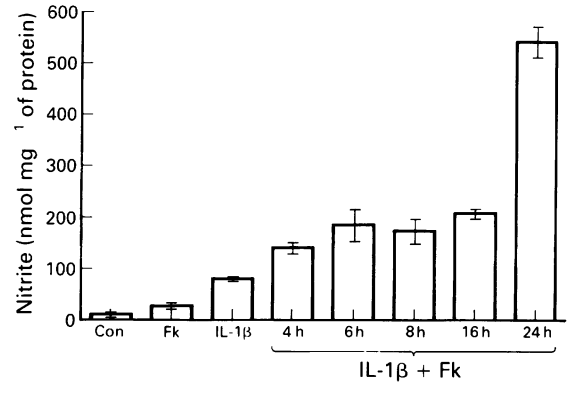


**Figure 11** Time-dependence of the synergistic induction of NO synthase by IL-1β and forskolin. Mesangial cells were incubated for 24 h with vehicle (Con), forskolin, (Fk, 10 μM) IL-1β (2 nM) or IL-1β plus forskolin (IL-1β + Fk). The indicated time points refer to the presence of forskolin in the stimulation medium (forskolin was added at time point 0). Cells were washed after the indicated time and incubation was continued in the presence of IL-1β alone up to a total incubation time of 24 h. Thereafter, the medium was removed and used for nitrite determination. Results are the sum of nitrite produced over the 24 h stimulation period and are expressed as means ± s.d. of four experiments.

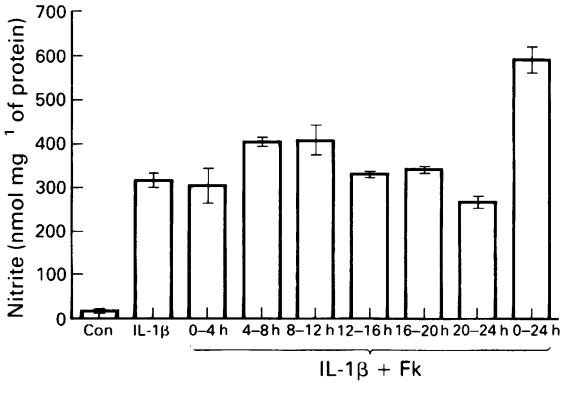


**Figure 12** Critical time period for the synergistic induction of NO synthase by IL-1β and forskolin. Mesangial cells were incubated for 24 h with vehicle (Con), IL-1β (2 nM) or IL-1β plus forskolin (Fk, 10 μM). The indicated time periods refer to the presence of forskolin in the stimulation medium (IL-1β was added at time point 0 and was present throughout the 24 h incubation period). Thereafter, the medium was removed and used for nitrite determination. Results are the sum of nitrite produced over the 24 h stimulation period and are expressed as means ± s.d. of four experiments.

against a role for protein kinase A-mediated covalent modifications of the cytokine-induced NO synthase in the observed potentiation of nitrite production. In summary, these data indicate that IL-1β and TNFα use signalling pathways different from the adenylate cyclase system in order to stimulate NO synthase in mesangial cells.

## Discussion

In many vascular beds *in vitro*, including the kidney, a role for NO in the control of arteriolar resistance has been proposed (Lüscher *et al.*, 1991). The contractile mesangial cell is a major determinant in the regulation of glomerular filtration rate. In coincubation experiments, it has been shown that NO release from glomerular endothelial cells increases cyclic GMP within mesangial cells and thereby inhibits angiotensin II-induced mesangial cell contraction (Shultz *et al.*, 1990; Marsden *et al.*, 1990). Therefore, NO generated by the constitutive endothelial NO synthase may be an important signalling molecule in the cross-communication between glomerular cells, contributing to normal glomerular physiology. Moreover, we and others have demonstrated that proinflammatory cytokines such as IL-1β or TNFα and bacterial lipopolysaccharide induce a macrophage-type of NO synthase in mesangial cells with subsequent elevation of cellular cyclic GMP concentrations (Pfeilschifter & Schwarzenbach, 1990; Marsden & Ballermann, 1990; Pfeilschifter *et al.*, 1992). It seems quite possible that the excessive production of NO may provide mesangial cells with properties that make them partially act as macrophages and thus contribute to tissue injury observed in certain forms of glomerulonephritis (Pfeilschifter *et al.*, 1993; Cattell & Cook, 1993). The latter hypothesis gains support from recent observations showing that anti-inflammatory steroids (Pfeilschifter & Schwarzenbach 1990; Pfeilschifter, 1991a), transforming growth factors of the β-type (Marsden & Ballermann, 1990; Pfeilschifter & Vosbeck, 1991; Pfeilschifter *et al.*, 1992), platelet-derived growth factor (Pfeilschifter, 1991b) and the immunosuppressive drug cyclosporin A (Mühl *et al.*, 1993) potently antagonize cytokine-induced NO synthase induction in mesangial cells. The inhibition of cytokine induction of NO synthase may be one aspect of the beneficial action of glucocorticoids and cyclosporin A seen in certain renal diseases.

Cytokines such as IL-1, TNFα, γ-interferon or bacterial lipopolysaccharide have been shown to induce NO synthase in a huge variety of different cell types including macrophages, smooth muscle cells, hepatocytes, astrocytes and endothelial cells (for review see Moncada *et al.*, 1991; Nathan, 1992). Recently, it has been reported that the cytokine-stimulated nitrite formation in rat liver macrophages (Kupffer cells) and in brain endothelial cells is potentiated by cyclic AMP (Gaillard *et al.*, 1992; Durieu-Trautmann *et al.*, 1993). However, to our knowledge, this is the first demonstration that cyclic AMP is able to induce NO synthase expression on its own and that it synergistically interacts with IL-1β and TNFα in this respect. Several lines of evidence argue in favour of this suggestion: (1) Elevation of intracellular cyclic AMP levels by stimulation with salbutamol at the receptor level, with cholera toxin at the G-protein level and with forskolin at the level of the catalytic subunit of adenylate cyclase, is always accompanied by an enhanced release of nitrite by mesangial cells. (2) Direct elevation of intracellular cyclic AMP by membrane-permeable analogues of cyclic AMP ($Bt_2$ cyclic AMP) causes nitrite synthesis. (3) Cytosolic fractions of $Bt_2$ cyclic AMP-treated cells display increased production of [$^3$H]-citrulline from [$^3$H]-arginine, a reaction catalyzed by NO synthase. (4) $Bt_2$ cyclic AMP increases mRNA steady state levels for inducible NO synthesis in mesangial cells. (5) $Bt_2$ cyclic AMP synergistically augments IL-1β or TNFα-induced nitrite production. The signalling mechanisms by which IL-1 and TNFα exert their effects on target cells are still largely unknown. The strong synergy

between cytokines and cyclic AMP suggests that there exist at least two distinct activation mechanisms for the induction of NO synthase, one is activated by cyclic AMP and the other is triggered by IL-1 or TNF and uses a signalling pathway different from the adenylate cyclase cascade. This is fully compatible with our previous studies on cytokine- and cyclic AMP-stimulated expression of group II phospholipase $A_2$ in mesangial cells (Pfeilschifter *et al.*, 1991; Mühl *et al.*, 1992).

Further studies will be required to provide insights into the mechanisms of cyclic AMP-stimulated NO synthase gene activation. From the potent inhibitory action of actinomycin D (Figure 7) and the lag period of several hours before the onset of NO synthase activity (Figure 1), a transcriptional activation of NO synthase expression seems to be a very likely explanation for the observed data. However, nuclear run-on experiments will be necessary to assess directly rates of transcription of the NO synthase gene. An alternative explanation would be a post-transcriptional stabilization of a putatively unstable NO synthase mRNA by cyclic AMP or cyclic AMP-dependent protein kinase. In this connection it is noteworthy that a kinase-phosphatase system is involved in the regulation of specific proteins that bind to AU-rich sequences in the 3'-untranslated region of mRNAs. These AU-binding factors may protect mRNAs containing AUUUA motifs from degradation. Preliminary data from our laboratory suggest that IL-1β and cyclic AMP synergistically interact to increase NO synthase expression at transcriptional level. Furthermore, message stability studies suggest that cyclic AMP exposure prolongs the half-life of NO synthase mRNA in mesangial cells (Kunz, Mühl, Walker & Pfeilschifter, unpublished observations). Work is going on in our laboratory to elucidate the exact mechanisms that promote expression of NO synthase mRNA in cyclic AMP- and cytokine-treated mesangial cells.

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# Toxic inhibition of smooth muscle contractility by plant-derived sesquiterpenes caused by their chemically reactive α-methylenebutyrolactone functions

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**1** Previous studies have shown that extracts of feverfew (*Tanacetum parthenium*) and parthenolide, a sesquiterpene α-methylenebutyrolactone obtained from it, inhibit smooth muscle contractility in a time-dependent, non-specific and irreversible manner.

**2** The hypothesis that this toxic effect is due specifically to the presence in the sesquiterpene lactone of the potentially reactive α-methylene function was tested on rabbit isolated aortic ring preparations. This was done (a) by comparing the effects of two plant-derived sesquiterpene lactones purified from yellow star thistle (*Centaurea solstitialis*): cynaropicrin (an α-methylenebutyrolactone) and solstitialin 13-acetate (lacking the α-methylene function), and (b) by chemically inactivating the α-methylene functions in cynaropicrin and parthenolide by reaction with cysteine.

**3** The results show that the characteristic smooth muscle inhibitory profile is demonstrated by the two α-methylenebutyrolactones (parthenolide and cynaropicrin), but not by the compound lacking this functional group (solstitialin 13-acetate), or by those previously active compounds in which it has been inactivated with cysteine.

**4** Thus the α-methylene function is critical for this aspect of the toxic pharmacological profile of the sesquiterpene butyrolactones, which are natural products widely distributed in the Compositae family of flowering plants.

**Keywords:** Smooth muscle; irreversible antagonism; natural products; sesquiterpene lactones; feverfew; yellow star thistle; Compositae/Asteraceae; sulphydryl groups

## Introduction

Feverfew (*Tanacetum parthenium*, Compositae, alternatively known as Asteraceae) is a garden plant which is widely used in the UK for self-medication of arthritis and migraine but the pharmacological basis for its claimed effects is not known (Berry, 1984; Editorial, Lancet 1985). Recent studies showed that extracts of fresh leaves contain sesquiterpene α-methylenebutyrolactones such as parthenolide which cause irreversible, time-dependent and non-specific inhibition of aortic smooth muscle contractility (Barsby *et al.*, 1992; 1993). In contrast, extracts of dried powdered leaves available from health food shops do not inhibit aortic contractility and do not contain α-methylenebutyrolactones (Barsby *et al.*, 1993).

In order to understand the molecular mechanisms underlying the inhibition of smooth muscle contractility, it is necessary to establish which chemical groups in the sesquiterpene α-methylenebutyrolactones are responsible. The exocyclic α-methylene function is capable of reacting with thiols such as cysteine residues of amino acids, thereby forming Michael adducts (Rodriguez *et al.*, 1976; Berry, 1984; Groenewegen *et al.*, 1986). The hypothesis that this may be responsible for the toxic actions of certain sesquiterpene lactones can be tested by comparing compounds of closely similar structure but which differ in the presence or absence of α-methylenebutyrolactone functional groups.

We have therefore studied the effects on rabbit aortic rings of two other plant-derived sesquiterpene lactones which have been purified from yellow star thistle (*Centaurea solstitialis*): cynaropicrin (an α-methylenebutyrolactone) and solstitialin 13-acetate (lacking the α-methylene function). We have also investigated the consequences of chemically inactivating the α-methylene functions in cynaropicrin and parthenolide by reaction with cysteine. Yellow star thistle is a member of a genus known to be especially rich in sesquiterpene lactones, and many have now been identified (Rodriguez *et al.*, 1976; Herz, 1977), some of which may cause the toxicity of various species of Compositae to livestock (Rodriguez *et al.*, 1976).

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## Methods

Rings of 2 mm thickness were carefully cut from the thoracic segment of the aorta taken from male New Zealand White rabbits (2.0–3.0 kg) and suspended for isometric recording under a load of 2 g in 3 ml tissue baths containing well-oxygenated Krebs solution as described earlier (Barsby *et al.*, 1992). Contractions were induced by the addition of $10^{-6}$ M phenylephrine or 40 mM KCl.

Parthenolide was isolated from *T. parthenium* and characterized as described (Dolman *et al.*, 1992), and was dissolved in methanol for biological testing. Cynaropicrin and solstitialin 13-acetate were isolated from *C. solstitialis* and characterized as reported previously (Wang *et al.*, 1991); they were dissolved in dimethylsulphoxide (DMSO) for biological testing. As before, these compounds were characterized by $^{1}H$ and $^{13}C$ nuclear magnetic resonance (n.m.r.) and the purity was greater than 97% by thin layer chromatography (t.l.c.).

The structures of the three sesquiterpene lactones are shown in Figure 1. For some experiments, parthenolide and cynaropicrin were dissolved at 25 mg ml$^{-1}$ in methanol or DMSO containing 100 mg ml$^{-1}$ cysteine and allowed to react overnight before testing. Parallel stocks were prepared without cysteine. To establish whether indeed satisfactory addition of cysteine to the α-methylene function had occurred, chemical high performance liquid chromatography (h.p.l.c.) analysis of the product of the reaction with parthenolide was performed (amounts of cynaropicrin-cysteine were insufficient). This was done using the procedure described by

Dolman et al. (1992) in which the adducts formed from the reaction of 5–10 mg samples of the parthenolide or parthenolide-cysteine with excess 9-thiomethylanthracene in chloroform are quantified using a 10 μm μPorasil column eluted at 3 ml $min^{-1}$ with 55% chloroform–45% hexane and the detector set at 0.08 sensitivity, 369 nm.

## Results

Representative records of the responses of the aortic rings to application of phenylephrine are shown in Figure 2. Addition of parthenolide or cynaropicrin at a final bath concentration of 100 μg $ml^{-1}$ caused a slow and prolonged decline in tension of the rings, whereas addition of solstitialin 13-acetate or the equivalent volumes of the vehicles (methanol or DMSO) did not (Figure 2). After repeated washout of the drugs and an extended recovery period of 30–90 min, further tests with phenylephrine were made. No responses were obtained in tissues treated with parthenolide or cynaropicrin, whereas full responsiveness was retained after the other treatments (Figure 2). Similar results were obtained using potassium chloride to induce contractions, although traces are not shown here.

Cynaropicrin and parthenolide were treated with cysteine as described in Methods. Addition of these chemically modified sesquiterpene lactones to aortic rings did not have

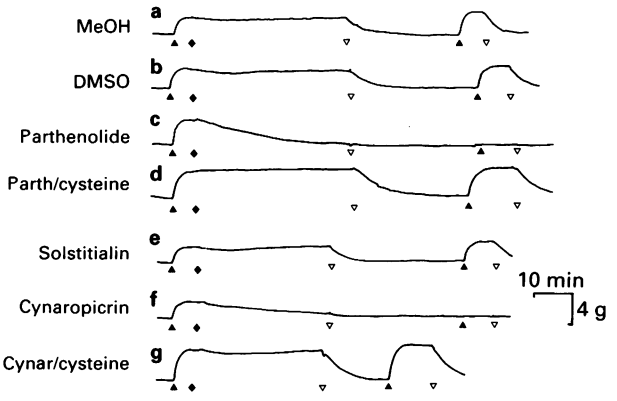


**Figure 2** Effects of sesquiterpene lactones on the contractile function of rabbit aortic rings. Panels (a) to (g) show the effects of the addition of (a) methanol, (b) dimethylsulphoxide, (c) parthenolide, (d) parthenolide reacted with cysteine, (e) solstitialin 13-acetate, (f) cynaropicrin, (g) cynaropicrin reacted with cysteine. Addition of $10^{-6}$ M phenylephrine was at ▲ and washout was at ▽. The lactones or vehicle were added once at ◆, after plateau contractions were attained. Similar results were obtained in tests on *n* other rings, as shown in Table 1.

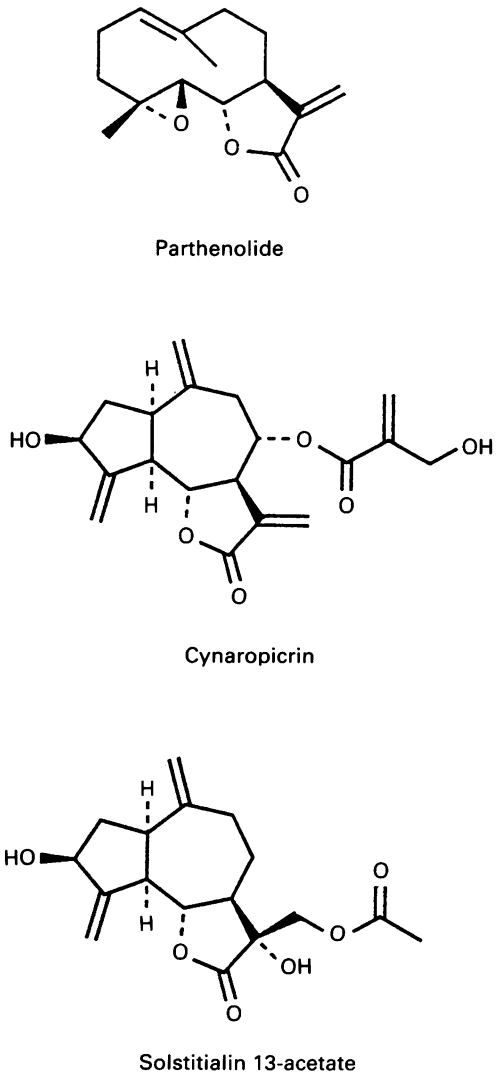


**Figure 1** Structures of the three sesquiterpene lactones.

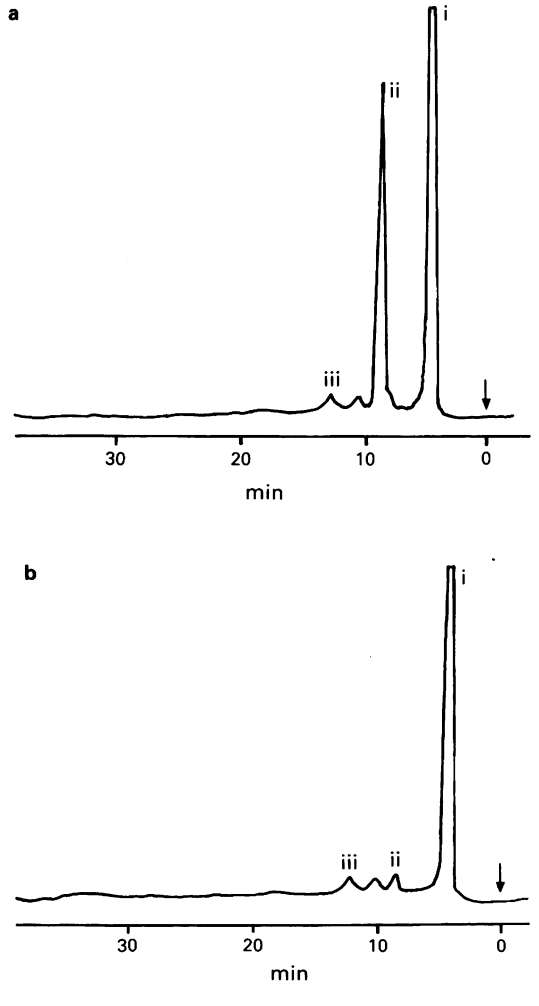


**Figure 3** Elution profiles from chemical-h.p.l.c. assay of parthenolide before and after reaction with cysteine. Panel (a) shows parthenolide, (b) is from a sample of parthenolide after treating with cysteine. Peak (i) is excess 9-thiomethylanthracene, peak (ii) is the parthenolide 9-thiomethylanthracene adduct, peak (iii) is a reagent impurity. Full scale deflection is 0.08 absorbance units at 369 nm.

**Table 1** Inhibition of contractility of rabbit aortic rings by sesquiterpene lactones: effect of lack of α-methylenebutyrolactone functions or their inactivation by cysteine

| *Test substance*† | *Loss of tone in rings precontracted with phenylephrine (mg min$^{-1}$)* | *% inhibition of subsequently applied phenylephrine* | *Number of tests* |
|---|---|---|---|
| Methanol vehicle | 4.9 ± 2.5 | − 3.2 ± 5.7 | 5 |
| Methanol + cysteine | 0.9 ± 0.9 | − 1.7 ± 6.1 | 3 |
| DMSO vehicle | 10.5 ± 3.8 | − 5.8 ± 3.7 | 7 |
| DMSO + cysteine | 24.8 ± 6.7 | 3.2 ± 3.9 | 4 |
| Parthenolide | 132.7 ± 20.7* | 98.2 ± 1.3* | 6 |
| Parthenolide + cysteine | 14.0 ± 5.3 | 4.0 ± 4.2 | 6 |
| Cynaropicrin | 84.7 ± 8.0* | 94.8 ± 2.0* | 9 |
| Cynaropicrin + cysteine | 7.3 ± 3.1 | − 4.0 ± 2.7 | 7 |
| Solstitialin 13-acetate | 9.1 ± 4.6 | − 9.2 ± 4.0 | 6 |

†Rings were set up in 3 ml baths as described and contractions to phenylephrine and KCl obtained until reproducible. The rings were then contracted with $10^{-6}$ M phenylephrine. Then 5 min later (at which time the response had reached a plateau) 12 μl of vehicle or test substance (final concentration 100 μg ml$^{-1}$) was added and allowed to remain in contact with the phenylephrine-contracted tissue for 30 min. The rate of change of tension of the rings (loss of tone) is shown in column 2. The preparations were then washed thoroughly to remove agonist and inhibitor, and allowed to rest for 30–90 min. The agonist was then retested, and the magnitude of the subsequent response related to the previous value (shown in column 3). Values are means ± s.e.mean for tests on the numbers of different ring preparations shown in column 4. *Indicates statistically significant difference from corresponding vehicle control and from corresponding cysteine treatment by Student's unpaired $t$ test, $P<0.01$.

any effect on agonist-induced tone, and did not alter subsequent agonist responsiveness after washout (Figure 2). Thus reaction with cysteine abolished the inhibitory activity of these two lactones on the aortic rings. Chemical-h.p.l.c. assay was used in the case of the parthenolide-cysteine adduct to check that the reactive α-methylene function had been removed. Figure 3 shows that the characteristic peak due to the presence of an adduct formed by reaction of 9-thiomethylanthracene with the free α-methylene function of parthenolide is no longer found if the parthenolide had been treated with cysteine.

The combined data for this series of experiments are collected in Table 1.

## Discussion

These experiments provide strong evidence that the smooth muscle inhibitory effects of parthenolide and other sesquiterpene α-methylenebutyrolactones are due to the chemically reactive α-methylenebutyrolactone function. Its neutralization with cysteine abolishes inhibitory activity. Moreover, solstitialin 13-acetate, which does not contain the α-methylene function is not effective, whereas the closely related lactone, cynaropicrin, is fully inhibitory.

Clearly, these plant-derived substances are toxic to smooth muscle, because their application at 100 μg ml$^{-1}$ (about 400 μM) produces irreversible loss of tone and subsequent inability to contract to agonists. We have found that washing the rings for up to 15 h does not lead to any return of contractility (unpublished experiments). This fact, together with the slow onset of inhibitory action and the known capacity of α-methylenebutyrolactones to react with sulphydryl groups, suggests that this toxic pharmacological effect is due to covalent modification of some as yet unidentified protein which is essential to the smooth muscle contractile apparatus.

The chemical reactivity of α-methylenebutyrolactones has been invoked as the explanation for other effects of these very widely distributed natural products (Rodriguez *et al.*, 1976; Berry, 1984). For example, they demonstrate potent anti-inflammatory actions in rodents (Hall *et al.*, 1980), are cytotoxic (Rodriguez *et al.*, 1976; Berry, 1984), cause allergic contact dermatitis (Rodriguez *et al.*, 1976; Berry, 1984) and inhibit secretion from platelets (Groenewegen *et al.*, 1986). However, it is not known whether the sesquiterpene lactones are responsible for the claimed therapeutic benefits of the feverfew plant, although they may be responsible for some of its side effects (Berry, 1984). There are also indications from *in vitro* neurotoxicity studies (Wang *et al.*, 1991; Cheng *et al.*, 1992) that α-methylenebutyrolactones might be responsible for the central neurotoxic effects observed in equine nigropallidal encephalomalacia. This disease occurs in horses grazing on meadows overgrown with yellow star thistle (Cordy, 1978).

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# Comparison of two new inhibitors of catechol *O*-methylation on striatal dopamine metabolism: a microdialysis study in rats

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**1** Effects of two new inhibitors of catechol *O*-methylation (CGP 28014 and entacapone; 30 mg kg$^{-1}$, i.p.) were compared by means of brain microdialysis in rats treated with L-3,4-dihydroxyphenylalanine (L-dopa)/carbidopa (50/50 mg kg$^{-1}$, i.p., respectively) or saline.

**2** In saline-treated rats, CGP 28014 maximally (max) increased striatal dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) effluxes by 41% and 49%, respectively, whereas homovanillic acid (HVA) levels were decreased by 71%.

**3** In the presence of L-dopa/carbidopa, a peripherally active inhibitor of catechol *O*-methyltransferase (COMT) entacapone had a short-lasting increasing effect on L-dopa efflux. Compared to the effects of L-dopa/carbidopa alone 3-*O*-methyldopa (3-OMD) levels were effectively reduced (max 79%) by entacapone, but not by CGP 28014.

**4** Entacapone, in contrast to CGP 28014, increased striatal dopamine efflux (max 492% of that after L-dopa/carbidopa alone). Also DOPAC levels were increased by entacapone (255% at 180 min), but not significantly by CGP 28014 (159% at 180 min).

**5** Both compounds initially decreased HVA efflux. The effect of CGP 18014 was longer-lasting. By the end of the measurement, entacapone even increased HVA levels (max 259%).

**6** Our results demonstrate that entacapone is a peripheral COMT inhibitor and support the view that CGP 18014 is mainly a centrally acting inhibitor of *O*-methylation.

**Keywords:** COMT inhibition; microdialysis; L-dopa; 3-OMD; dopamine metabolism; entacapone; CGP 28014

## Introduction

The most potent inhibitors of catechol *O*-methyltransferase (COMT; EC 2.1.1.6) presently available are nitrocatechols (e.g., nitecapone, entacapone and tolcapone). $IC_{50}$ and $K_i$ values of these substances for liver and brain COMT are in the low nanomolar range (Bäckström *et al.*, 1989; Borgulya *et al.*, 1989; Nissinen *et al.*, 1992). Nitecapone and entacapone are considered as mainly peripherally active drugs. Characteristically, they prevent 3-*O*-methyldopa (3-OMD) formation from L-3,4-dihydroxyphenylalanine (L-dopa), without affecting the levels of centrally formed metabolites of dopamine, e.g. 3-methoxytyramine (3-MT) and homovanillic acid (HVA) (Männistö *et al.*, 1990; 1992a; Nissinen *et al.*, 1992; Kaakkola & Wurtman, 1993). Tolcapone is active both in the periphery and in the brain (Maj *et al.*, 1990; Zürcher *et al.*, 1990; Männistö *et al.*, 1992a; Kaakkola & Wurtman, 1993) reducing the levels of the corresponding metabolites of both L-dopa and dopamine.

Waldmeier and co-workers (1990a) introduced CGP 28014, which appeared to be a poor inhibitor of striatal COMT enzyme *in vitro* with an $IC_{50}$ value in the millimolar range. Nevertheless, CGP 28014 reduces 3-MT and HVA levels in rat striatal and hypothalamic homogenates (Waldmeier *et al.*, 1990a; Männistö *et al.*, 1992b; Törnwall *et al.*, 1993), although not as effectively as the same dose of tolcapone (Männistö *et al.*, 1992a; Törnwall *et al.*, 1993).

Controversial results have been reported about the effect of CGP 28014 on peripheral 3-OMD formation. Waldmeier *et al.* (1990b) reported a clear decrease in 3-OMD levels from 30 min to 6 h after addition of 30 mg kg$^{-1}$ of CGP 28014 (p.o.) on 50 mg kg$^{-1}$ of L-dopa without dopa decarboxylase inhibition. Männistö *et al.* (1992a) noted no significant decrease in striatal and hypothalamic 3-OMD levels at 1 h after addition of 10 or 30 mg kg$^{-1}$ of CGP 28014 (i.p.) in L-dopa/carbidopa (50/50 mg kg$^{-1}$, respectively)-treated rats. At 3 h, however, 3-OMD levels were reduced. No dose-related decrease in 3-OMD levels after CGP 28014 has been observed in L-dopa treated healthy volunteers (Bieck *et al.*, 1990).

In this study, we compared the effects of CGP 28014 and peripherally acting entacapone on brain dopamine metabolism by means of brain microdialysis in L-dopa/carbidopa and saline-treated rats. The levels of 5-hydroxyindole acetic acid (5-HIAA) efflux was also studied, since in previous studies, CGP 28014, at doses of 1 to 10 mg kg$^{-1}$, has sometimes raised the tissue levels of striatal 5-HIAA and tryptophan (Waldmeier *et al.*, 1990a).

## Methods

### *Animals*

Male Wistar rats (Wist/Kuo, 280–350 g; from the colony of Department of Pharmacology and Toxicology, University of Helsinki) were housed 4 per cage at 21 ± 1°C in 12 h light and dark cycles (light on 07 h 00 min). Water and food were available *ad libitum*.

### *Surgery and brain dialysis*

The rats were anaesthetized with intraperitoneally (i.p.) given α-chloralose and urethane (50 and 500 mg kg$^{-1}$, respectively diluted in isotonic saline). Further anaesthesia was provided if needed. Body temperature was maintained at 37°C with a homeothermic blanket unit (Harvard apparatus Ltd., Edenbridge, Kent, England).

Each rat was placed in a Kopf stereotaxic apparatus, the skull was exposed and a dialysis probe was implanted through a burr hole with the tip extending 6.5 mm below the dura. Concentric custom-constructed probes (Parry *et al.*,

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1990), 210 μm in outer diameter, 4 mm of exposed membrane were used. The probe was perfused with artificial CSF solution (composition, mM: $Na^+$ 147, $K^+$ 3.5, $Ca^{2+}$ 1.0, $Mg^{2+}$ 1.2, $Cl^-$ 129, $PO_4^{3-}$ 1.0 and $HCO_3$ 25, gassed with $O_2/CO_2$ (95/5%) to pH 7.35) at a flow rate of 1 μl $min^{-1}$ (the effect of CGP 28014 alone) or 2 μl $min^{-1}$ (other experiments), using a CMA microperfusion pump (Carnegie Medicin, Solna, Sweden). To allow recovery from the surgical procedure and to obtain stable dialysate dopamine levels first samples for determinations were collected after 120 min wash-out period. Dialysate samples were then collected for 20 min periods in vials containing 5 μl (1 μl $min^{-1}$ flow) or 10 μl (2 μl $min^{-1}$ flow) of 0.5 M perchloric acid to minimize decomposition.

To assess the variability between probes, *in vitro* recoveries of the amines were studied in room temperature at a perfusion rate of 1 or 2 μl $min^{-1}$. Probes were placed in a beaker containing 10 ng $ml^{-1}$ solutions of amines or metabolites of interest. Relative recoveries were as follows (mean ± s.e. mean; $n = 4$) for flow of 1 μl $min^{-1}$: L-dopa, 11.4 ± 2.2%; 3-OMD, 14.3 ± 3.4%; dopamine, 12.9 ± 1.6%; 3,4-dihydroxyphenylacetic acid (DOPAC), 18.3 ± 1.9%; HVA, 23.3 ± 3.1%; 5-HIAA, 14.4 ± 3.1% and for flow of 2 μl $min^{-1}$: L-dopa, 5.1 ± 0.1%; 3-OMD, 9.0 ± 0.7%; dopamine, 6.5 ± 0.5%; DOPAC, 7.4 ± 0.3%; HVA, 14.4 ± 1.1%; 5-HIAA, 10.8 ± 1.0%.

All drugs were injected i.p. after a 1-h (3 × 20 min) baseline period. CGP 28014 (30 mg $kg^{-1}$), entacapone (30 mg $kg^{-1}$) and carbidopa (50 mg $kg^{-1}$) were administered 40 min before L-dopa methylester (50 mg $kg^{-1}$) after which further samples were collected for 400 min. When CGP 28014 was given without L-dopa and carbidopa, samples were collected for 280 min after CGP 28014 (30 mg $kg^{-1}$) or saline injection. All doses refer to acids or bases.

### *Biochemical analysis*

The concentrations of L-dopa, 3-OMD, dopamine, DOPAC, HVA, and 5-HIAA were analyzed from dialysate samples by high performance liquid chromatography (h.p.l.c.) with electrochemical detection. The system consisted of an isocratic Waters model 6000A pump with dual SSI suppressors in series, a Waters 712 WISP autoinjector (Waters Assoc., Milford, MA, U.S.A.) and a Hewlett Packard 3396a recording integrator (Palo Alto, CA, U.S.A.). An analytical cell 5011 of an ESA 5100A coulometric detector (ESA Inc., Bedford, MA, U.S.A.) set at + 0.10 V/− 0.30 V with a conditioning cell 5021 set at + 0.50 V were used. The column used was LiChrospher 100 RP-18 (5 μm) (E. Merck, Darmstadt, Germany). The mobile phase contained 10% methanol in 0.1 M phosphate buffer, 20 mM citric acid, 0.15 mM EDTA and 2.2 mM octanesulphonic acid at pH 2.7. The flow rate of a mobile phase was 0.9 ml $min^{-1}$ and injection volume 20 μl. The detection limits for the compounds were as follows (pmol 20 $\mu l^{-1}$): dopamine, 0.01; DOPAC, 0.02; HVA, 0.02; L-dopa, 0.02; 3-OMD, 0.1; 5-HIAA, 0.02.

### *Drugs and chemicals*

Carbidopa (Orion Pharmaceutica, Espoo, Finland) was suspended in 5% gum arabic. Levodopa methylester hydrochloride (L-dopa) was from Sigma Chemical Company, MO, U.S.A. CGP 28014 (N- (2-pyridone-6-yl)-*N'*, *N'*-di-n-propylformamidine) and entacapone [OR-611; N,N-diethyl-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl) acrylamide; batch 105] were synthesized in Orion Pharmaceutica, Espoo, Finland by Ms Aino Pippuri, M. Sci. The purity of the compound was checked by thin layer chromatography (t.l.c.) and nuclear magnetic resonance (n.m.r.) and was always better than 99%. CGP 28014 and entacapone were dissolved in a single drop of Polysorbate 80 (European Pharmacopoeia, vol. III) and then diluted with 0.9% NaCl in distilled water.

The standards for h.p.l.c. analysis and octanesulphonic acid were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Other chemicals were analytical grade and were obtained from Merck (Darmstadt, Germany).

### *Statistics*

The mean of three baseline samples was calculated for each rat and the successive data were given as a percentage of this baseline (100%) value to reduce animal-to-animal variety. In case of L-dopa and 3-OMD, only absolute values (pmol 20 $min^{-1}$), not corrected for probe recovery, were used since the baselines for these substances were undetectable. Arithmetic mean, s.d. and s.e.mean of each group were then calculated. The data was analyzed by analysis of variances for repeated measures (ANOVA) using a Systat statistical software (Wilkinson, 1990). Pairwise comparisons of means at different time points were made with the Tukey-Kramer method.

## Results

### *Effect of CGP 28014 (30 mg $kg^{-1}$) alone on striatal extracellular levels of endogenous dopamine, DOPAC, HVA and 5-HIAA*

Basal concentrations of the amines were as follows (mean ± s.e.mean; pmol 20 $min^{-1}$; $n = 12$): dopamine, 0.048 ± 0.006; DOPAC, 36.2 ± 2.9; HVA, 24.1 ± 1.7; 5-HIAA 6.6 ± 0.5 (Figure 1). Basal levels of L-dopa and 3-OMD were undetectable.

In saline-treated rats, dopamine efflux remained stable during the 280 min dialysis time, whereas moderate decline was observed in the levels of DOPAC (ANOVA: $F = 19.5$; $P < 0.05$), HVA ($F = 33.0$; $P < 0.05$) and 5-HIAA ($F = 58.3$; $P < 0.05$) (Figure 1).

CGP 28014 increased striatal dopamine efflux (ANOVA: $F = 9.7$; $P < 0.05$). In detailed comparison at separate time points, dopamine efflux was increased from 40 min onwards (Figure 1). CGP 28014 increased striatal dopamine efflux maximally by 41% (at 200 min) when compared to saline-treated rats.

Administration of CGP 28014 increased DOPAC efflux (ANOVA: $F = 18.2$; $P < 0.01$) from 40 min onwards (Figure 1). The maximal increase of DOPAC levels was 49% (at 120 min).

HVA efflux was decreased (ANOVA: $F = 153.0$; $P < 0.001$) from 20 min onwards by CGP 28014 (Figure 1). The maximal decrease in HVA levels occurred at 100 min (71%).

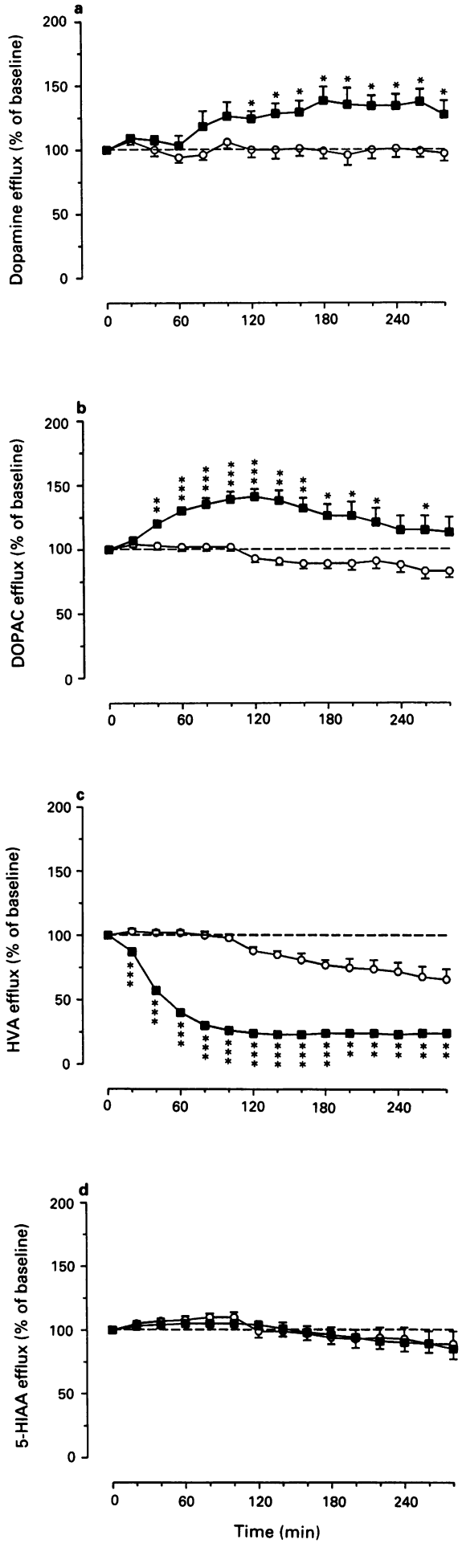
Dialysate 5-HIAA levels were not affected by CGP 28014 treatment (Figure 1).

### *Effects of L-dopa/carbidopa on striatal extracellular levels of L-dopa, 3-OMD, dopamine, DOPAC and HVA*

Compared to baseline levels, L-dopa/carbidopa (50/50 mg $kg^{-1}$) treatment alone increased striatal L-dopa (ANOVA: $F = 13.9$; $P < 0.01$), 3-OMD ($F = 12.5$; $P < 0.01$), DOPAC ($F = 7.9$; $P < 0.05$) and HVA ($F = 11.0$; $P < 0.05$) effluxes (Figures 2 and 3). L-Dopa and 3-OMD levels were detectable from 60 min and 40 min onwards, respectively. Basal concentrations were as follows (mean ± s.e.mean; pmol 20 $min^{-1}$; $n = 17$–23): dopamine, 0.054 ± 0.005; DOPAC, 20.9 ± 3.0; HVA, 15.5 ± 2.2.

### *Effect of CGP 28014 (30 mg $kg^{-1}$) on striatal extracellular levels of L-dopa, 3-OMD, dopamine, DOPAC and HVA in L-dopa/carbidopa (50/50 mg $kg^{-1}$) treated rats*

Addition of CGP 28014 to L-dopa/carbidopa treatment did not modify striatal L-dopa, 3-OMD or dopamine efflux (Figures 2 and 3). Additional CGP 28014 elevated DOPAC



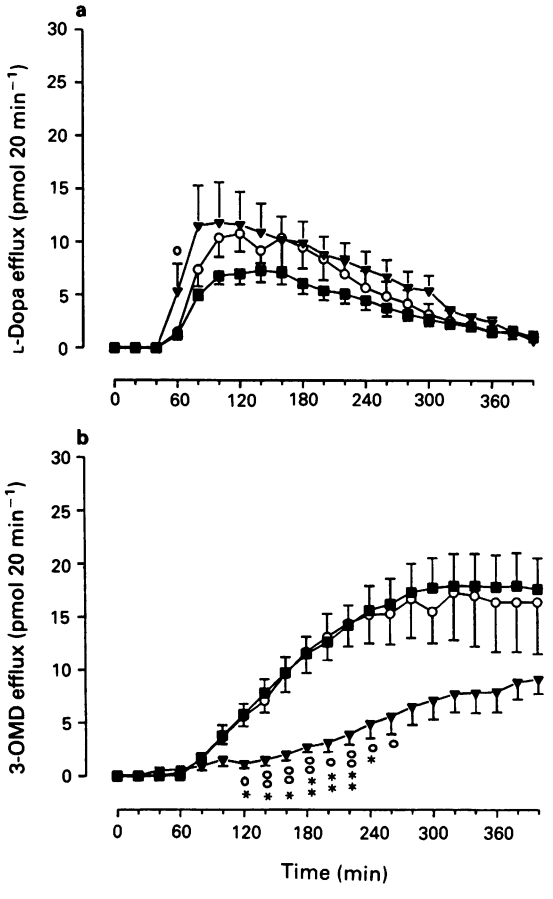




**Figure 2** The effect of CGP 28014 (30 mg kg$^{-1}$, i.p., ■), entacapone (30 mg kg$^{-1}$, i.p., ▼) or saline (○) on striatal efflux of L-dopa (a) and 3-*O*-methyldopa (3-OMD) (b) in L-dopa/carbidopa (50/50 mg kg$^{-1}$ i.p., respectively) treated rats. CGP 28014, entacapone or saline and carbidopa were given 40 min before L-dopa. Dialysis samples were collected for 20-min periods and assayed by h.p.l.c. with electrochemical detection. Data are mean ± s.e.mean ($n = 5-8$). The basal concentrations for the amines were undetectable, thus the results are given as absolute values (pmol 20 min$^{-1}$) without correction for probe recovery. Detailed statistics by Tukey-Kramer method are as follows: $^{*}P<0.05$, $^{**}P<0.01$ vs. L-dopa/carbidopa-treated rats, $^{\circ}P<0.05$, $^{\circ\circ}P<0.01$ vs. L-dopa/carbidopa + CGP 28014-treated rats.

levels slightly, but not significantly above those induced by L-dopa/carbidopa alone (Figure 3).

At 40, 60, 80 and 100 min, CGP 28014 decreased striatal HVA efflux ($P<0.001$, $P<0.001$, $P<0.01$ and $P<0.05$, respectively). The maximal decreasing effect of CGP 28014 on HVA levels occurred at 100 min, when HVA levels were 48% of those induced by L-dopa/carbidopa alone. From 240 min onwards, HVA efflux was even slightly, but not significantly, increased by CGP 28014 compared to L-dopa/carbidopa treatment alone (Figure 3).

**Figure 1** The effect of CGP 28014 (30 mg kg$^{-1}$, i.p., ■) or saline (○) on the striatal efflux of dopamine (a), 3,4-dihydroxyphenylacetic acid (DOPAC) (b) homovanillic acid (HVA) (c) and 5-hydroxyindole acetic acid (5-HIAA) (d). Dialysis samples were collected for 20-min periods and assayed by h.p.l.c. with electrochemical detection. Data are given as percentages of the mean of three baseline values (mean ± s.e.mean; $n = 6$). For basal concentrations of the amines see text. Dashed lines indicate the 100% baseline. Detailed statistics by Tukey's test are as follows: $^{*}P<0.05$, $^{**}P< 0.01$, $^{***}P<0.001$ vs. saline-treated rats.

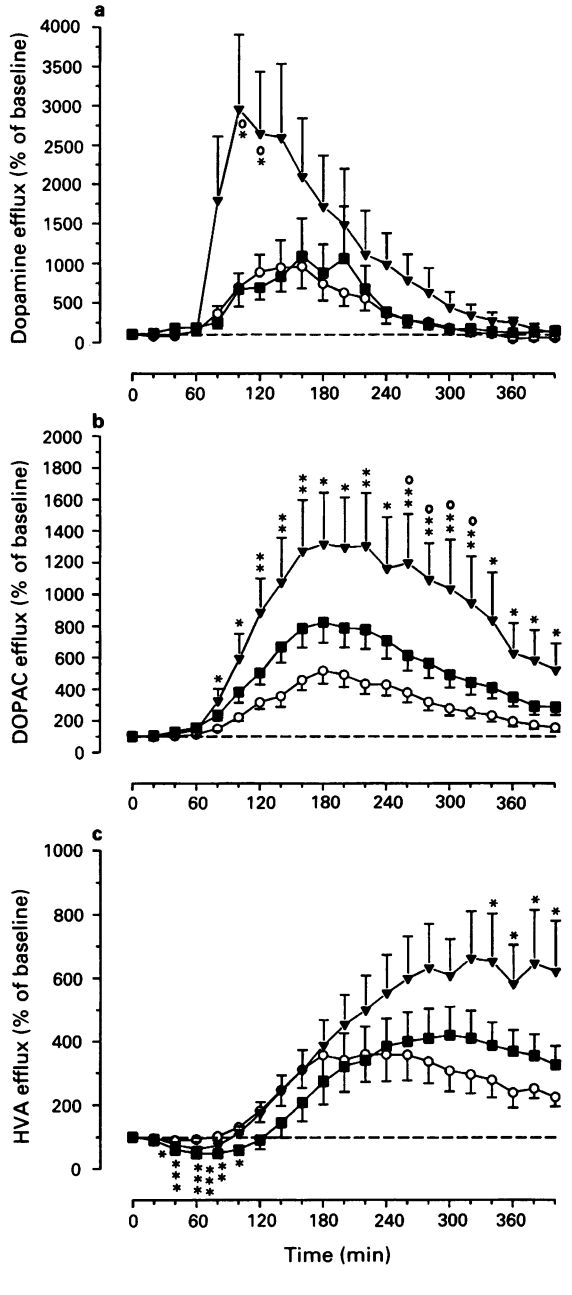



**Figure 3** The effect of CGP 28014 (30 mg kg$^{-1}$, i.p., ■), entacapone (30 mg kg$^{-1}$, i.p., ▼) or saline (○) on striatal efflux of dopamine (a), 3,4-dihydroxyphenylacetic acid (DOPAC) (b) and homovanillic acid (HVA (c) levels in L-dopa/carbidopa (50/50 mg kg$^{-1}$ i.p., respectively) treated rats. CGP 28014, entacapone or saline and carbidopa were given 40 min before L-dopa. Dialysis samples were collected for 20-min periods and assayed by h.p.l.c. with electrochemical detection. Data are given as percentages of the mean of three basal levels (mean ± s.e.mean; $n = 5-9$). For basal concentrations of the amines see text. Dashed lines indicate the 100% baseline. Detailed statistics by Tukey-Kramer method are as follows: $^{*}P<0.05$, $^{**}P<0.01$; $^{***}P<0.001$ vs. L-dopa/carbidopa-treated rats, $^{\circ}P<0.05$ vs. L-dopa/carbidopa + CGP 28014-treated rats.

### *The effect of entacapone (30 mg kg$^{-1}$) on striatal extracellular levels of L-dopa, 3-OMD, dopamine, DOPAC and HVA in L-dopa/carbidopa (50/50 mg kg$^{-1}$) treated rats*

Addition of entacapone (30 mg kg$^{-1}$) to the L-dopa/carbidopa treatment increased striatal L-dopa efflux significantly at 60 min ($P<0.05$), when compared to that after CGP 28014 and L-dopa/carbidopa (Figure 2).

Entacapone lowered 3-OMD levels from 120 min onwards (ANOVA: $F = 5.3$; $P<0.05$). Maximal decrease (79%) in 3-OMD levels was observed at 140 min and 160 min (Figure 2).

Entacapone increased dopamine efflux at 100 min (425% of those after L-dopa/carbidopa alone; $P<0.05$) and 120 min (298%; $P<0.05$). At these time points, the increase in dopamine was significant also compared to that after L-dopa/carbidopa and CGP 28014 (Figure 3).

L-Dopa/carbidopa-induced DOPAC levels were elevated from 80 min onwards by additional entacapone (ANOVA: $F = 4.2$; $P<0.05$). Between 260 min and 320 min the enhancement was significant ($P<0.05$) also when compared to the DOPAC levels after L-dopa/carbidopa and CGP 28014 (Figure 3).

At 40 min and 60 min, entacapone decreased striatal HVA efflux ($P<0.05$ and $P<0.001$, respectively), which was followed by gradual increase (Figure 3). From 340 min HVA efflux was increased compared to that after L-dopa/carbidopa alone (max 259%, $P<0.05$).

## Discussion

The effects of nitrocatechol-type COMT inhibitors on basal efflux of endogenous dopamine have been quite modest (Brannan *et al.*, 1992; Kaakkola & Wurtman, 1992). In contrast, to our knowledge this is the first time that a compound proposed to have central inhibitory activity on catechol *O*-methylation (by CGP 28014) elevates significantly striatal basal dopamine efflux. Increased DOPAC and decreased HVA effluxes after CGP 28014 in the absence of L-dopa/carbidopa treatment, evidently reflect diminished conversion of DOPAC to HVA. The results are in accordance with previous studies, where CGP 28014 increased DOPAC and decreased HVA levels also in the striatal tissue (Waldmeier *et al.*, 1990a).

In the presence of L-dopa/carbidopa treatment, the short-lasting increasing effect of entacapone on striatal L-dopa efflux is in accordance with earlier studies (Männistö *et al.*, 1992a). Increased central availability of L-dopa is obviously a consequence of decreased peripheral metabolism of the drug either by dopa decarboxylase or COMT. Thus, the lack of effect of CGP 28014 on L-dopa efflux favours the poor peripheral COMT-inhibiting property of the drug.

The above explanation is further supported by the lack of decreasing effect of CGP 28014 on the efflux of striatal 3-OMD. 3-OMD is a COMT-induced metabolite of L-dopa, that equilibrates quite easily between the periphery and the brain (Männistö *et al.*, 1992b). Most of the 3-OMD is formed outside the brain, which view was also presently supported by lowered brain 3-OMD levels after peripheral COMT inhibition by entacapone. The earlier controversial results about the effects of CGP 28014 on peripheral 3-OMD formation may be related to the presence or absence of an inhibitor of dopa decarboxylase. CGP 28014 lowered 3-OMD levels only when dopa decarboxylase was intact and the conversion of excess L-dopa to dopamine was possible. When dopa decarboxylase is inhibited, most of the L-dopa is metabolized to 3-OMD by COMT. This manifest COMT activity is evidently not inhibited by CGP 28014 or inhibition occurs very slowly (Männistö *et al.*, 1992a).

The inhibition of peripheral COMT (e.g. by entacapone) is apparently enough to increase the efficacy of L-dopa on brain dopamine concentrations (Brannan *et al.*, 1992; Männistö *et al.*, 1992a; Kaakkola & Wurtman, 1993). This has been reflected also in the behavioural studies in rats and mice (Etemadzadeh *et al.*, 1989; Törnwall *et al.*, 1992). However, recent results by Kaakkola and Wurtman (1993) suggest that central inhibition of COMT (e.g. by tolcapone) can further potentiate the effect of exogenous L-dopa on brain dopamine efflux. Indeed, centrally active COMT inhibitor (Ro 41-0960), but not entacapone, has increased the L-dopa-induced loco-

motor activity in nomifensine and clorgyline pretreated rats (Törnwall *et al.*, 1992). Thus, it seems peculiar that centrally active CGP 28014 could not further increase L-dopa/carbidopa-induced dopamine efflux.

Some recent results from our laboratory may throw light on the situation. At doses 50/50 mg $kg^{-1}$, L-dopa/carbidopa combination decreased spontaneously motility in rats and mice (Törnwall *et al.*, 1992; Lang & Männistö, unpublished results). Di Chiara *et al.* (1976) have postulated that L-dopa-induced decreased motility is mediated mainly at the presynaptic level (via autoreceptor stimulation). Interestingly, in our recent study, we used pimozide to stimulate presynaptic endogenous dopamine synthesis and metabolism without causing any obvious increase in dopamine release. In these circumstances, CGP 28014 did not decrease striatal 3-MT formation whereas tolcapone was still effective (Törnwall *et al.*, 1993). However, CGP 28014 was as effective as tolcapone in reducing 3-MT levels after amphetamine, a substance that evidently increases dopamine in the synaptic cleft. Thus, there may be a possibility that dopamine formed by presynaptic dopaminergic neurones from exogenous L-dopa, utilizes COMT-containing metabolic routes that cannot be reached by CGP 28014.

In L-dopa-treated rats, DOPAC levels were relatively resistant to the effects of CGP 28014 compared to the effects of entacapone. Similar effects have been observed in studies on brain homogenates (Männistö *et al.*, 1992a). The modest DOPAC increasing effect of CGP 28014 may have two explanations: (1) CGP 28014 may inhibit the access of dopamine into monoamine oxidase-B (MAO-B) containing cells (Männistö *et al.*, 1992a) and/or (2) unaltered central entry of L-dopa does not allow dopamine elevation (by dopa decarboxylase) and successive DOPAC (by MAO) formation.

CGP 28014, when given without L-dopa/carbidopa, suppressed effectively HVA levels for the whole time of dialysis (4 h 40 min), whereas HVA levels in L-dopa/carbidopa-treated rats were decreased for a shorter time period (3 h 20 min). Using the same doses and routes of administration of CGP 28014, L-dopa and carbidopa, Männistö *et al.* (1992a) found decreased striatal tissue HVA levels both at 1 h and 3 h after the triple treatment. However, this effect was not as strong as that of tolcapone. In the absence of exogenous L-dopa, Waldmeier *et al.* (1990a) found that striatal tissue HVA levels 4–6 h after administration of 20 mg $kg^{-1}$ (i.p.) of CGP 28014 were only 70–80% of the controls indicating a quite short-lasting effect of the drug at this dose level. However, a long-lasting effect of CGP 28014 on HVA levels has been observed when massive oral doses (300 mg $kg^{-1}$) of the drug have been used (Waldmeier *et al.*, 1990a). Dialysate HVA levels after entacapone were increased reflecting enhanced dopamine synthesis induced by augmented input of L-dopa into brain.

Entacapone proved to be a peripherally acting COMT inhibitor as reflected by reduced peripheral metabolism of L-dopa (Männistö *et al.*, 1992b; Nissinen *et al.*, 1992). However, the mechanism by which CGP 28014 suppresses 3-MT and HVA levels in the brain is still unknown. As the substance itself, and its known metabolite, 2-amino-6-hydroxypyridine, are poor inhibitors of COMT enzyme *in vitro* (Waldmeier *et al.*, 1990a), alternative mechanisms have been proposed (Waldmeier *et al.*, 1990a; Männistö *et al.*, 1992a; Törnwall *et al.*, 1993). According to one suggestion, CGP 28014 acts as an inhibitor for central $uptake_2$, a carrier system that transports amines from the synaptic cleft into glial or postsynaptic neuronal cells. This transport is the prerequisite not only for the action of COMT but also for MAO-B (Trendelenburg, 1991). Indeed, the inhibition of both major postsynaptic metabolic pathways at this single step would explain the increased dopamine efflux after CGP 28014 in the absence of exogenous L-dopa. Modestly elevated DOPAC levels after CGP 28014 and L-dopa/carbidopa would also fit this explanation. The inhibition of central $uptake_1$ (e.g. by nomifensine) increases dopamine and decreases DOPAC effluxes in the brain even more dramatically (Kaakkola & Wurtman, 1992), suggesting the primary importance of presynaptic neurones in the termination of basal dopaminergic transmission.

In conclusion, the lack of effect of CGP 28014 on peripheral 3-OMD formation and central L-dopa entry suggests that CGP 28014 is a mainly centrally acting inhibitor of one of the processes affecting *O*-methylation. Our results also suggest, that the mere central inhibition of *O*-methylation cannot significantly exceed the beneficial effects of peripheral COMT inhibition on brain dopamine levels in intact rats.

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# Tacrine-induced increase in the release of spontaneous high quantal content events in *Torpedo* electric organ

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**1** The anticholinesterases, tacrine (100 μM) and physostigmine (60 μM) had different effects on the amplitude distribution and kinetics of miniature endplate currents (m.e.p.cs) recorded extracellularly from the electric organ of *Torpedo marmorata*.

**2** Tacrine increased the ratio of giant miniatures (larger than 4 mV of amplitude) to more than 20% of recorded spontaneous events. In the presence of physostigmine such events represented only 4%.

**3** Both tacrine and physostigmine increased the rise time and the decay phase of normal-sized m.e.p.cs when compared to control conditions. Both effects were significantly greater for tacrine.

**4** We have tested the specificity of the tacrine effect on ectoenzyme activities associated with plasma membranes of these pure cholinergic nerve endings. Tacrine does not act unspecifically on every ectoenzyme, because it is not able to block the ectoapyrase activity even at a concentration 100 fold greater than that required to inhibit 94% of AChE.

**5** We conclude that the differential effects of tacrine and physostigmine can be explained in terms of undetermined presynaptic actions of tacrine, while comparable effects of the two compounds can be explained through a shared anticholinesterase activity.

**Keywords:** Tacrine; physostigmine; miniature endplate currents; *Torpedo*; acetylcholine; acetylcholinesterase; apyrase; quantal transmitter release; Alzheimer's disease

## Introduction

Alzheimer's disease produces a loss of cholinergic neurones in the nucleus basalis of Meynert. To increase the levels of acetylcholine, clinical trials have been carried out with acetylcholinesterase blockers. Physostigmine and tacrine (9-amino-1,2,3,4-tetrahydroacridine) block acetylcholinesterases in the central nervous system. Physostigmine produces a brief improvement in cognitive function, due to its short half-life. The use of the longer-acting tacrine has positive results, primarily in long-term trials (Kumar & Becker, 1989).

The strategy of using physostigmine or tacrine to enhance cholinergic transmission is based on the ability of acetylcholinesterases to prolong the postsynaptic action of acetylcholine (ACh). In the central nervous system the direct measurement, at the synaptic level, of ACh release and the mean life of ACh molecules is difficult. An alternative experimental model is the vertebrate neuromuscular junction with which the quantal nature of ACh release was established. Tacrine stimulates neurosecretion at mammalian motor endplates (Thesleff *et al.*, 1990) and prolongs the decay phase of miniature endplate potentials (Braga *et al.*, 1991).

Variations in clinical efficacy of tacrine and physostigmine may be related less to their acetylcholinesterase antagonism than to their interactions with other systems such as presynaptic actions or cholinoceptors. Tacrine is in fact structurally related to the $Na^+$ channel blocker, 9-aminoacridine (Yamamoto & Yeh, 1984) and to the $K^+$ channel blocker, 4-aminopyridine (Yeh *et al.*, 1976; Wurtman *et al.*, 1991). Also some ligand-gated channels and receptors such as those for 5-hydroxytryptamine and adenosine are in some systems sensitive to block by tacrine (Drukarch *et al.*, 1987; Shutske *et al.*, 1989).

Electric organs of Elasmobranchs are embryologically derived from the skeletal muscle and have a great number of motor nerve endings, indeed there are huge amounts of acetylcholinesterases. Tacrine is also able to block acetylcholinesterase activity in the ray electric organ (Wu & Yang, 1989). Our goal is to explore the effects of tacrine and physostigmine on the spontaneous quantal release of acetylcholine from the electric organ of *Torpedo*.

## Methods

*Torpedo marmorata* specimens were caught at the Catalan mediterranean coast and maintained alive in artificial sea water. Fish were anaesthetized with tricaine (Sigma, St Louis, MO, U.S.A.) before surgical excision of electric organs. Fragments were immediately immersed in *Torpedo* physiological saline solution. The composition of this solution was (in mM): NaCl 280, KCl 3, $CaCl_2$ 3.4, $MgCl_2$ 1.8, glucose 5.5, urea 300 and sucrose 100, HEPES/NaOH-buffer 6.8, pH adjusted to 7.0 with $NaHCO_3$. From 5 to 10 prisms of the electric organ were cross-sectioned with a scalpel blade and thin sections (1 mm) were placed in a plexiglass recording chamber coated with a sylgard bottom.

Spontaneous release of ACh was recorded with focal extracellular low-resistance microelectrodes (Katz & Miledi, 1977), as adapted to the electric organ by Soria (1983) and Muller & Dunant (1987). The method allows long-term recording with little damage to the cells.

Electrodes were made in three steps using a Mecanex horizontal puller, employing borosilicate capillaries (Clark Electromedical Instruments). Electrode tips were fire-polished in a home-made microforge. Recording electrodes having tip diameters around 10 micrometers were filled with physiological solution with a resistance of about 400 KΩ.

Spontaneous miniature endplate currents (m.e.p.cs), recorded as potential changes of a focal electrode plugged into a high impedance amplifier (P16, Grass), were monitored on a Tektronix 5110 oscilloscope and recorded in parallel on a VCR tape recorder (Biologic). Analysis of signals was done by means of the pClamp 5.5.1 software, Axon Instruments Inc., and a TL-1 labmaster digitizing interface and event detector (Axon Ins.). Data in ASCII form were exported to Sigmaplot 4.0.

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Results are presented in mean ± s.e.mean. Differences between means were tested by Student's $t$ test. Differences between distribution functions were evaluated by the Kolmogorov-Smirnov (K-S) test.

*Torpedo* electric organ synaptosomes were obtained by differential centrifugation following the method described by Morel *et al.* (1977). The acetylcholinesterase activity was measured according to Ellman *et al.* (1961). Ectoapyrase activity was measured as described by Sarkis & Saltó (1991), and phosphate formation was determined according to Lanzetta *et al.* (1979).

All recordings were made at room temperature (20–23°C).

1,2,3,4-Tetrahydro-9-aminoacridine (tacrine), and physostigmine (eserine-salicylate) were purchased from Sigma, St. Louis, MO, U.S.A. All other reagents used and tricaine were of synthesis grade.

## Results

In the present work we show that tacrine (100 μM) efficiently inhibited the acetylcholinesterase (AChE) activity associated with isolated presynaptic plasma membranes of cholinergic nerve terminals of *Torpedo marmorata* electric organ (Massoulie *et al.*, 1991). As in the same preparation, Sarkis & Saltó (1991) reported the presence of another ectoenzyme which catalyzed the hydrolysis of ATP that is co-released with ACh during the stimulation of cholinergic synaptic vesicles, it was of interest to determine if tacrine at high concentration (100 μM) was able to modify the ectonucleotidase activity associated with nerve terminals. Tacrine does not act unspecifically on every ectoenzyme, because whereas at the concentration of 1 μM it blocks AChE activity fully (remaining activity is 2.4 ± 3.0%, $n = 4$), it is not able to block the apyrase activity even at a concentration 100 fold higher (remaining activity after application of tacrine 100 μM is 93.9 ± 4.8%, $n = 4$).

We focused the rest of our study on the spontaneous electrical activity of cholinergic nerve terminals in slices of *Torpedo* electric organ. Because we were looking for effects other than purely anticholinesterase action, the effects of tacrine on m.e.p.cs have been compared with the effects induced by a strong AChE inhibitor, physostigmine, at a similar concentration (60 μM). The amplitude and kinetics of the spontaneous events were modified by the action of these compounds (Figure 1).

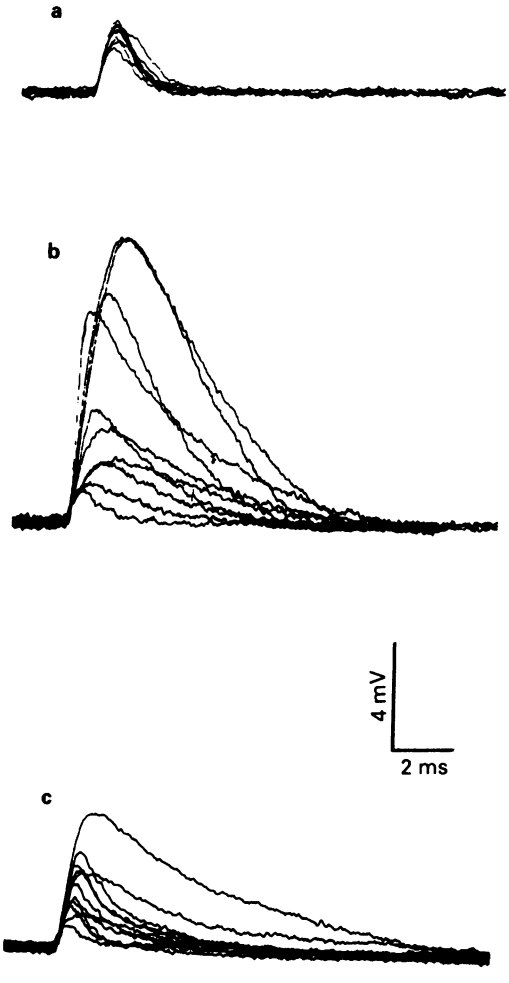


**Figure 1** Superimposed oscilloscope tracings showing spontaneous m.e.p.cs recorded in *Torpedo marmorata* electric organ: (a) an untreated control electric organ shows normal-sized, fast-rising and fast-decaying m.e.p.cs; (b) application of tacrine (100 μM) leads to the appearance of giant m.e.p.cs, and to a general increase of rise time and decay time constants; (c) in the presence of physostigmine (60 μM) spontaneous events have prolonged rising times and decaying time constants when compared with controls, but the frequency of slow-rising and giant m.e.p.cs is low in comparison with (b).

The mean amplitude of miniature endplate currents in control conditions was 1.66 ± 0.04 mV (449 m.e.p.cs, number of experiments ($n$) = 3). Tacrine (100 μM) increased this value to 2.54 ± 0.10 mV (420 m.e.p.cs, $P < 0.05$, $n = 3$) and physostigmine (60 μM) to 1.80 ± 0.05 mV (455 m.e.p.cs, $P < 0.05$, $n = 3$).

Histogram plots comparing the amplitude distribution of m.e.p.cs (see Van der Kloot, 1991) in the three experimental conditions, are shown in Figure 2a. With tacrine the appearance of a population of large size m.e.p.cs can be noted clearly. In control conditions only 0.7% of the m.e.p.cs had amplitudes greater than 4 mV. Tacrine resulted in a big increase in this kind of spontaneous event which represented 21% of the total recorded. In contrast, after physostigmine treatment such big m.e.p.cs only represented 4.5%. Because these giant m.e.p.cs had no brakes on their rising phases, we discarded the possibility that they arose from the simultaneous release of two or more quanta.

As tested by the K-S statistics, the distribution function of m.e.p.c. amplitudes recorded in presence of tacrine 100 μM was different from controls ($P < 0.001$) and from m.e.p.cs recorded in the presence of physostigmine 60 μM ($P < 0.001$). In contrast, m.e.p.c. amplitude distribution recorded after application of physostigmine is not different from that in control conditions.

The mean value of the rise time of m.e.p.cs in a non-treated preparation was 0.42 ± 0.06 ms, while in physostigmine solution it was 0.79 ± 0.02 ms and in the presence of tacrine, 1.28 ± 0.03 ms. The differences are significant in all cases at the $P < 0.05$ level. Histogram plots are shown in Figure 2b. Tacrine and physostigmine caused an increase in the frequency of slow-rising m.e.p.cs. In the distribution functions, differences between control and tacrine or physostigmine data are significant at the $P < 0.001$ level. The effect was more potent for tacrine, because m.e.p.cs distribution after addition of tacrine shifts significantly to prolonged rise times when compared with physostigmine ($P < 0.001$, K-S statistics).

The mean half decay of m.e.p.cs was 0.36 ± 0.01 ms in control m.e.p.cs, while after physostigmine treatment it was 1.67 ± 0.05 ms, after tacrine it was 2.24 ± 0.05 ms. These differences are significant ($P < 0.05$). Control and tacrine data are different ($P < 0.001$) when comparing the frequency distribution of the half decay time constants of m.e.p.cs. Control and physostigmine are also different at $P < 0.001$. Differences between tacrine and physostigmine are significant at a lower level ($P < 0.05$, K-S statistics). Half decay histograms are shown in Figure 2c.

The increases in both time constants affected apparently normal-sized m.e.p.cs, because when we excluded giant

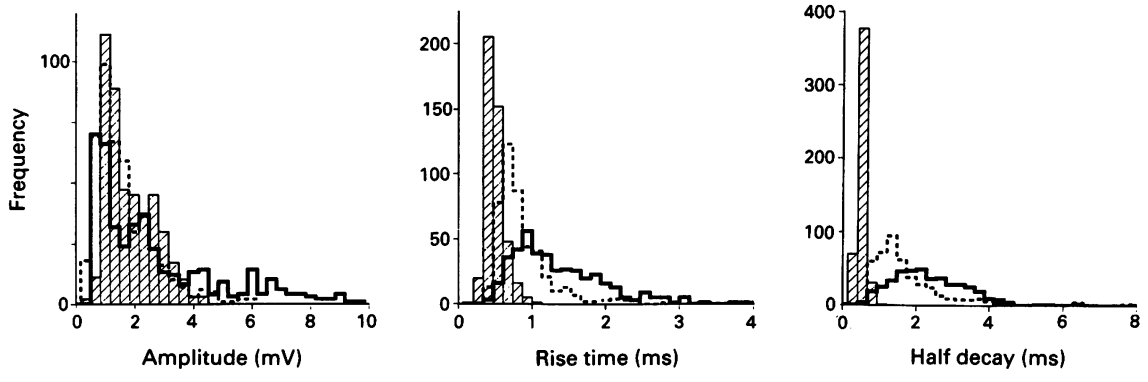


**Figure 2** Histograms showing the effects of tacrine and physostigmine on m.e.p.cs size and kinetics. (a) M.e.p.cs amplitudes; (b) m.e.p.cs rise time constants and (c) m.e.p.cs half decay time constants. Non-treated condition: filled histogram bars. Tacrine (100 μM): thick histogram outline. Physostigmine (60 μM): discontinuous histogram outline. Tacrine treatment induces clearly an increase in the number of m.e.p.cs larger than 4 mV and also a shift towards prolonged time constants. All histogram plots have a Normal distribution as tested by the Kolmogorov-Smirnov test. $n = 3$ different experiments.

**Table 1** Mean values (± s.e.mean) of m.e.p.cs measurements and the correlation coefficients between paired measurements

| | *Control* | *Tacrine* (100 μM) | *Physostigmine* (60 μM) |
|---|---|---|---|
| Mean amplitude (mV) | $1.66 \pm 0.04^{2}$ | $2.54 \pm 0.10^{1,2}$ | $1.80 \pm 0.05^{1}$ |
| Mean rise time (ms) | $0.42 \pm 0.06^{2}$ | $1.28 \pm 0.03^{1,2}$ | $0.79 \pm 0.02^{1}$ |
| Mean half decay (ms) | $0.36 \pm 0.01^{2}$ | $2.24 \pm 0.05^{1,2}$ | $1.67 \pm 0.05^{1}$ |
| Amplitude vs. rise time | $r = 0.185$ | $r = 0.271$ | $r = -0.148$ |
| Amplitude vs. half decay | $r = 0.153$ | $r = 0.142$ | $r = -0.168$ |

[1]Different from control at $P < 0.05$
[2]Different from physostigmine at $P < 0.05$
Note negative $r$ values in physostigmine treatment, that could account for a molecular action different from tacrine. $n = 3$ different experiments.

m.e.p.cs (larger than 4 mV) from the measurements the given mean values did not change significantly.

Correlation coefficients between the parameters, amplitude and rise time or amplitude and half-decay, were slightly positive for m.e.p.cs recorded in untreated tissue or after bathing with tacrine. On the contrary, physostigmine treatment led to a slightly negative correlation between the paired measurements (Table 1).

## Discussion

It is well known that tacrine has anticholinesterase activity (Freeman & Dawson, 1991, for review). We have confirmed this property in the electric organ by means of enzymatic activity determination and through the observation of the slower decay phase of m.e.p.cs. Prolonged rise times and decay phases of m.e.p.cs have been reported in relation to different anticholinesterase agents in the neuromuscular junction (Magleby & Stevens, 1972; Kordas, 1977; Fiekers, 1985). We have determined that the action of tacrine is not unspecific upon any ectoenzyme, since apyrase activity is not modified.

Tacrine, in contrast to physostigmine, has another effect on quantal ACh release, increasing the proportion of giant and slow-rising m.e.p.cs. Diverse treatments like 4-aminoquinoline (Molgó & Thesleff, 1982), clostridial toxins (Sellin & Thesleff, 1981; Dunant *et al.*, 1987) and chloride replacement by isethionate (Ashford & Wann, 1983) are able to increase the frequency of giant and slow-rising m.e.p.cs. Recently it has been shown in the neuromuscular junction that tacrine induces the appearance of giant m.e.p.cs. Thesleff *et al.* (1990) suggested that they may reflect the fusion of dense-cored granules present in the nerve endings. In the electric organ of *Torpedo*, large dense-cored vasoactive intestinal polypeptide containing vesicles have been described (Whittaker, 1990) which could be homologous with the dense-cored vesicles of the neuromuscular junctions. However, there is no electrophysiological correlation between the release of dense-core vesicles and m.e.p.cs. Alternatively, it has been proposed that under resting conditions a m.e.p.c. could represent the concerted release of several vesicles (Stevens, 1993); in this case the action of tacrine should be to increase the number of vesicles cooperating for a quantum, that would lead to an eventual increase of acetylcholine in the synaptic cleft.

Further work has to be done to determine the molecular actions of tacrine on the increase of the release of high quantal spontaneous events and on the possible relation to its effectiveness on patients with Alzheimer's disease.

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# Regulation of aromatic L-amino acid decarboxylase in rat striatal synaptosomes: effects of dopamine receptor agonists and antagonists

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**1** In this study we investigated the effects of dopamine receptor agonists and antagonists on rat striatal synaptosomal aromatic L-amino acid decarboxylase (AADC) activity.

**2** The results show that $10^{-5}$–$10^{-7}$ M *cis*-flupenthixol increased the striatal synaptosomal AADC activity (by 25% to 57%) in a time-dependent manner. SCH 23390 and remoxipride alone had little or no effect on striatal synaptosomal AADC activity, but in combination they increased AADC activity by 20%, suggesting that the increases in striatal synaptosomal AADC activity occurred only after blockade of both dopamine $D_1$ and $D_2$ receptors.

**3** Treatment with (+)-amphetamine and (±)-2-(N-phenylethyl-*N*-propyl)amino-5-hydroxytetralin hydrochloride ((±)-PPHT) produced a reduction of striatal synaptosomal AADC activity in a concentration- and time-dependent manner. SKF 38393 and (−)-quinpirole, however, exhibited no effect on striatal synaptosomal AADC activity, suggesting that only the mixed dopamine receptor agonists can reduce the AADC activity. Incubation with apomorphine at a concentration of $10^{-4}$ M inhibited the AADC activity by 74% and this inhibition cannot be antagonized by SCH 23390, remoxipride or *cis*-flupenthixol, suggesting that apomorphine-induced inhibition of striatal synaptosomal AADC activity was not mediated by dopamine receptors.

**4** *cis*-Flupenthixol can reverse the reduction of AADC activity induced by (+)-amphetamine and (±)-PPHT. The inhibition of AADC activity elicited by (±)-PPHT also can be reversed by SCH 23390 and remoxipride.

**5** The inhibition of striatal synaptosomal AADC activity induced by (±)-PPHT is calcium-dependent and protein kinase C may play a role in the regulation of striatal AADC activity.

**6** These studies show that striatal synaptosomal AADC activity is regulated by dopamine receptors and indicate that *in vitro* dopamine $D_1$ and $D_2$ receptors have a synergistic effect in this regulation.

**Keywords:** Aromatic L-amino acid decarboxylase; dopamine receptor; agonists; antagonists; striatum; synaptosomes

## Introduction

Aromatic L-amino acid decarboxylase (E.C. 4.1.1.28, AADC) is the enzyme which catalyzes the decarboxylation of L-phenylalanine to form 2-phenylethylamine (PE) and it is considered to have a regulatory role in the synthesis of this amine (Saavedra, 1974). AADC is also required for the formation of the catecholamines and 5-hydroxytryptamine (5-HT), but in these cases it is not rate limiting (Brodie *et al.*, 1962). Since brain AADC is thought not to be saturated with substrates and to be relatively nonselective (Bowsher & Henry, 1986), the regulation of the enzyme has not been intensively studied. There is now evidence, however, that some physiological conditions and some treatments can change AADC activity *in vivo*. In the rat retina, AADC activity increases in response to light (Hadjiconstantinou *et al.*, 1988) and these changes were mediated by dopamine $D_1$ receptors (Rossetti *et al.*, 1990). The change in AADC activity was associated with *de novo* synthesis of the protein (Hadjiconstantinou *et al.*, 1988). Both dopamine $D_1$ and $D_2$ receptor blockers such as SCH 23390, haloperidol and sulpiride increase striatal AADC activity in control mice and in mice treated with N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Hadjiconstantinou *et al.*, 1993). Using molecular biology methods, it was recently found that the AADC mRNA level of rat brain was increased after chronic treatment with the neuroleptics, haloperidol and loxapine (Buckland *et al.*, 1992a), which also produced an increase in $D_1$, $D_2$ and $D_3$ dopamine receptor mRNA level (Buckland *et al.*, 1992b). These findings suggest that AADC activity may be modulated by dopamine receptors.

Previous studies from this laboratory have shown that the administration of dopamine $D_1$ or $D_2$-like receptor blockers produced dose-dependent increases in AADC activity in striatal and mesolimbic areas in the rat (Zhu *et al.*, 1992). The dopamine receptor blockade increased the $V_{max}$ but did not affect the $K_m$ of the enzyme. The use of a protein synthesis inhibitor suggested that at least within 1 h after drug treatment, *de novo* protein synthesis was not responsible for the increases in AADC (Zhu *et al.*, 1993). AADC activity was reduced by the administration of the dopamine receptor agonist, bromocriptine (Zhu *et al.*, 1993). Moreover, there is an interaction between some dopamine receptor antagonists on the striatal AADC activity after joint administration of these compounds (Zhu *et al.*, 1993). These data suggest that *in vivo* AADC is a modulated enzyme.

AADC has been considered to be predominantly located in the soluble fraction of both kidney extracts (Lovenberg *et al.*, 1962) and rat brain (Sims *et al.*, 1973). There are reports, however, that a substantial proportion (50% or more) of AADC may be associated with membranes (Rodriguez de Lores Arnaiz & De Robertis, 1964; Sims *et al.*, 1973). Subsequently, Gardner & Richards (1981) have reported that 35% of AADC is associated with the synaptosomal pool. In the present study we have investigated the effects of some dopamine receptor agonists and antagonists on AADC activity in rat striatal synaptosomes and the possible mechanism in the AADC regulation.

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## Methods

### Animals

Male Wistar rats (Charles River Canada, Montreal, Quebec), weighing 230–250 g at the time of experimentation, were used. The animals were housed in hanging wire cages with free access to food and water on a 12 h light/dark cycle (lights on at 06 h 00 min) at a temperature of 19–21°C.

### Preparation of synaptosomes

Synaptosomal-enriched fractions were prepared from striatum by differential and Ficoll-sucrose density gradient centrifugation as described by Cotman (1974) and Wood *et al.* (1979) and modified in our laboratory. Rats were killed by decapitation and brains were removed and immediately placed in ice-cold saline followed by dissection on ice to remove striata. Striata of three rats were homogenized in 0.32 M sucrose (1%, w/v), using 10 up and down strokes at 900 r.p.m. in a glass-Teflon homogenizer (clearance 0.25 mm). The homogenate was centrifuged at 1,000 *g* for 5 min and the separated supernatant was again centrifuged at 15,000 *g* for 12 min. The pellet was collected and resuspended in 0.32 M sucrose and then applied to a Ficoll-sucrose density gradient, consisting of 4, 6 and 13% (w/v) Ficoll in 0.32 M sucrose. After centrifugation at 63,580 *g* for 45 min, the synaptosomal-enriched fraction was collected from the 6–13% interface. The particulate fraction thus obtained was resuspended in 4 volumes of 0.32 M sucrose and spun at 50,000 *g* for 20 min. The resultant pellet was resuspended in the medium to be used for incubation.

### Incubation of synaptosomes with drugs

The synaptosomal pellet was resuspended in Somjens saline (in mM: NaCl 123, KCl 3.51, $NaH_2PO_4$ 1, $MgSO_4$ 0.8, $NaHCO_3$ 26, $CaCl_2$ 1.2 and D-glucose 11, pH 7.35 and saturated with 95% $O_2$/5% $CO_2$) (Magoski & Walz, 1992). For the calcium-free experiments, $Ca^{2+}$ was omitted from the medium. For the incubation, 200 µl of the synaptosome preparation was mixed with 600 µl of oxygenated Somjens saline in the presence of different concentrations of drugs (dissolved in 200 µl water) in a total volume of 1 ml. The control group received 200 µl of water instead of drugs. All samples before incubation were undertaken at 0°C in an ice bath. After shaking, the samples were transferred to a water bath of 37°C and incubated for 1 h. The reaction was terminated by returning the samples to the ice bath following which the samples were immediately centrifuged (4°C) at 40,000 *g* for 20 min. The pellets were then resuspended in 0.01 M sodium phosphate buffer, and stored overnight at −70°C for AADC and protein assay.

### Aromatic L-amino acid decarboxylase assay

AADC activity was assayed by a modification of the method of Nagatsu *et al.* (1979) and Okuno & Fujisawa (1983). The assay was based on the enzymatic conversion of L-3,4-dihydroxyphenylalanine (L-DOPA) to dopamine with measurement of dopamine by high performance liquid chromatography with electro-chemical detection (h.p.l.c.-e.c.): 50 µl of the incubation suspension were used for the assay. The assay mixture, containing sodium phosphate buffer 50 mM, pH 7.2, L-DOPA 0.04 mM (D-DOPA for estimation of blank values), ascorbic acid 0.17 mM, pyridoxal-5-phosphate 0.01 mM, pargyline 0.1 mM, 2-mercaptoethanol 1 mM, EDTA 0.1 mM and enzyme in a total volume of 400 µl, was incubated at 37°C for 20 min and the reaction was terminated by addition of 600 µl ice cold perchloric acid (0.1 M) containing isoprenaline as an internal standard. The mixture was transferred to a small conical polypropylene test tube, centrifuged again (3,000 r.p.m. for 10 min) and 50 µl of supernatant used for h.p.l.c. assay. Protein concentration was determined (Lowry *et al.*, 1951) with bovine serum albumin used as the standard and the enzyme activity was expressed as nmol of dopamine 20 $min^{-1}$ $mg^{-1}$ of protein at 37°C.

### Drugs for incubation with synaptosomes

Drugs used in this study were obtained from following sources: (−)-quinpirole HCl, SCH 23390 (R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride), (±)-PPHT hydrochloride ((±)-2-(N-phenylethyl-*N*-propyl)amino-5-hydroxytetralin hydrochloride), SKF 38393 (1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol hydrochloride), H-7 HCl ((1-(5-isoquinolinesulphonyl)-2-methylpiperazine) and PDBu (phorbol 12,13-dibutyrate), Research Biochemicals Inc. (Natick, MA, U.S.A.); apomorphine hydrochloride, Sigma Chemical Co. (St. Louis, MO, U.S.A.); (+)-amphetamine sulphate, Smith, Kline and French Laboratories (Philadelphia, PA, U.S.A.). The following compounds were generous gifts: remoxipride [S-3-bromo-*N*-(1-ethyl-2-pyrrolidinyl)methyl-2,6-dimethoxybenzamide hydrochloride monohydrate], Astra Pharma Inc. (Mississauga, Ontario); *cis*- and *trans*-flupenthixol, H. Lundbeck & Co. (Kobenhavn-Valby, Denmark). Solutions were freshly prepared in nanopure water.

### Statistical methods

Data were analysed by one- and two-way analysis of variance (ANOVA) (Winer, 1971) and the Newman-Keuls tests for multiple comparisons, depending on the experimental design. Statistical analyses were performed on a Macintosh Microcomputer using the CLR ANOVA programme (Clearlake Research, Houston, TX, U.S.A.).

## Results

### The effects of dopamine receptor antagonists on AADC activity in striatal synaptosomes

cis-*Flupenthixol* The concentration-response curve for activation of striatal synaptosomal AADC by *cis*-flupenthixol is presented in Figure 1a. The results show that $10^{-6}$ and $10^{-7}$ M of *cis*-flupenthixol increased AADC activity by 56% and 57% above control values, respectively ($F_{6,25} = 5.8$, $P < 0.001$). Incubation with higher concentrations of *cis*-flupenthixol produced a lower (with $10^{-5}$ M) or no increase (with $10^{-4}$ M) in AADC activity (Figure 1a).

The time course of *cis*-flupenthixol ($10^{-6}$ M) activation of AADC activity in striatal synaptosomes shows that at 30 min of incubation AADC activity has been increased (37%) and that maximal activation (47%) occurred after 1 h of incubation. By 2 h of incubation, however, this compound reduced AADC activity by 40% below the control value (Figure 1b). This reduction in AADC activity observed with longer exposures to *cis*-flupenthixol (Figure 1b) agrees with the result of lower effects produced by higher doses (Figure 1a).

*SCH 23390 and remoxipride* Unlike *cis*-flupenthixol, both SCH 23390 and remoxipride had no effects on striatal synaptosomal AADC activity when they were separately incubated with synaptosomal preparations (Figure 2a,b), nor was any inhibition in AADC observed with doses as high as $10^{-4}$ M. When $10^{-8}$ M of each of these compounds was incubated together with striatal synaptosomes, they increased synaptosomal AADC activity by 20% above control value ($F_{6,26} = 5.02$, $P < 0.01$, Figure 2c). This was the only dose combination found to increase the AADC activity; other lower dose ($10^{-9}$ M) or higher doses (up to $10^{-4}$ M) did not change its activity (Figure 2c).

*The effects of dopamine receptor agonists on AADC activity in striatal synaptosomes*

*(+)-Amphetamine* The incubation of different concentrations of (+)-amphetamine with striatal synaptosomes for 1 h produced a reduction in AADC activity. The effects were observed with concentrations of $10^{-7}$ M–$10^{-4}$ M of (+)-amphetamine and the reductions ranged from 29 to 35% of their respective controls ($F_{6,48} = 3.26$; $P<0.05$, Figure 3a). The time-course of the effect of (+)-amphetamine was studied with a concentration of $10^{-5}$ M and showed that by 1 h of incubation the AADC activity was 26% of controls and further reductions were observed by 2 h of incubation ($F_{3,20} = 13.2$, $P<0.001$; Figure 3b). The results indicate that (+)-amphetamine inhibits striatal synaptosomal AADC in both a concentration- and time-dependent manner.

*(±)-PPHT* Incubation of striatal synaptosomes with (±)-PPHT at concentrations of $10^{-4}$ M to $10^{-7}$ M produced a concentration-dependent reduction in AADC activity. The reductions were 37%, 36%, 31% and 30% with respect to their respective controls ($F_{6,49} = 3.3$, $P<0.01$) (Figure 3a).

Like (+)-amphetamine, (±)-PPHT ($10^{-5}$ M) also inhibited striatal synaptosomal AADC activity in a time-dependent manner (Figure 3b). This effect, however, was observed at an earlier time than that of (+)-amphetamine, reaching significance 30 min after incubation with values of 20% below control ($F_{3,19} = 8.27$, $P<0.01$).

*SKF 38393 and (−)-quinpirole* The concentration-response curves for SKF 38393 and (−)-quinpirole were measured after 1 h of incubation. The experiment indicated that neither SKF 38393 nor (−)-quinpirole (added in concentrations ranging from $10^{-9}$ to $10^{-4}$ M) changed the AADC activity in striatal synaptosomes ($F_{6,31} = 1.69$, for SKF 38393; $F_{6,57} = 0.9$, for (−)-quinpirole; data not shown), suggesting that selective dopamine $D_1$ or $D_2$-like receptor agonist does not affect AADC activity in striatal synaptosomes.

*Apomorphine* The concentration-response of apomorphine in rat striatal synaptosomes was determined after 1 h incubation. The AADC activity of controls was 5.18 ± 0.19 nmol dopamine $mg^{-1}$ of protein, 20 $min^{-1}$. Addition of $10^{-9}$ to $10^{-5}$ M of apomorphine produced no changes in synaptosomal AADC activity. AADC activity was inhibited by apomorphine at a dose of $10^{-4}$ M to values of 1.34 nmol dopamine $mg^{-1}$ of protein, 20 $min^{-1}$ (reduced by 74%, $F_{6,42} = 21.85$, $P<0.001$; data not shown). The time course indicated that incubation with $10^{-4}$ M apomorphine produced a reduction in AADC activity that was observed within 30 min (with reductions from 6.18 to 4.36 nmol dopamine

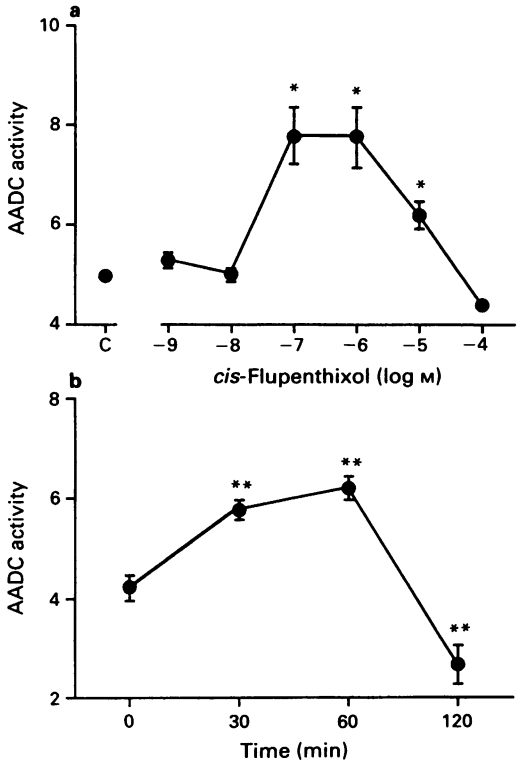


**Figure 1** Dose-response effects (a) and time course (b) of *cis*-flupenthixol on AADC activity (nmol of dopamine $mg^{-1}$ protein, 20 $min^{-1}$) in the rat striatal synaptosomes. The incubation time for dose-course was 1 h. The concentration of *cis*-flupenthixol used in the time-course was $10^{-6}$ M. The values in each experimental group were obtained from 6–8 samples. Data are mean ± s.e.mean. Significance was determined by one way ANOVA and Newman-Keuls test: $*P<0.05$; $**P<0.01$, compared with the control group (C group).

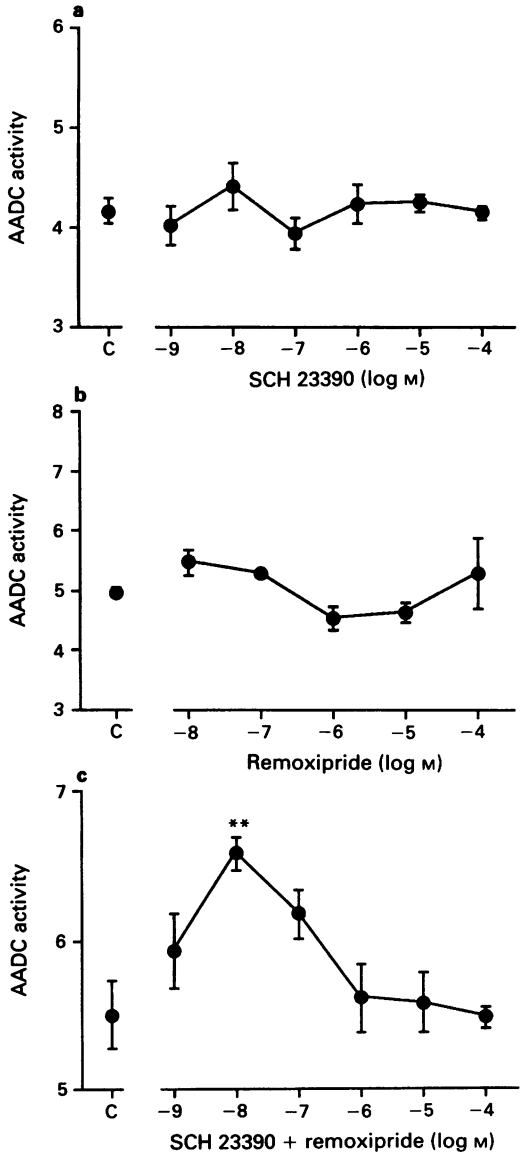


**Figure 2** Dose-response effects of SCH 23390 (a), remoxipride (b) and their combination (c) on AADC activity (nmol dopamine $mg^{-1}$ protein, 20 $min^{-1}$) in the rat striatal synaptosomes. The incubation time was 1 h. The values in each experimental group were obtained from 6 samples. Data are means ± s.e.mean. Significance was determined by one-way ANOVA and Newman-Keuls test: $**P<0.01$, compared with the control group (C).

$mg^{-1}$ of protein, 20 $min^{-1}$, 30% below controls). The AADC inhibition increased with time and by 2 h of incubation, almost all AADC activity (reaching values of 0.39 nmol dopamine $mg^{-1}$ of protein, 20 $min^{-1}$) was inhibited ($F_{6,28} = 94.5$, $P < 0.001$; data not shown). Treatment with SCH 23390, remoxipride or *cis*-flupenthixol did not reverse the inhibition of AADC activity induced by $10^{-4}$ M of apomorphine (data not shown). The findings suggested that the inhibition of AADC activity produced by apomorphine ($10^{-4}$ M) in striatal synaptosomes is unspecific or acts by a different mechanism.

### *The reversal of dopamine receptor agonist-mediated reduction in AADC activity by dopamine receptor blockers*

Exposure of synaptosomes to *cis*-flupenthixol abolished the (+)-amphetamine- or (±)-PPHT-induced reduction in AADC activity. The results showed that $10^{-5}$ and $10^{-6}$ M of *cis*-flupenthixol can effectively antagonize the effects of (+)-amphetamine ($10^{-5}$ M) on striatal synaptosomal AADC activity (Figure 4a). In another experiment, the action of (±)-PPHT ($10^{-5}$ M) was antagonized by $10^{-5}$–$10^{-8}$ M of *cis*-flupenthixol (Figure 4b).

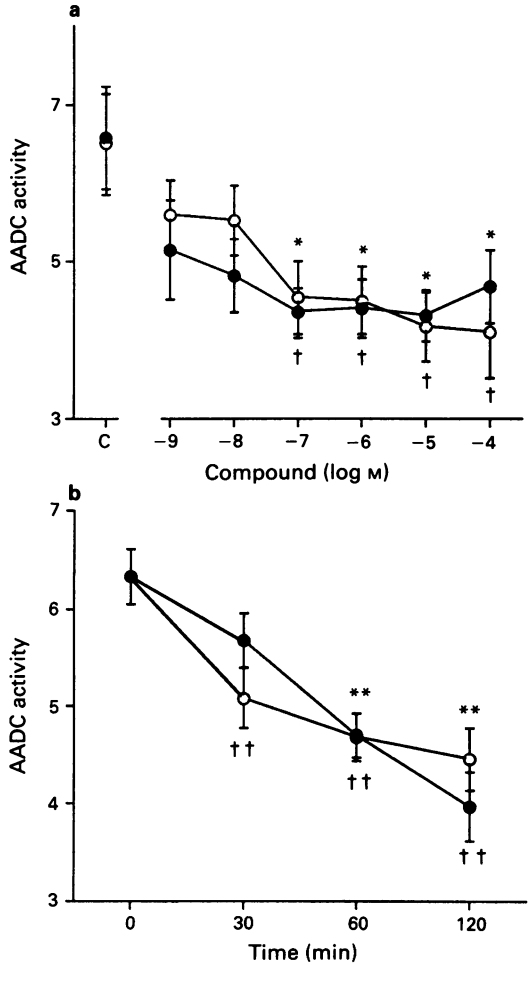


**Figure 3** Dose-response effects (a) and time course (b) of (+)-amphetamine (●) and (±)-PPHT (○) on AADC activity (nmol of dopamine $mg^{-1}$ protein, 20 $min^{-1}$) in the rat striatal synaptosomes. The incubation time for dose-course was 1 h. The concentrations of (+)-amphetamine and (±)-PPHT used in the time-course were $10^{-5}$ M. The values in each experimental group were obtained from 10 samples. Data are mean ± s.e.mean. Significance was determined by one way ANOVA and Newman-Keuls test: *,†$P < 0.05$; **††$P < 0.01$, compared with the control group (C and 0 groups).

Moreover, when SCH 23390 ($10^{-6}$ M) or remoxipride ($10^{-4}$–$10^{-6}$ M) was incubated alone with $10^{-5}$ M of (±)-PPHT and striatal synaptosomes, both counteracted the inhibition of AADC activity elicited by (±)-PPHT (Figure 5a,b).

### *The effects of calcium on AADC activity in striatal synaptosomes*

Somjens saline contains 1.2 mM calcium. Striatal synaptosomes were incubated with modified Somjens solution containing no calcium, low calcium (0.6 mM) and high calcium (2.4 mM). The effects of calcium on (±)-PPHT-induced inhibition of synaptosomal AADC activity were investigated and the results were analysed by two-way ANOVA. Incubation of striatal synaptosomes in Somjens solution in the absence of calcium or with lower (0.6 mM) calcium concentrations had no effect on synaptosomal AADC activity, compared to synaptosomes incubated in normal Somjens saline (1.2 mM calcium). The increase in calcium content of Somjens saline (2.4 mM) increased AADC activity by 41% ($F_{3,40} = 37.77$, $P < 0.001$) (Figure 6).

(±)-PPHT significantly reduced AADC activity in normal (1.2 mM calcium) medium as described above ($F_{1,40} = 16.52$, $P < 0.001$), but had no effects on synaptosomal AADC activity in calcium-free and lower calcium medium (0.6 mM) (Figure 6). In the higher calcium (2.4 mM) medium, incubation with (±)-PPHT counteracted the increase in AADC activity (Figure 6). These results indicate that (±)-PPHT-induced inhibition of AADC activity was calcium-dependent. There was a significant interaction between calcium and (±)-PPHT treatment ($F_{3,40} = 5.72$, $P < 0.001$).

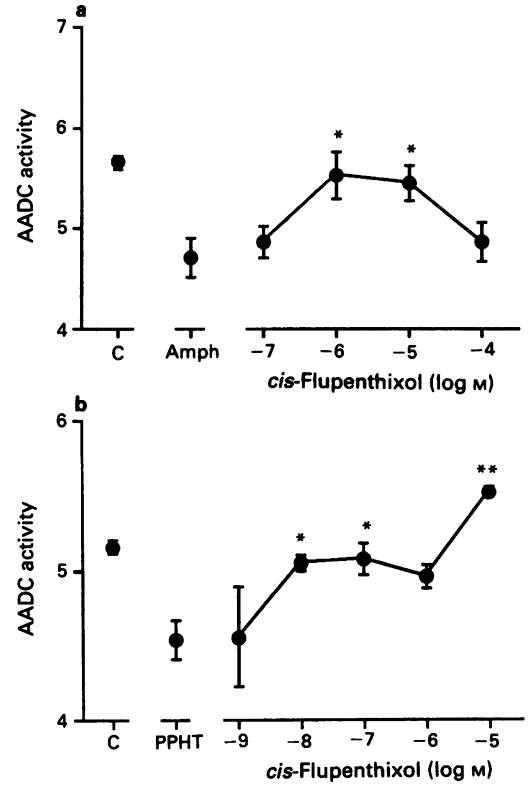


**Figure 4** The antagonism by *cis*-flupenthixol of (+)-amphetamine (Amph) (a) and (±)-PPHT (b)-induced inhibition of AADC activity (nmol of dopamine $mg^{-1}$ protein, 20 $min^{-1}$) in rat striatal synaptosomes. The incubation time was 1 h. The concentration of (+)-amphetamine and (±)-PPHT used in the experiments was $10^{-5}$ M. The values in each experimental group were obtained from 6 samples. Data are means ± s.e.mean. Significance was determined by one-way ANOVA and Newman-Keuls test: *$P < 0.05$; **$P < 0.01$, compared with the Amph and (±)-PPHT group, respectively.

### *Synaptosomal AADC activity after stimulation or inhibition of protein kinase C*

Striatal synaptosomal preparations were incubated with H-7 or PDBu, which respectively inhibit or activate protein kinase C activity. Activation of protein kinase C with PDBu alone significantly inhibited synaptosomal AADC activity at concentrations of $10^{-6}$–$10^{-7}$ M ($F_{5,24} = 3.68$, $P<0.05$, Figure 7b). H-7 alone had no effect on AADC activity ($F_{6,28} = 1.72$, $P<0.015$, Figure 7a), but $10^{-6}$ M H-7 attenuated the inhibition of synaptosomal AADC activity produced by $10^{-6}$ M PDBu ($F_{1,21} = 7.47$, $P<0.05$, as determined by two-way ANOVA).

Following a two-way experimental design, the addition of $10^{-5}$ M of (±)-PPHT alone reduced the synaptosomal AADC activity ($F_{1,10} = 43.93$, $P<0.001$) (Figure 7d). Treatment with $10^{-6}$ M H-7 neither affected AADC activity ($F_{1,10} = 1.05$, $P<0.33$), nor interfered with the inhibitory action of (±)-PPHT on synaptosomal AADC activity

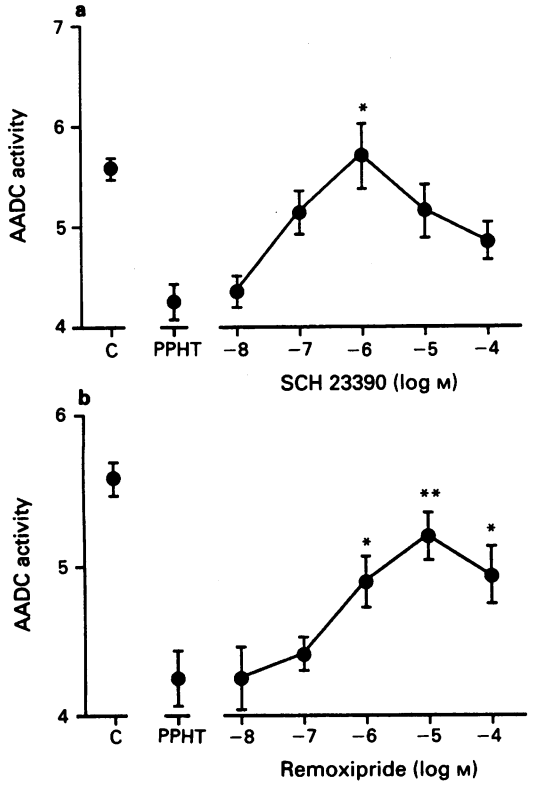


**Figure 5** The antagonism by SCH 23390 (a) and remoxipride (b) of (±)-PPHT-induced inhibition of AADC activity (nmol of dopamine $mg^{-1}$ protein, 20 $min^{-1}$) in rat striatal synaptosomes. The incubation time was 1 h. The concentration of (±)-PPHT used in the experiments was $10^{-5}$ M. The values in each experimental group were obtained from 6 samples. Data are means ± s.e.mean. Significance was determined by one-way ANOVA and Newman-Keuls test: $*P<0.05$; $**P<0.01$, compared with the (±)-PPHT group.

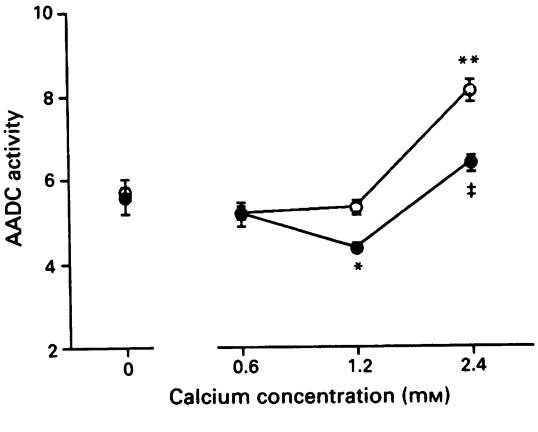


**Figure 6** The effects of different calcium concentrations on AADC activity (nmol of dopamine $mg^{-1}$ protein, 20 $min^{-1}$) in the presence (●) and absence (○) of (±)-PPHT ($10^{-5}$ M) in striatal synaptosomes. The incubation time was 1 h. The values in each group were obtained from 6 samples. Data are means ± s.e.mean. Significance was determined by two-way ANOVA and Newman-Keuls test: $*P<0.05$; $**P<0.01$, compared with the no-(±)-PPHT-1.2 mM group. $\dagger\dagger P< 0.01$, compared with the no-(±)-PPHT-2.4 mM group.

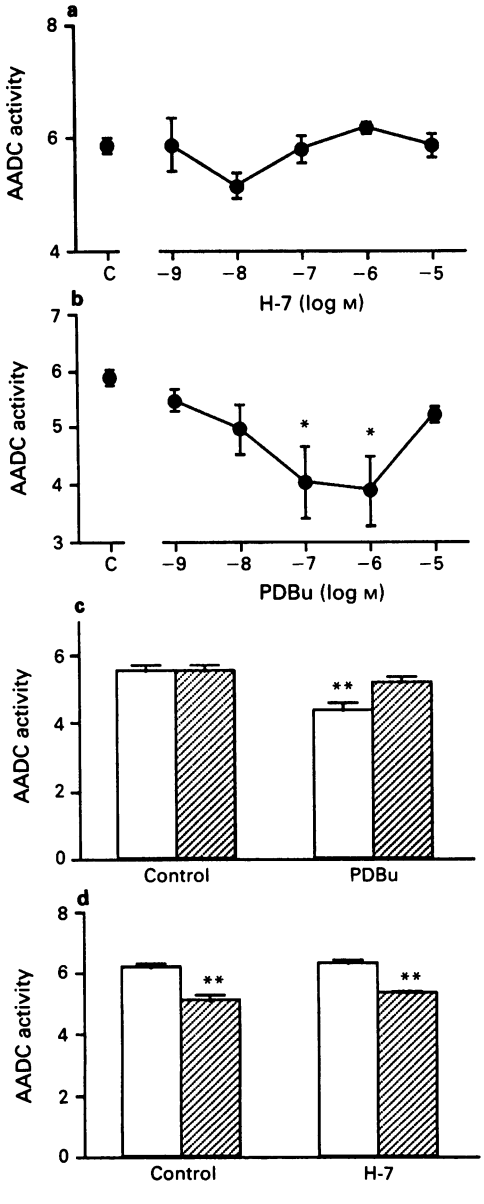


**Figure 7** Dose-response effects of H-7 (a), PDBu (b) and combinations of H-7 and PDBu (c, open columns represent control group; hatched columns represent H-7), H-7 and (±)-PPHT (d, open columns represent control group; hatched columns represent (±)-PPHT) on AADC activity (nmol dopamine $mg^{-1}$ of protein, 20 $min^{-1}$) in the rat striatal synaptosomes. The concentrations of H-7, PDBu and (±)-PPHT in (c) and (d) experiments were $10^{-6}$, $10^{-6}$ and $10^{-5}$ M respectively. Data are means ± s.e.mean (bars) from 9 samples. Statistical significances are determined by one-way ANOVA and Newman-Keuls test (a,b) or two-way ANOVA (c,d). $*P<0.05$, $**P<0.01$, compared with control group (c and d) groups.

($F_{1,10} = 0.1$, $P < 0.75$). This finding suggests that inhibition of synaptosomal AADC by (±)-PPHT is not related to protein kinase C.

## Discussion

This study has shown that treatment of rat striatal synaptosomes with the direct dopamine receptor agonist, (±)-PPHT and the indirect dopamine receptor agonist, (+)-amphetamine, inhibits AADC activity in rat striatal synaptosomes in a concentration- and time-dependent manner (Figure 3). A mixed dopamine receptor antagonist such as *cis*-flupenthixol not only increased AADC activity (Figure 1), but also reversed the inhibition of AADC produced by either (+)-amphetamine or (±)-PPHT (Figure 4). The dopamine $D_1$ receptor antagonist, SCH 23390 and the dopamine $D_2$ receptor antagonist, remoxipride alone had no effect on the synaptosomal AADC activity (Figure 2a,b), but when simultaneously administered, they increased the AADC activity (Figure 2c). Furthermore, both antagonized the (±)-PPHT-induced inhibition of AADC activity (Figure 5). These results extend our previous findings *in vivo* (Zhu *et al.*, 1992; 1993), and reveal that in rat striatal synaptosomes AADC is regulated by dopamine $D_1$-like and $D_2$-like receptors.

Both AADC and tyrosine hydroxylase are involved in the synthesis of dopamine. It is well known that apomorphine can act directly and indirectly on tyrosine hydroxylase. High concentrations of apomorphine ($10^{-4}$ M) produced a complete inhibition of tyrosine hydroxylase activity in a striatal enzyme preparation (Hurata & Shibata, 1991), striatal slices (Delanoy & Dunn, 1982) or in synaptosomal preparations (Christiansen & Squires, 1974), and DOPA producing cell lines (Bräutigram *et al.*, 1982). In most cases the inhibition of tyrosine hydroxylase caused by apomorphine can be completely antagonized by haloperidol and other neuroleptics *in vivo* (Kehr *et al.*, 1972; Walters & Roth, 1976; Westerink & Horn, 1979). The mechanism of the direct action of apomorphine on tyrosine hydroxylase is believed to be due to the catechol structure present in the apomorphine molecule, which competes for the pterin cofactor (Goldstein *et al.*, 1970; Birtan & Bustos, 1982). In this study we observed that a high concentration of apomorphine inhibited the striatal synaptosomal AADC activity and this inhibition was not mediated by dopamine receptors, since it was not reversed by dopamine receptor antagonists. In the control experiment we found that apomorphine ($10^{-4}$ M) had no direct effect on freely presented AADC activity (Zhu, 1993) so it is most likely that apomorphine is having an effect by an unspecific action.

Since it was first reported that both dopamine $D_1$ and $D_2$ receptor agonists were required to reverse the hypokinesia produced by reserpine in mice (Gershanik *et al.*, 1983), evidence has been accumulated suggesting that there is an interaction between dopamine $D_1$ and $D_2$ receptors. It has been proposed that this interaction possesses both synergistic or opposing effects (Clark & White, 1987). Most of the evidence about synergistic effects, however, was obtained from behavioural (Gershanik *et al.*, 1983; Rosengarten *et al.*, 1986), electrophysiological (White & Wang, 1986) and clinical studies (Lieberman *et al.*, 1981). Furthermore, the investigations were limited to the nucleus accumbens (White & Wang, 1986), globus pallidus (Carlson *et al.*, 1986; Walters *et al.*, 1987) and substantia nigra (Weick & Walters, 1987), and few investigations were done in the striatum (Stoof & Kebabian, 1981; 1984).

Although (±)-PPHT is considered as a selective dopamine $D_2$-like receptor agonist (Seeman *et al.*, 1985; Seiler *et al.*, 1986), there is evidence that when its concentration is over $10^{-6}$ M, it can act on dopamine $D_1$ high and low affinity sites (Berry, 1993). In the present study, it was found that among the dopamine receptor agonists, only nonselective agonists such as (+)-amphetamine and (±)-PPHT produced inhibition of striatal synaptosomal AADC activity (Figure 3), while no effects were observed with either the dopamine $D_1$-like receptor agonist, SKF 38393 or the dopamine $D_2$-like receptor agonist, (−)-quinpirole. Furthermore, only a mixed dopamine $D_1$ and $D_2$-like receptor antagonist such as *cis*-flupenthixol increased the striatal synaptosomal AADC activity (Figure 1). Blockade of dopamine $D_1$ receptors by SCH 23390 or dopamine $D_2$ receptors by remoxipride produced no changes in striatal synaptosomal AADC activity (Figure 2a,b). It was interesting to find that the combination of SCH 23390 and remoxipride dramatically increased the AADC activity (Figure 2c). The results clearly demonstrate that the activation and inhibition of AADC activity in striatal synaptosomes requires the participation of both dopamine $D_1$ and $D_2$ receptors. This is the first time that a biochemically synergistic interaction between dopamine $D_1$ and $D_2$ receptors in striatal synaptosomes has been reported.

The synergistic action of dopamine $D_1/D_2$ receptors has been interpreted as dopamine $D_1$ receptor activation enabling the functional expression of dopamine $D_2$ receptor stimulation (Clark & White, 1987). There has been a report that dopamine $D_1$ and $D_2$ receptor synergism at the level of a single cell may also occur via activation of the arachidonic acid cascade (Piomelli *et al.*, 1991). At present, however, the mechanism of the regulation of striatal AADC by dopamine receptors remains unknown. Since the administration of (+)-amphetamine inhibited striatal synaptosomal AADC activity (Figure 3), and *in vivo* acute and chronic administration of reserpine, which depletes endogenous dopamine storage (Bertler, 1961), increased the striatal AADC activity (Zhu, 1993), endogenous dopamine may act as a negative feedback for striatal AADC. Similar results were recently observed by Hadjiconstantinou *et al.* (1993). This finding that in striatal synaptosomes dopamine $D_1$ and $D_2$ receptors appear to be synergistic with respect to AADC activity may possess important clinical significance. Administration of a selective and potent dopamine $D_1$-like receptor agonist with a dopamine $D_2$-like receptor agonist may lead to therapeutic improvement in the treatment of Parkinsons's disease. Recently, apomorphine has been investigated as a rescue therapy for Parkinson's disease; it was administered subcutaneously and sublingually to reverse 'off' periods occurring during oral L-DOPA therapy (Deffond *et al.*, 1993), and as a continual therapy, delivered subcutaneously by minipump (Frankel *et al.*, 1990).

*In vivo* the dopamine $D_1$-like receptor antagonist, SCH 23390 and the dopamine $D_2$-like receptor antagonist, pimozide, as well as remoxipride alone increased the AADC activity in rat striatum (Zhu *et al.*, 1992; 1993). Synaptosomal preparations showed no increase in AADC activity after selective blockade of dopamine $D_1$ or $D_2$ receptors (Figure 2a,b). These differences may be due to the different experimental conditions, and the regulation of striatal synaptosomal AADC activity may be more specific than in *in vivo* brain tissues. In the intact animal, the dopaminergic nigrostriatal neurones have perikarya in the substantia nigra with their terminals in the striatum. The substantia nigra is an important action site for dopamine receptor antagonists. Also, dopamine receptors may be localized on dendrites, terminals, or glial cells adjacent to dopaminergic terminals (Creese, 1987). All these factors may affect the appearance of action of dopamine receptor antagonists *in vivo*. In contrast, in synaptosomes nerve terminals in sufficiently dilute suspension may be considered as an isolated structure where interactions with other brain regions do not exist.

The inhibition of striatal synaptosomal AADC activity induced by (+)-amphetamine and (±)-PPHT can be reversed by the mixed dopamine $D_1$ and $D_2$-like receptor antagonist *cis*-flupenthixol (Figure 4). Although SCH 23390 and remoxipride alone had no effect on AADC activity (Figure 2a,b), they can reverse the inhibition of AADC activity induced by (±)-PPHT (Figure 5). These findings suggest that the changes in striatal synaptosomal AADC

activity by (+)-amphetamine and (±)-PPHT may be receptor-mediated. Although the precise mechanism to explain this regulation has not been identified, one interesting finding is that the absence of calcium or the presence of a lower than normal concentration of calcium has no effect on the synaptosomal AADC activity, but it does prevent the inhibition of AADC activity by (±)-PPHT (Figure 6). On the other hand, higher concentrations of calcium increased the AADC activity, but did not block the inhibition of AADC activity induced by (±)-PPHT. This suggests that the regulation of striatal synaptosomal AADC by dopamine receptors is calcium-dependent. At present, it may be speculated that stimulation or blockade of dopamine receptors in striatal synaptosomes by dopamine receptor agonists or antagonists produced a change in the second messenger system, which in turn caused other changes like phosphorylation and led to the changes in AADC activity.

Other experiments were conducted to examine whether phosphorylation was involved in the regulation of AADC via dopamine receptors. The results show that PDBu, a phorbol ester which selectively activates protein kinase C (Castagna *et al.*, 1982), significantly reduced synaptosomal AADC activity (Figure 7b). This reduction in AADC activity was prevented by the simultaneous administration of H-7, a protein kinase inhibitor (Hidaka *et al.*, 1984) (Figure 7c), although H-7 alone did not influence the AADC activity (Figure 7a). This suggests that protein kinase may participate in the regulation of striatal AADC *in vitro*, which is supported by the finding that AADC regulation requires the presence of calcium (Figure 6), since activation of protein kinase C is dependent on calcium (Nishizuka, 1986). However, when H-7 was co-administered with the dopamine receptor agonist (±)-PPHT, H-7 could not prevent the reduction of synaptosomal AADC activity produced by (±)-PPHT (Figure 7d). This latter experiment indicates that although protein kinase C may participate in the regulation of AADC, it may not be related to the regulation of AADC by dopamine receptors. Other neurotransmitters or compounds, which act on protein kinase C via their second messenger system, may therefore regulate AADC activity.

Our previous papers (Zhu *et al.*, 1992; 1993) indicated that at least within 1 h after administration of dopamine receptor antagonist the change of striatal AADC is not due to *de novo* protein synthesis. Other investigators demonstrated that 3 or 6 h after injection of dopamine receptor antagonists, protein synthesis was responsible for the increase of striatal AADC activity (Hadjiconstantinou *et al.*, 1988; 1993). These observations suggest that there may be different mechanisms for regulation of AADC: a short-term regulation of AADC that occurs within 1 h after dopamine receptor blockade and that is not dependent on protein synthesis (Zhu *et al.*, 1992; 1993), and a long-term regulation that is dependent on protein synthesis (Hadjiconstantinou *et al.*, 1988; 1993). Since phosphorylation of tyrosine hydroxylase is the main mechanism for its short term regulation (Masserano *et al.*, 1989), it is reasonable to assume that phosphorylation may be responsible for the short term regulation of striatal AADC activity. The data demonstrate that a phorbol ester can reduce the AADC activity in striatal synaptosomes and this reduction may be mediated by activation of protein kinase C. In conclusion, protein kinase C may play a role in the regulation of striatal AADC. This preliminary result, however, cannot rule out the participation of other protein kinases such as adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase or $Ca^{2+}$/calmodulin-dependent protein kinase, both of which as well as protein kinase C, participate in the regulation of tyrosine hydroxylase (Joh *et al.*, 1978; Vulliet *et al.*, 1984; Raese *et al.*, 1979). Consistent with the present findings, the AADC activity in the mouse striatum and midbrain has been reported to be increased by the intracerebroventricular injection of either forskolin, which activates adenylate cyclase and increases cyclic AMP, or 8-Br-cyclic AMP (Young *et al.*, 1993), suggesting that a cyclic AMP-dependent protein kinase may phosphorylate AADC and increase its activity.

In conclusion, the present *in vitro* study provides evidence that the regulation of striatal AADC occurs in synaptosomes and that it may play a role in the mechanism of the effects of drugs that interact with dopamine receptors. The data also suggest that synaptosomes may provide a useful system to study the effects of drugs on AADC activity.

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# Chloride secretion in response to guanylin in colonic epithelia from normal and transgenic cystic fibrosis mice

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**1** Guanylin, a 15 amino acid endogenous gut peptide, increased the short circuit current (SCC) in the epithelium of the mouse colon, but only when applied to the apical and not the basolateral surface.

**2** By use of selective blockers of epithelial ion transport and modification of the bathing solution, it was concluded that guanylin increased electrogenic chloride secretion but also had a minor effect on electrogenic sodium absorption. In addition there were small residual currents which remained unresolved.

**3** The threshold concentration of guanylin causing a SCC increase was less than 50 nM, but at concentrations 40 times greater no indication of a maximally effective concentration was found.

**4** Two guanylin isomers with the same amino acid sequence but with the disulphide bridges joined in an alternate fashion showed no activity. Thus only guanylin with the greatest structural homology to heat stable enterotoxin (STa) showed biological activity.

**5** The action of guanylin was virtually eliminated in colonic epithelia from transgenic cystic fibrosis (CF) mice. As these animals lack the chloride channel coded by the CF gene sequence, it is likely that the final effector process in murine colonic epithelia involves the CFTR (cystic fibrosis transmembrane conductance regulator) chloride channel.

**6** Opportunistic infections of the gut generating STa lead to diarrhoeal conditions via an action of the toxin on apical guanylin receptors. Thus, as discussed, the CF heterozygote may have a genetic advantage in this circumstance.

**Keywords:** Guanylin; chloride secretion; sodium absorption; epithelia (colonic); cystic fibrosis; heat stable enterotoxin

## Introduction

Guanylin is a recently discovered peptide of 15 amino acids isolated from the rat small intestine (Currie *et al.*, 1992). Since the mRNA for the peptide is most abundant in the colon (Weigand *et al.*, 1992) we decided to examine the effects of the peptide on the epithelium of this organ. The peptide is of interest because of structural homology with heat stable enterotoxin (STa) which is produced by *E. coli* and is responsible for some forms of secretory diarrhoea. The structure of guanylin in relation to the structure of STa is shown in Figure 1. Guanylin can displace radiolabelled STa from binding sites in cultured epithelial cells, two populations of binding sites being detectable (Forte *et al.*, 1993). The peptide causes electrogenic chloride secretion in monolayers of $T_{84}$ epithelial cells and increases guanosine 3':5'-cyclic monophosphate (cyclic GMP) content. STa and presumably guanylin are considered to activate GC-C, a plasma membrane form of guanylyl cyclase (Wong & Garbers, 1992; Li & Goy, 1993). This paper gives details of the effects of guanylin on murine colonic epithelium, this species being chosen so that a comparison could be made of the effects of the peptide on both the normal epithelium and that from transgenic cystic fibrosis (CF) animals. It is known that intestinal epithelia from CF mice fail to show chloride secretion in response to agents which elevate adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Ratcliff *et al.*, 1993). The CF gene codes for a protein, CFTR, which behaves as a small conductance epithelial chloride channel (for a review, see Cuthbert, 1992). It is this channel which fails to function properly in CF so that if chloride secretion is normal in CF tissues exposed to guanylin then the peptide might have some application in treatment. Alternatively if chloride secretion is deficient in CF colons treated with guanylin then it is probable that the final effector mechanism in the secretory process is the CFTR chloride channel. CF heterozygotes (carriers) producing fewer channel molecules may therefore withstand better the opportunistic infections by organisms generating STa. A second objective of this study was to examine the relative activities of the two other possible guanylin isomers which while possessing the same amino acid sequence differed in the way the disulphide bridges are joined.

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## Methods

### *Animals and tissues*

Embryonic stem cells carrying a disruptive mutation in exon 10 of the cftr locus were injected into C57Bl/6 host blastocytsts to derive chimeric animals. Heterozygote $F_1$ animals were intercrossed to generate homozygous CF offspring, the genotypes of the $F_2$ offspring being ascertained by Southern blot analysis (Ratcliff *et al.*, 1993). CF and wild type mice were treated in identical fashion. Mice were killed by exposure to 100% $CO_2$ and the large intestine removed in its entirety and small lengths around 0.5 cm long were cut from the distal colon starting at about 1 cm from the anal end. Maximally four pieces were taken from mice weighing 25–30 g. Each piece was opened longitudinally and the muscle layers dissected away under a microscope. The tissues were mounted in Ussing chambers with a 20 $mm^2$ window using Parafilm washers to cushion the tissue. Transepithelial potentials were monitored by fine polythene tubes filled with Krebs Henseleit solution (KHS) which ended within a mm of the tissue surface. These tubes led, via a 3 M KCl solution and calomel cells to the input stage of a voltage clamp (WPI

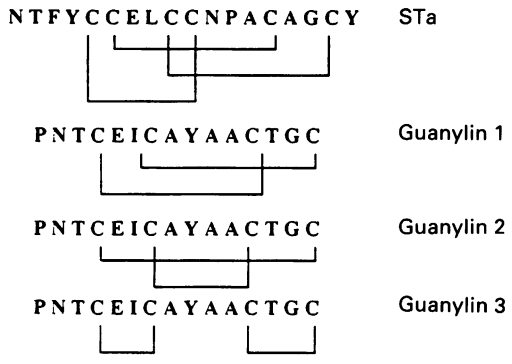


**Figure 1** Structure of STa and three guanylin peptides showing the position of the disulphide bonds. The single letter amino acid code is used to define the peptide sequence.

Dual Voltage Clamp). Current was passed via Ag/AgCl electrodes and gel filled tubes containing 3 M KCl to current ports in the chambers far removed from the tissue. Each side of the tissue was bathed in 20 ml KHS solution maintained at 37°C and gassed with 95% $O_2$/5% $CO_2$.

In a few instances preparations were made from the murine ileal epithelium at its mid region. Exactly the same procedure was used as for the colon.

### *Solutions*

The KHS solution used had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.1. This solution had a pH of 7.4 when gassed with 95% $O_2$/5% $CO_2$ at 37°C. In some experiments modified bathing solutions were used. When chloride was replaced by gluconate the following changes were made: NaCl was replaced with Na gluconate, KCl and $CaCl_2$ with the sulphate salts except a ten fold excess of the calcium salt was used to maintain sufficient ionised calcium in the presence of gluconate (Boron & Boulpaep, 1983). When it was necessary to remove all permeable anions, the same substitutions were made as above except that NaCl and $NaHCO_3$ were replaced with Na isethionate. In this circumstance it was not necessary to add excess calcium. The pH of this solution was controlled with Tris buffer, 10 mM, pH 7.4 and the solution was gassed with pure $O_2$.

### *Preparation of peptides*

The guanylin isomers were prepared by solid phase peptide synthesis using a Novabiochem Crystal continuous flow synthesizer and the Fmoc strategy. Pairs of cysteine residues were orthogonally protected with trityl (Trt) or acetamidomethyl (acm) protecting groups. Cleavage of the assembled sequences from the solid phase with trifluoroacetic acid (TFA) gave the bis-acm dithiol tetra cysteine peptides. Air oxidation of the thiol groups yielded the first disulphide bridge. Oxidation of the bis-acm disulphide with iodine in acetic acid formed the second disulphide bridge. Peptides were purified by h.p.l.c. using a Dynamax $C_{18}$ column in acetronitrile-water containing 0.1% TFA, and were characterized by amino acid analysis and liquid secondary ion mass spectra (LSIMS).

### *Materials*

Drugs were obtained from the following sources: niflumic acid and amiloride from Sigma Chemical Co., Poole, Dorset; frusemide from Hoechst, Hounslow and acetazolamide from Lederle Laboratories, Gosport, Hants. All other chemicals used were of reagent grade.

## Results

### *Characteristics of the responses to guanylin*

Guanylin caused a rapid increase in the SCC of mouse colon epithelium *in vitro* which was maintained as long as the peptide was present (up to 1 h). This action of the peptide was reversible, so that repeated responses could be obtained. In this study we allowed 90 min between consecutive applications of peptide to ensure all the peptide was removed and the epithelium had returned to a steady state. Under these conditions, a 50% reduction in the size of the responses was seen up to 4.5 h, but in some individual tissues no diminution occurred at all.

A series of experiments, all performed with guanylin 1 μM is shown in Figure 2 to illustrate the main characteristics of the responses to the peptide. Addition of the peptide to both surfaces of the epithelium produced no greater response than with apical application alone, while basolateral addition was without effect (Figure 2a). Amiloride, the epithelial sodium channel blocker, produced a minor reduction in SCC when applied during the plateau response to guanylin but produced no effect when added before the peptide. Frusemide, the NaK2Cl cotransport inhibitor, reduced the response to guanylin when added before the peptide and reduced the SCC

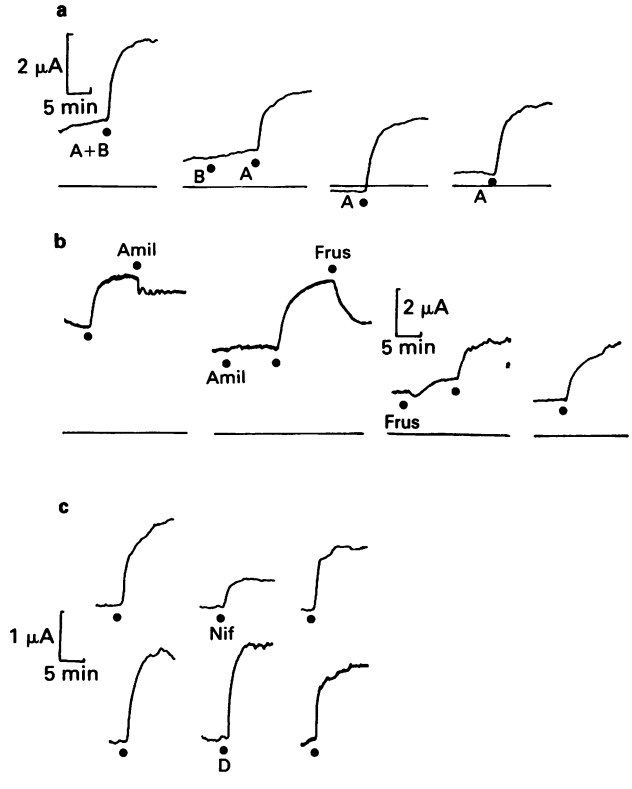


**Figure 2** Effects of guanylin on SCC in mouse colon. At each unlabelled symbol guanylin, 1 μM, was added to the apical bathing solution of a mouse colonic epithelium, area 20 $mm^2$, with an interval of 90 min between each addition with washing between. In (a) and (b) the horizontal lines indicate the value of zero SCC. The mean basal SCC values for the two preparations shown in (c) were 1.9 μA (upper) and 0.5 μA (lower). In (a) the symbols A, B, A + B indicate when guanylin was added to the apical, basolateral or both solutions. In (b) amiloride (Amil), 100 μM, was added to the apical bathing solution at the times indicated. Similarly Frus indicates when frusemide, 1 mM, was applied basolaterally. In (c) the upper and lower sets of records are from two adjacent pieces of epithelium. In the response labelled Nif, niflumic acid 100 μM, was present in the apical bathing solution 15 min before guanylin was added, while D indicates when an equivalent amount of solvent (DMSO) had been added 15 min before the peptide.

when applied during the plateau phase of the response (Figure 2b). However, the response to frusemide was not impressive, a substantial residual current remaining. Finally, the chloride channel blocker, niflumic acid, attenuated the response to guanylin when applied apically, while the solvent for this agent was without effect on the response (Figure 2c). Niflumic acid had a minor effect on basal SCC, but no greater than the effect of an equivalent amount of dimethylsulphoxide (DMSO) in which it was dissolved. Niflumic acid also inhibited the effect of the known chloride secretagogue, lysylbradykinin, on the mouse colon epithelium.

### *Nature of the transported ions(s)*

In two sets of experiments chloride ions were replaced by impermeable anions (gluconate and isethionate) either with or without simultaneous removal of bicarbonate. When chloride was substituted with gluconate in the continued presence of bicarbonate responses to guanylin, 1 μM, were unimpaired. Acetazolamide and amiloride both caused minor reductions of SCC during the plateau response to guanylin. However, amiloride had no effect on the basal current in the absence of guanylin (Figure 3a).

In the second series, both chloride and bicarbonate were replaced by isethionate and the bathing solution buffered to pH 7.4 with Tris. In this situation the responses to guanylin were reduced to about 30% of the control value in chloride containing solution. Nevertheless, there was still a distinct increase in current, which could not have been due to chloride secretion (Figure 3b).

In the four experiments illustrated in Figure 3, amiloride caused inhibition of the SCC indicating that some part, at least, of the response in chloride-free solutions was due to electrogenic sodium transport, since when amiloride was added before guanylin there was no rapid fall in SCC, indicating that sodium transport was not active in the absence of the peptide.

Further experiments were limited by the availability of the peptide but taking the findings of this and the previous section together it is possible to be definitive about the nature of the effects of guanylin on ion transport as discussed later.

### *Concentration-response relationship to guanylin*

The maintenance of the plateau response in the presence of guanylin made it possible to determine a concentration-response relationship by cumulative addition to the apical bathing solution. A partial concentration-response relationship is given in Figure 4 with no indication that the maximal effect had been reached, indeed the curve is steepening at the maximum concentration used (2 μM). Lack of peptide prevented further additions at higher concentrations. The threshold concentration for the SCC effect was less than 50 nM.

### *Response of mouse ileal epithelium*

As guanylin was first isolated from the rat small intestine (Currie *et al.*, 1992) it might be presumed to have some epithelial function at that site. Therefore guanylin, 1 μM, was applied apically, every 90 min to stripped ileal preparations. The results are shown in Figure 5 for three separate ileal preparations subjected to this protocol. Responsiveness declined considerably at the second exposure to the peptide but more importantly the responses to guanylin were not maintained, the SCC often returning to baseline values within a few minutes in the continued presence of the peptide (data not shown). We have not taken any steps in this study to prevent the degradation of the peptide when applied to tissues and it is possible that activity in the ileum may have been enhanced if such precautions had been taken. Nevertheless an endogenously released peptide would not have such protection physiologically, although nothing is yet

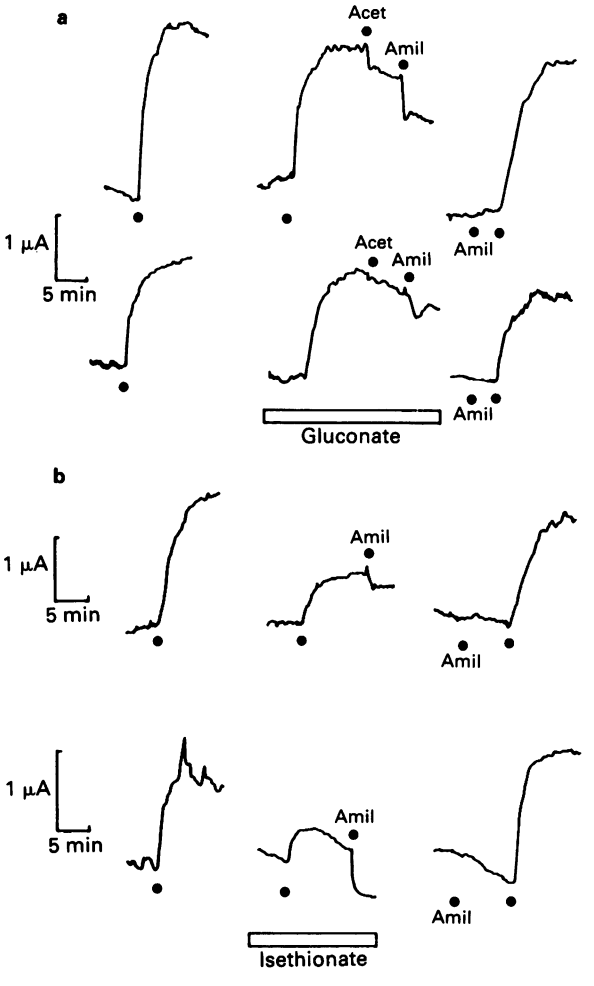


**Figure 3** SCC responses in mouse colon epithelium to guanylin. At each unlabelled symbol guanylin, 1 μM, was added to the apical bathing solution. Responses were repeated at 90 min intervals. In (a) both preparations were from the same mouse while in (b) separate epithelia from two mice were used. During the middle response of each set of three responses the bathing solution was modified. In (a) chloride was replaced with gluconate with bicarbonate still present, while in (b) the chloride and bicarbonate were replaced by isethionate. Acet indicates when acetazolamide, 450 μM, was present in both bathing solutions, while Amil indicates the addition of amiloride, 100 μM, to the apical solution.

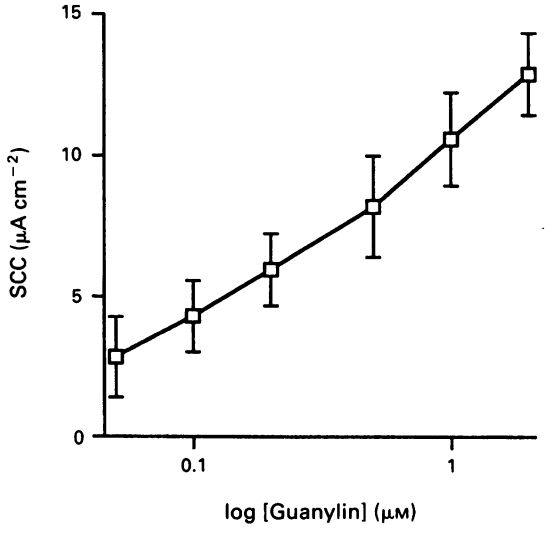


**Figure 4** SCC increases in response to guanylin at different concentrations on murine colonic epithelia. Each point shows the mean value ± s.e.mean for three separate preparations.

known of its metabolism. It is evident that guanylin's activity is less impressive on the murine ileum than the colon.

### *Effects of guanylin in CF mouse colon epithelium*

CF mouse colon epithelium was prepared in the same way as for normal colons and exposed to guanylin, 1 μM, on the apical side every 90 min with washing between each application. The responses of normal epithelia and those from CF transgenic mice were compared and the data are given in Figure 6. They show that there is a highly significant ($P < 0.005$, Student's $t$ test) reduction in the response of CF tissues at all time points. In normal colons the mean value of the control responses was about 50% of its initial value at 4.5 h in this series, although as stated earlier some control tissues show no decrement in the responses to guanylin over 6 h. The residual responses in CF tissues were small making them difficult to investigate systematically. The initial approach was to examine if the residual guanylin response in CF colon was sensitive to amiloride, but this entailed a more detailed study of the mouse colon to amiloride. With a maximally effective concentration (100 μM) not all colons showed a response. In normal colons the amiloride-sensitive SCC was $4.8 \pm 1.2$ μA cm$^{-2}$ (mean ± s.e.mean) (34 observations, 16 animals, 76% responders) whereas in CF colons the values were $3.5 \pm 1.0$ μA cm$^{-2}$ (38 observations, 16 animals, 76% responders). To examine specifically the nature of guanylin/amiloride interactions in CF colons, four preparations were exposed to guanylin every 90 min for 4.5 h with washing in between. For two tissues, amiloride was present when guanylin was added, while in the other two it was present only after the response to guanylin had developed. The first two responses of each tissue are shown in Figure 7. In the top trace amiloride removed SCC equivalent to the whole of the current generated by guanylin, while the bottom trace with an identical format showed no response to amiloride. In the middle traces it is shown that amiloride sometimes affected the basal current whereas in others it had no effect. Thus guanylin can still cause a minor SCC increase even in the presence of amiloride in CF colons (Figure 6).

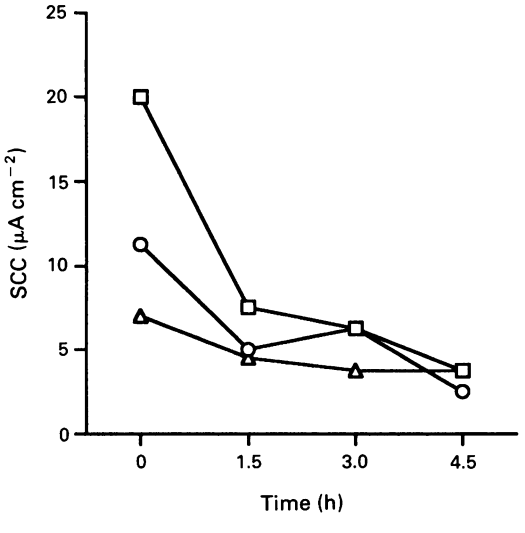


**Figure 5** Illustrates the SCC responses of three separate murine ileal epithelial tissues (each 20 mm$^2$) to guanylin, 1 μM, applied every 90 min with washing between.

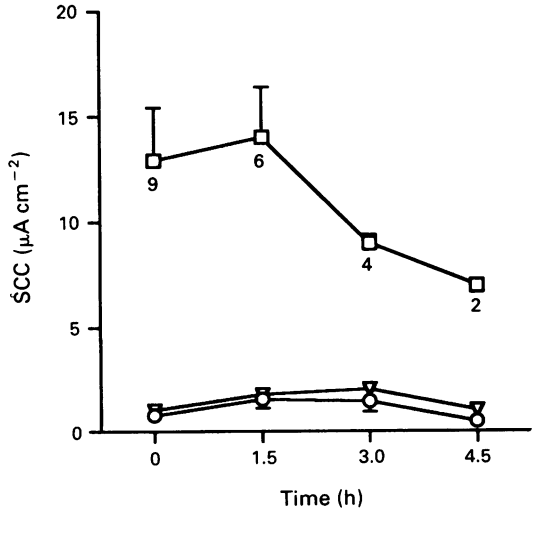


**Figure 6** SCC responses in normal and CF murine colonic epithelia to guanylin, 1 μM, every 90 min with washing between; (□) normal tissues, with mean values ± s.e. and the number of observations for each value shown; (○) mean ± s.e. for CF tissues, each for five observations; (△) mean responses in two CF tissues in the presence of amiloride, 100 μM.

### *Activity of guanylin analogues*

Guanylin isomers in which the amino-acid backbone was conserved but with different disulphide bridges were synthesized (see Figure 1). Throughout the study so far we have used only guanylin 1 (referred to throughout as guanylin), the isomer with the closest analogy to heat stable enterotoxin (STa). In two experiments, the relative activities of guanylin 2 and guanylin 3 were compared to that of guanylin. No activity of guanylin 2 or 3 was detected, either as an agonist or antagonist of the effects of guanylin, given the constraint that limited amounts of material were available.

Figure 8 gives an example of the type of experiment carried out, in this instance with guanylin 3. Paired colonic

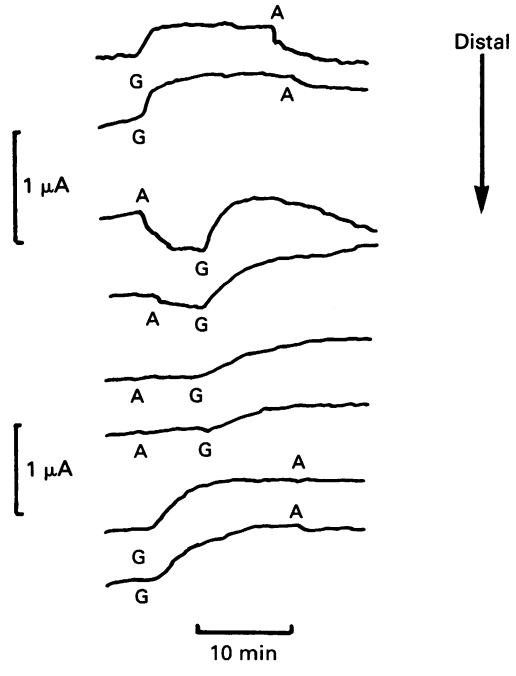


**Figure 7** Responses to guanylin, 1 μM, and amiloride, 100 μM, both applied apically in four CF colonic epithelia all derived from the same mouse and designated by G and A respectively. Each pair of responses was obtained consecutively 90 min apart. The most distal tissue is represented by the upper pair of traces, the other three pieces were adjacent and consecutively more proximal. Note in some tissues amiloride was given before guanylin and in others afterwards and that the calibration for the lower two tissues is different from the upper pair.

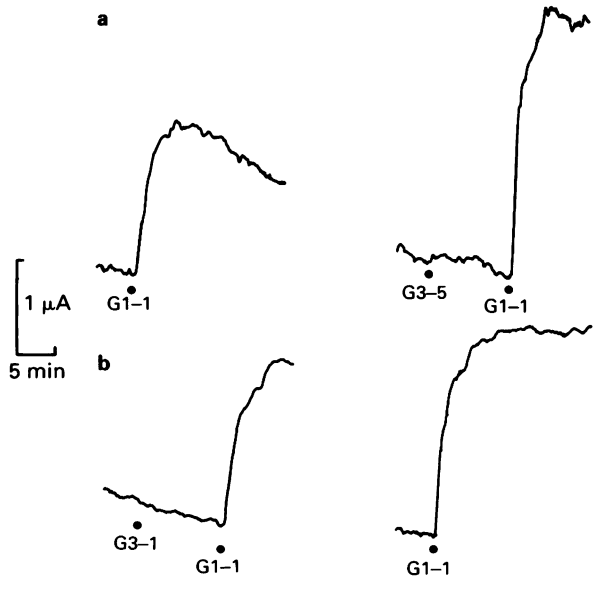


**Figure 8** SCC responses to two guanylin analogues, guanylin (G1) and G3. The concentrations applied to the apical surface of colonic epithelia, in μM, are indicated by the symbol for the peptide used. Two adjacent pieces of epithelia were used. In one (a) G1 was applied first and then, 90 min later, G3 followed by G1. In the other (b) the tissue was exposed to G3 then G1 and 90 min later to G1. Note G3 neither affected SCC alone nor inhibited the action of G1.

preparations from adjacent sections of the gut were used in a crossover test. One preparation received guanylin and the other guanylin 3, then to the preparation that received guanylin 3 the active peptide was added 10 min later in the continued presence of the inactive peptide. After washing the tissues the protocol was repeated 90 min later but changing the tissue which was exposed first to the inactive peptide. It is apparent that guanylin 3 has no effect on SCC at a concentration of 5 μM, neither does it markedly affect the action of guanylin at this concentration. Similarly with guanylin 2 (1 μM) there was no indication that this peptide had either agonist or antagonist activity at the level of the guanylin receptor(s). While it was not possible to use significantly higher concentrations of guanylins 2 or 3, any action they might have is insignificant when given that the threshold for the guanylin effects is $< 50$ nM.

## Discussion

The direction of the SCC responses to guanylin, the partial inhibition of the responses by frusemide and niflumic acid, indicating the involvement of the NaK2Cl-transporter and chloride channels respectively, suggest that the peptide increases chloride secretion in the mouse colon. The hypothesis confirms an expectation by analogy with the well known anion-secretory activity of heat stable enterotoxin (STa) (Field *et al.*, 1978) with which guanylin has a partial structural homology. STa, generated *in vivo* by opportunistic bacterial infections of the gut, acts only from the apical side of the epithelium, as does guanylin. Furthermore, $T_{84}$ monolayers (a human tumour-derived cell line with the characteristics of colonic epithelial crypt cells) also show chloride secretion with guanylin but only when applied apically (Forte *et al.*, 1993). The mouse colon is distinctly more complex than $T_{84}$ monolayers because in addition to anion secretory crypt cells the surface cells are presumed to be absorptive, by analogy with other species.

The mouse colon appears to have intermediate properties to that of the rabbit and the rat. The former always shows an amiloride sensitive SCC while the latter only demonstrates this after treatment with mineralocorticoids (Will *et al.*, 1981). Five examples are given (Figures 2 and 3) showing an effect of amiloride during the plateau response to guanylin, yet in each example the sodium transport blocker had no effect in the absence of the peptide, suggesting that the latter itself stimulated electrogenic sodium absorption. As with normal colons, only some CF preparations showed amiloride-sensitivity in the basal state. In the presence of amiloride, guanylin was still able to elicit a small SCC increase of a similar size to that seen in its absence. The small size of these currents make investigation difficult and furthermore, amiloride addition may polarize the epithelium in a way which alters the ionic gradient for other small, unresolved currents. In summary the evidence is that guanylin stimulates sodium absorption as well as chloride secretion in normal mouse colon but the evidence that the former is still present in CF colon is equivocal.

In the absence of chloride, but with bicarbonate present, the response to guanylin was not modified. Acetazolamide produced only a minor reduction in SCC in this situation, perhaps not an unexpected result since the solutions were gassed with 5% $CO_2$ and the hydration of $CO_2$ within the cells probably remained high. In some species (rabbit) the colonic epithelium can secrete bicarbonate, especially in the absence of chloride (Grasl & Turnheim, 1984) and it seems probable that the same is true for the mouse. Bicarbonate ions may be secreted through chloride channels or residual intracellular chloride may be recycled across the apical membrane in parallel with a chloride bicarbonate exchanger. When both chloride and bicarbonate were replaced by isethionate the response to guanylin was reduced to around 30% of the control values. Part of the residual response seems to be due to sodium absorption, but in some individual tissues there remained a significant current which is unexplained. It is notable that frusemide never gave complete inhibition of the guanylin SCC response, while it did so in $T_{84}$ monolayers (Forte *et al.*, 1993) again indicating the presence of some unresolved current(s). This is what might be expected since $T_{84}$ monolayers consist exclusively of crypt cells.

The location of guanylin receptors in gut epithelium is unknown but if they occur in both crypt and surface cells it may be that the chloride secretory effect arises in the latter, while the effect on sodium transport is mediated by an action on surface epithelial cells. In the rat GC-C mRNA is expressed in the crypt epithelium but to a lesser exent in surface epithelial cells, while guanylin mRNA is expressed strongly in surface cells and in the upper 20% of the crypts (Li & Goy, 1993). Nevertheless taking all the present evidence together it indicates that guanylin causes chloride secretion in the mouse colon and that under some conditions bicarbonate may be secreted in place of chloride. Also there is strong evidence that guanylin can cause electrogenic sodium absorption but these two transporting activities do not necessarily arise from stimulation of the same epithelial cells.

The inability of guanylins 2 and 3 to produce any discernible effect on SCC suggests that the restriction of the peptide conformation by the disulphide bridges as in guanylin and STa are crucial for biological activity.

Of particular interest is the finding that the action of guanylin is virtually eliminated in the colonic epithelium from CF transgenic mice. This indicates that agents acting via the guanylin receptor to increase cyclic GMP levels are not likely to be useful agents in the treatment of CF. However it is known that cGK (cyclic GMP-dependent protein kinase) can phosphorylate CFTR, the protein coded for by the CF gene, *in vitro*. Yet cyclic GMP is unable to increase chloride conductance in human cultured airway epithelium (Berger *et al.*, 1993). CFTR contains seven serines which can be phosphorylated but it is not necessary that all of these are modified in order to activate the chloride channel (Cheng *et al.*, 1991) so cGK may phosphorylate only non crucial sites.

There has a been a report that cyclic GMP stimulates chloride secretion in normal but not CF human intestinal epithelia but also that the intestine contains a different isoform of cGK, which may be capable of activating CFTR (de Jonge *et al.*, 1989; O'Loughlin *et al.*, 1991). CFTR not only acts as a chloride channel but its expression in cells has a permissive effect on other channels, for example the ORDIC (outwardly rectifying depolarization induced channel) channels fail to be activated by protein kinase A (PKA) in the absence of CFTR (Egan *et al.*, 1992). Thus there are three possible explanations of the failure of guanylin to activate chloride secretion in murine CF colon.

The first, and most likely, is the absence of CFTR which is normally activated by an intestinal form of cGK. Alternatively it is possible that cyclic GMP is able to activate PKA (Forte *et al.*, 1992) which in a native tissue would activate CFTR. Finally, but highly improbable, is the possibility that guanylin activates a non-CFTR channel which needs CFTR permissively to be activated.

There has been a great deal of speculation as to why the CF gene has not been eliminated by natural selection and the conclusion that there is a heterozygote advantage has been advanced (Hansson, 1988; Field & Semrad, 1993). In CF the CFTR chloride channel fails to be activated by cyclic AMP so that in diseases such as cholera, where the toxin activates adenylate cyclase, heterozygotes may show a lesser diarrhoeal response because less CFTR is expressed. A similar argument is applicable to the STa toxin which is considered to act upon apically located guanylin receptors. It should now be possible to test this hypothesis experimentally by comparing responses in wild type homozygotes with those of heterozygotes.

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# Electrophysiological effects of S 16257, a novel sino-atrial node modulator, on rabbit and guinea-pig cardiac preparations: comparison with UL-FS 49

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**1** S 16257 is a new bradycardic agent. Its electropharmacological profile has been compared to that of the known bradycardic compound UL-FS 49 (Zatebradine). Intracellular recordings of action potentials (APs) were performed with conventional glass microelectrodes.

**2** In the rabbit isolated sino-atrial node (SAN) tissue, S 16257 and UL-FS 49 (1 μM, 3 μM and 10 μM) were equipotent in slowing spontaneous APs firing predominantly by decreasing the rate of diastolic depolarization (at 3 μM, $-23.8 \pm 3.9\%$ and $-27.9 \pm 2.6\%$, respectively). For the two compounds a maximal effect was obtained at 3 μM. In these preparations, action potential duration at 50% of total repolarization ($APD_{50}$) was more affected by UL-FS 49 than S 16257 at any concentration tested (at 3 μM, $+8.9 \pm 2.9\%$ and $+29.1 \pm 3.7\%$ for S 16257 and UL-FS 49, respectively; $P \leqslant 0.01$).

**3** To estimate the direct effects on AP duration, driven cardiac preparations were exposed to these agents. In guinea-pig papillary muscles, paced at a frequency of 1 Hz, increasing concentrations of S 16257 or UL-FS 49 (0.1 to 10 μM, 30 min exposure for each concentration) slightly prolonged AP repolarization. This prolongation was more marked for UL-FS 49 (at 1 μM, $+6.1 \pm 0.6\%$ and $+11.2 \pm 1.3\%$ elevation of $APD_{50}$, for S 16257 and UL-FS 49, respectively).

**4** Application of UL-FS 49 (3 μM) to rabbit Purkinje fibres, triggered at a frequency of 0.25 Hz, induced a marked prolongation of $APD_{50}$ and $APD_{90}$ ($+149.4 \pm 51.2\%$ and $+86.0 \pm 15.4\%$, respectively). S 16257 (3 μM) induced only a weak prolongation of AP ($+14.1 \pm 5.0\%$ and $+14.8 \pm 3.3\%$ for $APD_{50}$ and $APD_{90}$, respectively) significantly smaller than in the case of UL-FS 49.

**5** These results show that S 16257 slows the rate of spontaneous AP firing in isolated SAN mainly by a reduction of the diastolic depolarization of the cells, which suggests an inhibition of the pace-maker current ($I_f$). S 16257 and UL-FS 49 are equipotent in their bradycardic effect but S 16257 is more specific as it induces less increase in myocardial repolarization time.

**Keywords:** S 16257; UL-FS 49; specific bradycardic agent; action potential; sino-atrial node; Purkinje fibres; papillary muscles

## Introduction

Myocardial ischaemia, whatever may be its clinical expression, always results from an imbalance between oxygen supply and demand. This imbalance may lead to irreversible myocardial damage. As heart rate is one of the major determinants of myocardial oxygen consumption (Laurent *et al.*, 1956; Sonnenblick *et al.*, 1968), agents able to reduce sinus heart rate are of major interest for the treatment of ischaemic heart diseases. This can be achieved with β-adrenoceptor antagonists or some calcium channel blockers; however, these agents may exert concomitant negative inotropic and hypotensive effects (Opie, 1989; Kern *et al.*, 1989), potentially deleterious during ischaemia. Recently the pharmacological properties of a novel class of substances, specific bradycardic agents (SBAs), have been described (Kobinger & Lillie, 1987). Such agents induce sinus bradycardia at concentrations that are devoid of additional haemodynamic effects (Krumpl *et al.*, 1986; 1988; Franke *et al.*, 1987; Raberger *et al.*, 1987a; Van Woerkens *et al.*, 1992). SBAs have been shown to act by reducing the oxygen demand of the heart and by increasing the diastolic period which induces an elevation of the subendocardial blood flow (Harron *et al.*, 1982; Raberger *et al.*, 1987b; Indolfi *et al.*, 1989). One of the most potent SBAs described so far, UL-FS 49 (Zatebradine), has been reported to slow the action potentials (APs) firing of the pacemaker cells, via an inhibition of the hyperpolarization activated $I_f$ current (Van Bogaert & Gothals, 1987; 1992; Van Bogaert *et al.*, 1990).

In the present paper, we describe the electropharmacological profile, in isolated cardiac preparations, of a new sinus node inhibitor, S 16257 (7,8-dimethoxy 3-{3-{[(1*S*)-(4,5-dimethoxybenzocyclobutan-1-yl) methyl] methylamino}propyl} 1,3,4, 5-tetrahydro-2*H*-benzazepin 2-one). The effects of S 16257 are compared to those of UL-FS 49, on sinoatrial node cells, papillary muscles and Purkinje fibres.

## Methods

### *Isolated cardiac preparations*

Governmental and institutional guidelines for the care and the use of animals were followed at all times.

Male New Zealand White rabbits and Hartley guinea-pigs were stunned by a blow on the head. After exanguination, hearts were rapidly removed and placed in an oxygenated Tyrode solution at 4°C. The cardiac preparations were excised from the right ventricle (papillary muscle or Purkinje fibre) or right atrium (sinus node tissue) and mounted in a tissue bath (3.5 ml). The isolated preparations were superfused with oxygenated Tyrode solution at a constant flow rate (5 ml $min^{-1}$). The temperature was kept at 36°C ± 0.5.

For guinea-pig papillary muscle, the mural end of the preparation was pinned to the base of the experimental chamber and the tendinous end was connected to a Gould UC2 force transducer via a fine silk thread to record the isometric tension. The muscles were carefully stretched until the peak of the length-tension relationship was reached. Rab-

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bit sino-atrial node tissue and Purkinje fibre were carefully pinned to the base of the experimental chamber.

### *Electrical recordings*

The papillary muscles and the Purkinje fibres were stimulated at a basal frequency of 1 Hz with rectangular pulses of 2 ms duration, twice threshold intensity, applied through two platinum electrodes. The stimuli were delivered to the stimulating electrodes from a Grass S88 stimulator through a Digitimer Ds2 stimulus isolation unit. All the preparations were equilibrated with the solution for at least 2 h before starting the study.

Transmembrane potentials were recorded with conventional glass microelectrodes filled with 3 M KCl (tip resistance of 18–22 MΩ) and connected to a high input impedance Biologic VF180A amplifier. The membrane potential was displayed on a Tektronix 2230 digital storage oscilloscope. Membrane potential and isometric force (for papillary muscles) were continuously recorded on a Gould RS3400 pen chart recorder. Storage and analysis of both signals were also performed with specific software (Clovis, CLOD Sarl), installed on a personal computer, equipped with a 12 bit analog-digital DAS50 converter. All experimental results were obtained from continuous impalements of single cells throughout the whole experiment.

### *Experimental protocol*

Each sino-atrial node preparation was exposed to S 16257 or UL-FS 49 at one fixed concentration, for 40 min. Then a 60 min wash out of the drug was performed. The concentrations tested for both substances were 1 μM, 3 μM and 10 μM.

Papillary muscles were exposed to cumulative concentrations of S 16257 or UL-FS 49 in the following sequence: 0.1 μM, 0.3 μM, 1 μM, 3 μM and 10 μM, 30 min exposure for each concentration.

After reducing the rate of pacing from 1 Hz to 0.25 Hz, Purkinje fibres were exposed to S 16257 or UL-FS 49, at 3 μM, for 40 min. Then a wash-out of the drug was performed (minimum of 1 h), but in some preparations no reversibility was obtained, even after 2 h of wash-out. Then the preparations were exposed to S 16257 or UL-FS 49 at 10 μM for 40 min.

### *Drugs and solutions*

The control Tyrode solution contained the following (in mM): NaCl 130, KCl 5.6, $CaCl_2$ 2.15 and 1.8 for guinea-pig and rabbit preparations, respectively, $NaH_2PO_4$ 0.6, $NaHCO_3$ 20, $MgCl_2$ 1.1 and glucose 11. The pH of the Tyrode solution was 7.4 after bubbling with $O_2/CO_2$ (95:5, v/v).

UL-FS 49 (7,8-dimethoxy 3-{3-{[2-(3,4-dimethoxyphenyl)-ethyl]methylamino}-propyl}1,3,4,5-tetrahydro-2*H*-benzazepin 2-one dihydrochloride) and S 16257 (7,8-dimethoxy 3-{3-{[(1*S*)-(4, 5-dimethoxybenzocyclobutan-1-yl) methyl] methylamino} propyl}1,3,4,5-tetrahydro-2*H*-benzazepin 2-one hydrochloride) were synthesized in the Institute. Both molecules as a powder were initially dissolved in distilled water (0.1 mM). Further dilutions were carried out in Tyrode solution.

### *Statistical analysis*

Values are expressed as means ± s.e.mean. Statistical significance was evaluated by a two-way analysis of variance with repeated measures. One way complementary analysis followed by a Newman-Keuls test was performed at fixed times of superfusion (or at fixed concentrations for papillary muscles). Differences were considered significant for $P \leqslant 0.05$. Five to 11 experiments were performed for each group.

## Results

### *Bradycardic effects of S 16257 and UL-FS 49 on rabbit SAN preparations*

Superfusion of rabbit isolated sino atrial node preparations with S 16257 induced a reduction of spontaneous action potentials (APs) firing. A maximal bradycardic effect was obtained after a 40 min exposure to 3 μM, for both agents (− 23.8 ± 3.9% and − 27.9 ± 2.6%, for S 16257 and UL-FS 49, respectively). As shown in Figure 1, during application of S 16257, the reduction of APs firing was of same order of magnitude as that induced by UL-FS 49, at any concentration tested (for example, − 12.3 ± 5.2% and − 8.6 ± 1.3% after a 40 min exposure to 1 μM S 16257 and UL-FS 49, respectively).

### *Reduction of the diastolic depolarization rate by S 16257 and UL-FS 49, in rabbit SAN preparations*

As shown in Table 1 and Figure 2, the bradycardic effect of S 16257 was predominantly mediated by a reduction in the rate of diastolic depolarization, thus increasing the cycle

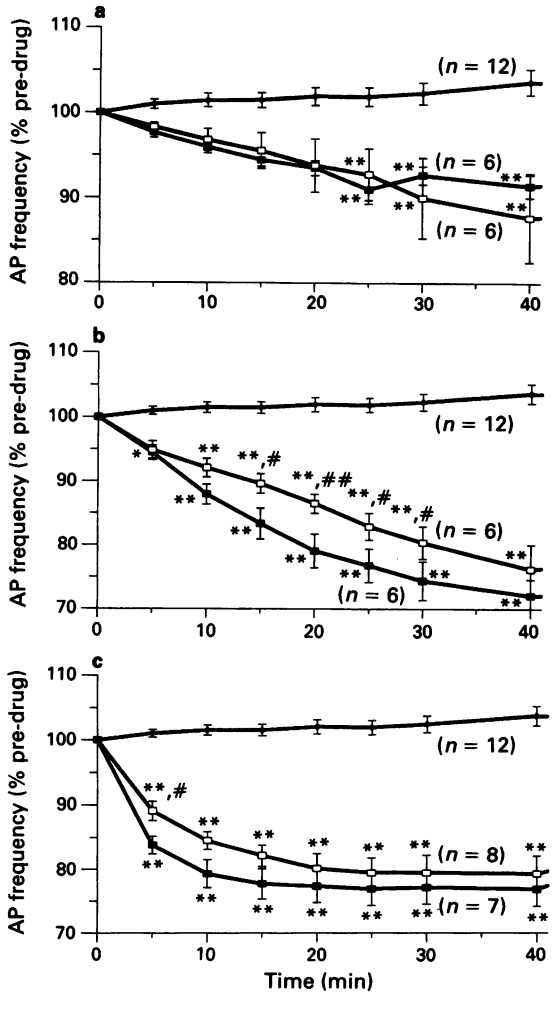


**Figure 1** Reduction of spontaneous action potentials (APs) firing in rabbit sino-atrial node during a 40 min exposure to S 16257 (□) or UL-FS 49 (■). Bradycardic agents were applied at 1 μM (a), 3 μM (b) and 10 μM (c). Data are expressed as means ± s.e.means for *n* preparations. Values are expressed as a percentage of the initial rate just before application of the drugs. (◆) Drug-free experiments (same control group for a, b and c). $^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.01$: significance of differences from drug-free superfusate. $^{\#}P \leqslant 0.05$; $^{\#\#}P \leqslant 0.01$: significance of differences between the two treated-groups.

**Table 1** Comparative effects of S 16257 and UL-FS 49 on action potential parameters of rabbit pace-maker cells

| | *DDR* (mV s$^{-1}$) | | *MDP* (− mV) | | *Thr Pot* (− mV) | |
|---|---|---|---|---|---|---|
| | Pre-drug | Δ 40 min | Pre-drug | Δ 40 min | Pre-drug | Δ 40 min |
| *Drug-free* | | | | | | |
| ($n = 12$) | 54.5 ± 8.6 | 0.3 ± 0.8 | 70.3 ± 1.2 | − 0.3 ± 1.6 | 60.2 ± 1.9 | 0.1 ± 1.4 |
| *S 16257* | | | | | | |
| 1 μM ($n = 6$) | 67.9 ± 9.1 | − 21.2 ± 3.6** | 68.0 ± 2.7 | − 1.3 ± 4.0 | 55.3 ± 3.5 | 0.8 ± 4.0 |
| 3 μM ($n = 6$) | 53.8 ± 5.0 | − 36.1 ± 1.8** | 71.5 ± 2.6 | 1.0 ± 1.1 | 60.2 ± 3.5 | 6.5 ± 2.1* |
| 10 μM ($n = 8$) | 83.0 ± 11.0 | − 42.7 ± 5.1** | 66.3 ± 1.4 | − 1.5 ± 1.6 | 57.0 ± 1.3 | 1.6 ± 1.8 |
| *UL-FS 49* | | | | | | |
| 1 μM ($n = 6$) | 76.0 ± 11.6 | − 32.1 ± 3.9**†† | 70.8 ± 1.1 | 0.8 ± 2.3 | 56.2 ± 3.8 | 4.2 ± 3.3 |
| 3 μM ($n = 6$) | 72.2 ± 15.9 | − 46.5 ± 7.3**† | 69.3 ± 2.2 | 1.0 ± 2.3 | 55.7 ± 2.4 | 7.5 ± 1.4* |
| 10 μM ($n = 7$) | 78.0 ± 13.5 | − 10.7 ± 12.7†† | 67.4 ± 2.0 | − 5.0 ± 1.5 | 57.7 ± 2.2 | − 3.1 ± 2.3 |

Bradycardic agents were applied at 1 μM, 3 μM or 10 μM, for 40 min. Data are expressed as means ± s.e.mean for $n$ preparations. DDR: diastolic depolarization rate; MDP: maximal diastolic potential; Thr Pot: threshold potential.
*$P<0.05$; **$P<0.01$: significance of differences from drug-free superfusate.
†$P<0.05$; ††$P<0.01$: significance of differences between the two treated groups.

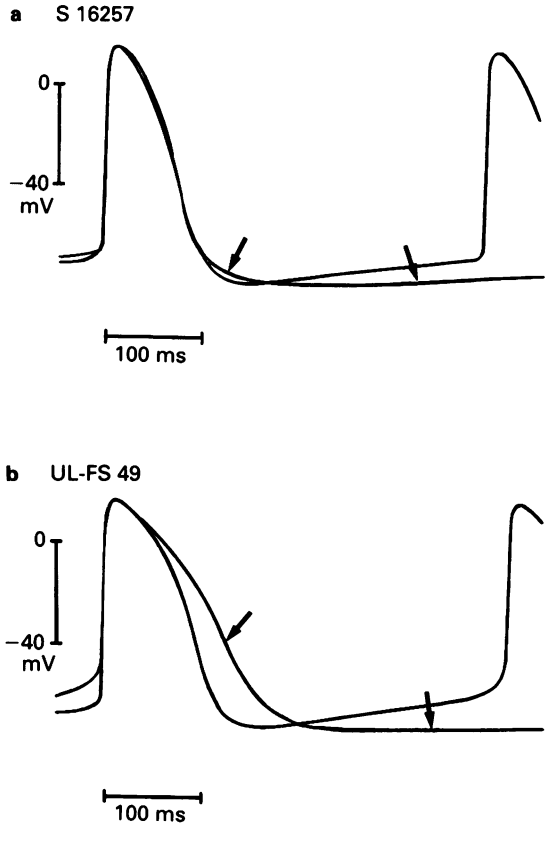


**Figure 2** Representative recordings of spontaneous action potentials (APs) during a 40 min exposure of rabbit sinus node preparations to S 16257 (a) and UL-FS 49 (b) at 3 μM. Arrows show APs after the 40 min exposure to bradycardic agents. The other traces are the respective baseline APs before drug application.

length of the beating preparations. The slope of the diastolic depolarization phase was reduced from 67.9 ± 9.1 to 46.7 ± 5.6 mV s$^{-1}$ and from 76.0 ± 11.6 to 43.9 ± 11.2 mV s$^{-1}$ for S 16257 (1 μM) and UL-FS 49 (1 μM), respectively. At the higher concentration, an increase of diastolic depolarization rate (DDR) was observed with UL-FS 49. This reduction in DDR occurred without any significant change in maximal diastolic potential. Threshold potential was slightly modified, as shown in Table 1: 6.5 ± 2.1 mV and 7.5 ± 1.4 mV more negative after a 40 min exposure to S 16257 (3 μM) and UL-FS 49 (3 μM), respectively.

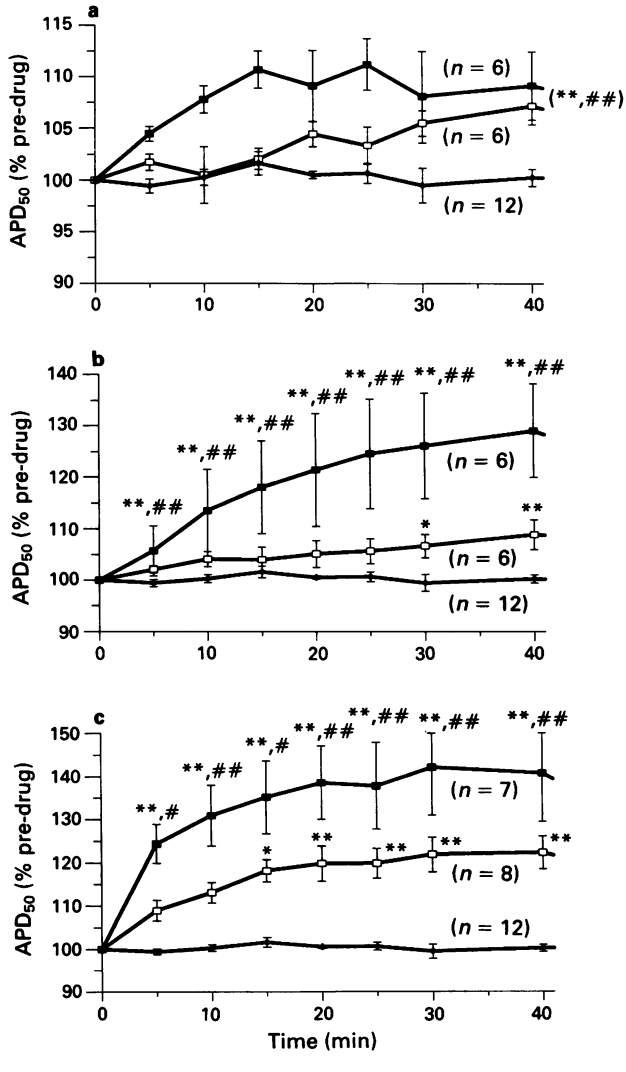


**Figure 3** Prolongation of spontaneous action potential duration at 50% of repolarization ($APD_{50}$) in rabbit sino-atrial node, during a 40 min exposure to S 16257 (□) or UL-FS 49 (■). Bradycardic agents were applied at 1 μM (a), 3 μM (b) and 10 μM (c). Data are expressed as mean ± s.e.mean for $n$ preparations. Data are expressed as the percentage of the initial value just before application of the drugs. (◆) Drug-free experiments (same control group for a, b and c). (**,**)$P \leqslant 0.01$: significance of differences from drug-free superfusate profile and between the two treated-groups profile. *$P \leqslant 0.05$; **$P \leqslant 0.01$: significance of differences from drug-free superfusate at fixed times. *$P \leqslant 0.05$; **$P \leqslant 0.01$: significance of differences between the two treated-groups at fixed times.

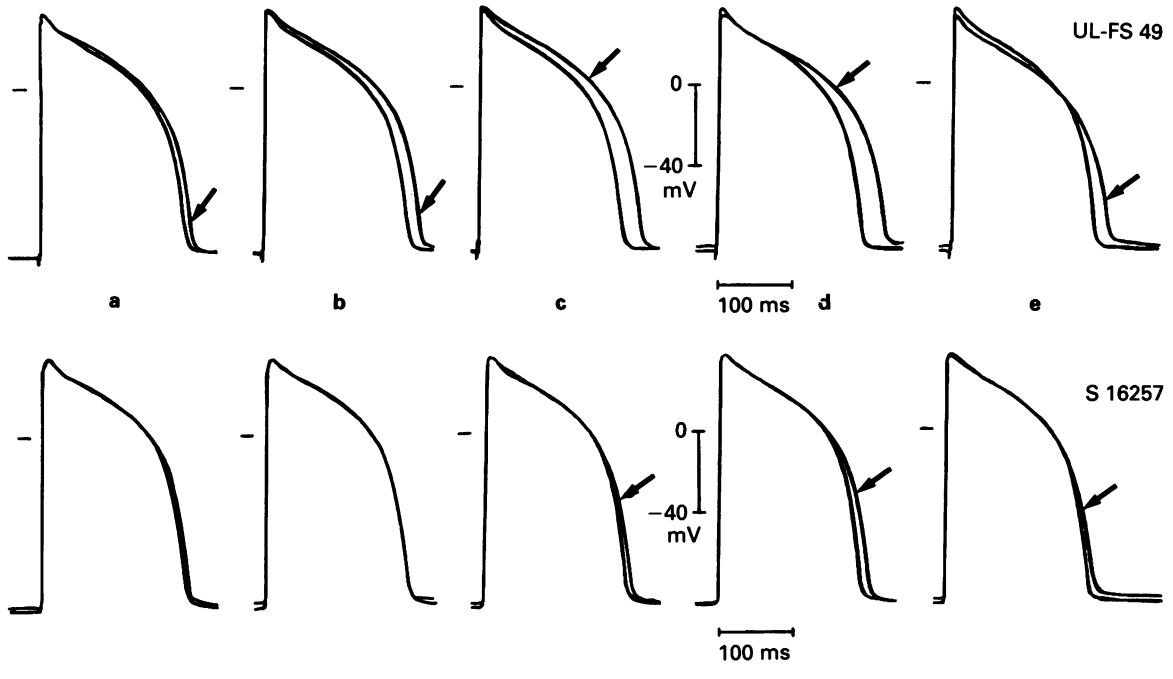


**Figure 4** Comparative effects of S 16257 and UL-FS 49 on action potentials (APs) of guinea-pig papillary muscles. Concentrations of bradycardic agents were applied cumulatively (from 0.1 μM to 10 μM, 30 min exposure for each concentration). Representative recordings of APs after a 30 min exposure to UL-FS 49 (top panels) and S 16257 (bottom panels) at 0.1 μM (a), 0.3 μM (b), 1 μM (c), 3 μM (d) and 10 μM (e). Arrows show APs after the 30 min exposure to bradycardic agents. The other traces are the respective baseline APs before drug application.

### *Prolongation of AP repolarization by S 16257 and UL-FS 49, in rabbit SAN preparations*

As shown in Figures 2 and 3, AP duration measured at 50% repolarization ($APD_{50}$) was augmented dose-dependently during application of the bradycardic agents. This AP prolongation was more pronounced with UL-FS 49 at any concentration tested. For example, during superfusion with 3 μM S 16257 and UL-FS 49, increases of $APD_{50}$ of 8.9 ± 2.9% and 29.1 ± 3.7% respectively, were observed.

### *Prolongation of AP duration by S 16257 and UL-FS 49, in guinea-pig papillary muscles*

To eliminate the contribution of changes in frequency in AP prolongation, increasing concentrations of S 16257 and UL-FS 49 were applied to driven guinea-pig papillary muscles (from 0.1 to 10 μM, 30 min exposure for each concentration). During the application of these compounds, no modification of AP amplitude, resting potential and $dV/dt_{max}$, in comparison with drug-free experiments, were noted (control values for drug-free experiments: 122.4 ± 0.8 mV, − 84.1 ± 0.3 mV and 214.2 ± 10.1 V $s^{-1}$, for the three parameters, respectively). All AP duration parameters were increased significantly. As illustrated in Figure 4, this augmentation of the repolarization time was maximal during exposure to 3 μM (elevations of $APD_{90}$ of + 9.0 ± 0.9% and + 13.0 ± 1.1% for S 16257 and UL-FS 49, respectively). From 0.3 to 3 μM, this effect was more pronounced during UL-FS 49 exposure ($P \leqslant 0.01$). In control conditions, during hours of perifusion a run down of the isometric tension was observed (from 187.7 ± 27.0 mg to 128.4 ± 20.0 mg after 150 min perifusion). This reduction was less pronounced during application of UL-FS 49 (0.3 and 1 μM) and 1 μM S 16257 (no difference between the two treated groups).

### *Prolongation of AP duration by S 16257 and UL-FS 49, in rabbit Purkinje fibres*

Rabbit Purkinje fibres, paced at a low rate (0.25 Hz), were exposed to 3 μM S 16257 or UL-FS 49 solutions, for closer examination of the effect of the bradycardic agents on AP duration. As shown in Figure 5, $APD_{50}$ and $APD_{90}$ of these preparations were markedly augmented by UL-FS 49 (3 μM). S 16257 had only a weak effect on AP repolarization time. The time needed for recovery when the drug was washed out was related to the effect on APD observed during application. In some preparations exposed to UL-FS 49, no reversibility was observed, even after 2 h of wash out. Application of an increased concentration (10 μM), after wash out of the drug (3 μM), induced a more marked prolongation of AP, as shown in Figure 6.

## Discussion

The present paper describes the electropharmacological profile of a new specific bradycardic agent, S 16257. The results show that S 16257 reduced the spontaneous action potentials (APs) firing of the pacemaker cells to the same extent as the well known SBA, UL-FS 49. There are four ways of reducing the heart sinus rate: (a) by prolonging AP repolarization time; (b) by reducing maximal diastolic potential (more electronegative potential); (c) by shifting the threshold potential to a more positive level; and (d) by slowing the rate of diastolic depolarization. Experiments performed in rabbit sinoatrial node (SAN) preparations showed that S 16257, as UL-FS 49, acted mainly by reducing the slope of diastolic depolarization, without significant change in maximal diastolic potential. Although the threshold potential was slightly modified by both drugs (potential more electronegative in some preparations), this variation could not account for the

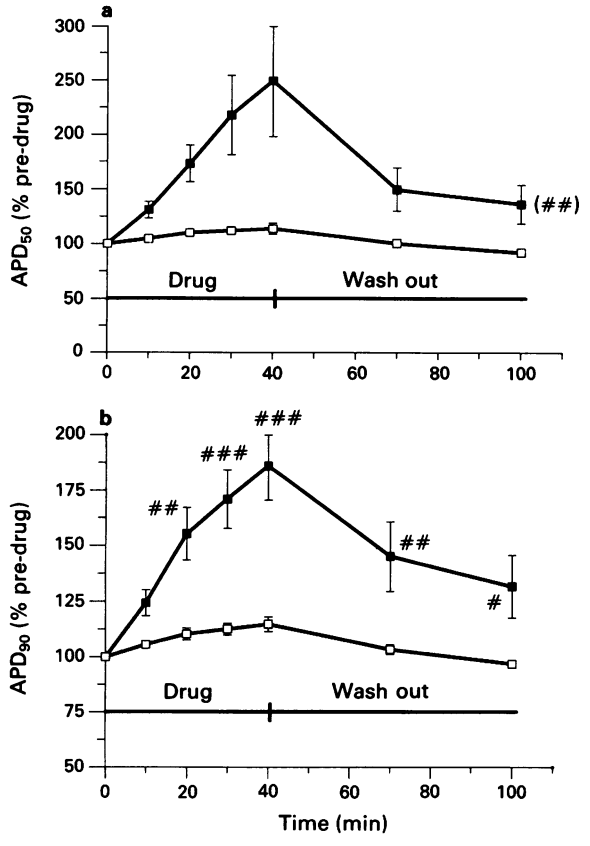


**Figure 5** Prolongation of action potential duration at 50% (a) and 90% (b) of repolarization ($APD_{50}$ and $APD_{90}$, respectively) in rabbit Purkinje fibres, during a 40 min exposure to S 16257 (□) or UL-FS 49 (■). Bradycardic agents were applied at 3 μM. Data are expressed as mean ± s.e.mean for *n* preparations. Data are expressed as the percentage of the initial value just before application of the drugs. (**)$P \leq 0.01$: significance of differences between the two treated-groups profiles. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$: significance of differences between the two treated-groups at fixed times.

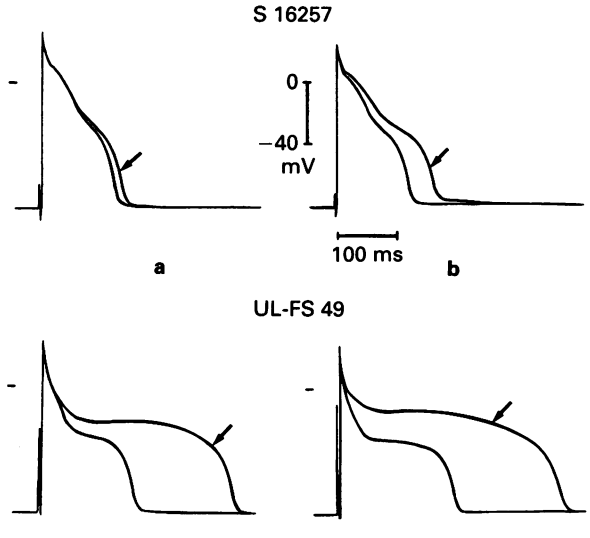


**Figure 6** Comparative effects of S 16257 and UL-FS 49 on action potentials (APs) of rabbit Purkinje fibres. Representative recordings of APs after a 40 min exposure to S 16257 (top panels) and UL-FS 49 (bottom panels) at 3 μM (a, left panels) and at 10 μM (b, right panels), in two preparations. Arrows show APs after the 40 min exposure to bradycardic agents. The other traces are the respective baseline APs just before drug application (control and wash-out of 3 μM for (a) and (b), respectively).

bradycardic effect because it did not move in the way of reducing spontaneous APs frequency. Furthermore, this phenomenon could limit bradycardia to physiological values (about 25–30% reduction). However, we must point out that the threshold potential was not easy to determine, since in some cells the AP onset lacked a precise inflection point between diastolic depolarization and upstroke of the AP, and was characterized by a progressive acceleration of the rate of depolarization. The observed modification in threshold may be the result of a change in the way that recorded cells were driven by the other nodal cells. The bradycardic action obtained with UL-FS 49 on rabbit SAN preparations agreed with the results of experiments performed in other laboratories (Kobinger & Lillie, 1984; Lillie & Kobinger, 1986; Doerr & Trautwein, 1990).

S 16257, in rabbit SAN preparations, had less effect than UL-FS 49 on AP repolarization at any concentration tested, despite a quite similar bradycardic action. The marked increase in AP duration during application of UL-FS 49 could contribute to some extent to the bradycardia induced by this agent.

Because the duration of the AP in cardiac cells is very sensitive to the rate, the increase in $APD_{50}$ during exposure to UL-FS 49, could be the consequence of the increase in cycle length, although this AP prolongation was very limited with S 16257, for a similar bradycardia. Therefore, we exposed driven guinea-pig papillary muscles to these agents with the aim of evaluating their direct effects on cardiac AP repolarization. These preparations are not the most sensitive for AP prolongation but are the most well known cardiac preparations. In these cardiac muscles, S 16257 and UL-FS 49 caused a concentration-dependent increase in AP duration from 0.1 μM to 1 μM. We found significantly more pronounced effect with UL-FS 49, at any concentration tested.

The cardiac AP duration may be enhanced by an increase of inward currents, e.g. $Ca^{2+}$ current, and/or a decrease of outward currents, e.g. $K^+$ current. Among ionic currents implicated in AP repolarization, the potassium delayed rectifier current ($I_K$) is one of the major determinants of AP duration (Anumonwo *et al.*, 1991). Therefore a moderate class III antiarrhythmic effect of UL-FS 49 could not be excluded. Carmeliet (1985) have described prolongation of AP in guinea-pig papillary muscles with a moderate class III agent, sotalol, to a similar extent to that induced by UL-FS 49 in our study. Although this alteration is small in these preparations, prolongation of AP was demonstrated with sotalol in man (Echt *et al.*, 1982). Generally $I_K$ is more prevalent in Purkinje fibres than in ventricular cells (Surawicz, 1992) and effects of inhibiting agents are more marked in these preparations (Carmeliet, 1985; Li *et al.*, 1990; Gwilt *et al.*, 1991; Abrahamsson *et al.*, 1993). Therefore we have exposed rabbit Purkinje fibres, driven at a slow rate, to the bradycardic agents. Our results indicate that UL-FS 49 markedly prolonged the duration of phase 2 ($APD_{50}$) and phase 3 ($APD_{90}$) of AP repolarization, with a more pronounced effect on phase 2. Because block of $I_K$ prolongs the duration of AP predominantly by lengthening phase 2 and this effect is more pronounced in Purkinje fibres and at longer cycles (Surawicz, 1992), inhibition of $I_K$ would be expected. The exact ionic mechanism(s) underlying this effect of UL-FS 49 could be clarified by using the patch clamp technique.

In conclusion, S 16257 is a novel potent SBA, acting by reducing AP firing of pacemaker cells. Reduction of the diastolic rate of depolarization suggests a similar mechanism of action to that of UL-FS 49 (Van Bogaert & Goethals, 1987; 1992; Van Bogaert *et al.*, 1990): inhibition of the inward hyperpolarization-activated current which is one of the most important currents in pacemaking (Di Francesco, 1991; Irisawa *et al.*, 1993). Studies of inhibition of the $I_f$ current by S 16257 in isolated cells, by use of the patch clamp technique, are in progress.

In comparison with UL-FS 49, S 16257 has less effect on AP repolarization of all cardiac preparations used. This

difference of action on AP should mean that it is safer to use S 16257 than UL-FS 49. Indeed, early after depolarizations (EADs) and EADs-triggered activity have been suggested as a possible cause of polymorphic ventricular tachyarrhythmias, better known as 'Torsades de pointes' (El-Sherif *et al.*, 1988; Wit, 1990; Libersa *et al.*, 1992) and are more likely to occur when action potential duration is prolonged and when the heart rate is slow (Damiano & Rosen, 1984; Levy, 1989).

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# Metabolism of methylarginines by human vasculature; implications for the regulation of nitric oxide synthesis

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**1** The metabolism of methylarginines by human cultured endothelial cells and human saphenous vein was studied *in vitro*. The human endothelial cell line (SGHEC-7), primary cultures of human umbilical vein endothelial cells (HUVEC) and human saphenous vein were incubated with [$^{14}$C]-monomethyl-L-arginine ([$^{14}$C]-L-NMMA) and the cytosolic extract analysed by high performance liquid chromatogaphy (h.p.l.c.) with on-line radioisotope detection.

**2** SGHEC-7, HUVEC and human saphenous vein metabolized [$^{14}$C]-L-NMMA to a compound which co-eluted with [$^{14}$C]-citrulline. A second metabolite which co-eluted with [$^{14}$C]-arginine was evident on the radiochromatograms of HUVEC cytosol and saphenous vein extracts.

**3** The intracellular levels of [$^{14}$C]-L-NMMA and [$^{14}$C]-citrulline in SGHEC-7 cells incubated with [$^{14}$C]-L-NMMA (0.5 $\mu$Ci ml$^{-1}$: 8.9 $\mu$M) for 1 h were 113 ± 22 and 67.6 ± 6.2 pmol mg$^{-1}$ cell protein respectively ($n = 7$). Co-incubation with $N^GN^G$dimethyl-L-arginine (ADMA; 100 $\mu$M) but not $N^GN'^G$dimethyl-L-arginine (SDMA; 100 $\mu$M) reduced the intracellular level of [$^{14}$C]-citrulline to 26.3 ± 3.7 pmol mg$^{-1}$ cell protein ($P<0.01$; $n = 3$) without reducing the intracellular level of [$^{14}$C]-L-NMMA.

**4** The intracellular levels of [$^{14}$C]-citrulline in SGHEC-7 cells incubated with [$^{14}$C]-L-NMMA for 1 h were reduced following co-incubation with $N^G$nitro-L-arginine methylester (L-NAME; 1 mM), $N^G$nitro-L-arginine (L-NOARG; 1 mM) and L-canavanine (1 mM) to 47.1 ± 6.2, 24.7 ± 3.6 and 12.5 ± 2.8% of control levels ($P<0.001$; $n = 9$). ADMA (1 mM; $n = 3$) reduced intracellular [$^{14}$C]-citrulline levels to 4 ± 4% of control ($P<0.01$) but SDMA (1 mM; $n = 3$) had no effect.

**5** The accumulation of endogenously synthesized ADMA in the culture supernatant of SGHEC-7 cells was increased by co-incubation with L-NMMA (1 mM) from 1.98 ± 0.08 to 2.74 ± 0.36 nmol mg$^{-1}$ cell protein, an increase of 40%.

**6** These results demonstrate that human vasculature possesses an enzyme which has similar properties to dimethylarginase; human endothelial cells and human saphenous vein metabolize L-NMMA to citrulline via a process inhibited by ADMA but not SDMA. The increase in endothelium-derived ADMA following co-incubation with L-NMMA is consistent with competition between ADMA and L-NMMA for dimethylarginase. Inhibition of this enzyme might increase the intracellular concentration of ADMA, an endogenously produced compound that inhibits nitric oxide synthesis.

**Keywords:** Nitric oxide; endothelial cells; methylarginines; dimethylarginase; human vasculature

## Introduction

Nitric oxide (NO) is a ubiquitous intra- and intercellular messenger, synthesized from L-arginine by a family of NO synthase isoenzymes. It is involved in the control of vascular tone (Vallance *et al.*, 1989) and blood pressure (Rees *et al.*, 1989), platelet function (Radomski *et al.*, 1987), neurotransmission (Garthwaite *et al.*, 1988) and host defence (Moncada *et al.*, 1991). Guanidino-substituted arginine derivatives including $N^G$-monomethyl-L-arginine (L-NMMA) (Rees *et al.*, 1990) inhibit the L-arginine:NO pathway and are useful tools with which to probe the role of NO *in vitro* and *in vivo* (Moncada *et al.*, 1991). L-NMMA causes arteriolar vasoconstriction in animals (Gardiner *et al.*, 1990) and man (Vallance *et al.*, 1989), inhibits non-adrenergic non-cholinergic neurotransmission (Gillespie *et al.*, 1989; Kim *et al.*, 1991) and diminishes the host response to leishmania (Liew *et al.*, 1990).

Certain nitric oxide synthase inhibitors occur endogenously (Vallance *et al.*, 1992); L-NMMA and $N^GN^G$-dimethyl-L-arginine (asymmetric dimethylarginine; ADMA) are found as components of polypeptides (Nakajima *et al.*, 1971; Kakimoto *et al.*, 1975), occur as free amino acids in brain and other tissues (Ueno *et al.*, 1992), circulate in plasma (Park *et al.*, 1988; Vallance *et al.*, 1992) and are excreted in the urine (Vallance *et al.*, 1992). Recently it has been demonstrated that human endothelial cells synthesize methylarginines and the predominant form is ADMA, which is produced in quantities that may affect NO synthesis (Fickling *et al.*, 1993). However, the mechanisms determining the intracellular levels of ADMA are not known.

ADMA appears to be synthesized by methylation of arginine residues in proteins and subsequent hydrolysis of the proteins (Cantoni, 1975). Thus the amounts of ADMA produced by endothelial cells may reflect rates of protein methylation and degradation. The removal of ADMA from the cell might occur by metabolism or extrusion. Rat kidney contains dimethylarginase (previously known as dimethylarginine dimethylaminohydrolase) (Ogawa *et al.*, 1989), which metabolizes ADMA and L-NMMA to citrulline, and indirect evidence suggests that bovine endothelial cells may contain a similar enzyme (Hecker *et al.*, 1990a). If this enzyme is present in cells which make ADMA, its activity might determine the intracellular levels of this NO synthase inhibitor. In the present study we have examined the metabolism of methylarginines in human cultured endothelial cells and human saphenous vein and have studied the effects of known inhibitors of NO synthase on this process.

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## Methods

### *Endothelial cell culture*

SV40-transfected human umbilical vein endothelial cells (SGHEC-7) were cultured as previously described (Fickling *et al.*, 1992) in medium M199:RPMI 1640 in a ratio of 1:1 supplemented with 2.5 $\mu g\,ml^{-1}$ endothelial cell growth supplement, 0.09 $mg\,ml^{-1}$ heparin, 2.5% (v/v) foetal calf serum and 2.5% (v/v) newborn calf serum. Cells were grown as monolayers in 9 cm petri dishes and used between passage 16 and 22. Primary cultures of human umbilical vein endothelial cells (HUVEC) were isolated with collagenase (0.5 $mg\,ml^{-1}$) as previously described (Jaffe *et al.*, 1973) and grown on gelatin-coated 9 cm petri dishes in SGHEC-7 culture medium (as above) modified to include 10% (v/v) foetal calf serum, 10% (v/v) newborn calf serum and 20 $\mu g\,ml^{-1}$ endothelial cell growth supplement. In initial studies, HUVEC and SGHEC-7 cells were grown to confluence and then depleted of arginine by incubation in arginine- and serum-free medium for 24 h before use since this manoeuvre has been reported to increase the metabolism of L-NMMA in bovine endothelial cells (Hecker *et al.*, 1990a). In subsequent experiments SGHEC-7 cells were seeded in 6 well plates for 24 h before use in standard SGHEC-7 medium since in these studies incubation in arginine-free medium did not alter the metabolism of [$^{14}$C]-L-NMMA by these cells (unpublished observations).

### *Metabolism of [$^{14}$C]-L-NMMA and [$^{14}$C]-citrulline by HUVEC and SGHEC-7 cells*

Confluent 9 cm dishes of HUVEC and SGHEC-7 were washed twice with 10 ml of warm (37°C) Krebs solution of the following composition (mM): NaCl 131, KCl 5.5, $CaCl_2$ 2.5, $MgCl_2$ 1, $NaHCO_3$ 25, $NaH_2PO_4$ 1, glucose 5.5, HEPES 20. Cells were then incubated at 37°C or kept on ice for 1 h in 2 ml of Krebs solution containing either [$^{14}$C]-L-NMMA (0.5 $\mu Ci\,ml^{-1}$) or [$^{14}$C]-citrulline (0.25 $\mu Ci\,ml^{-1}$). After 1 h the cells were washed twice with 10 ml of cold (4°C) Krebs solution; washing with Krebs containing 10 mM L-NMMA confirmed that non-specific binding was negligible (unpublished observations). Cells were scraped from the dish in 1.5 ml of methanol/water (90/10; v/v), freeze-thawed 3 times and centrifuged for 10 min at 10,000 *g* in a bench top microcentrifuge. The precipitate was kept for protein estimation by the Bio-Rad protein assay with bovine serum albumin as a standard. The supernatant was evaporated at 140°C under nitrogen, following which the residue was resuspended in distilled water for analysis using high performance liquid chromatography (h.p.l.c.). To determine substrate specificity incubations with [$^{14}$C]-L-NMMA were carried out in the presence of ADMA (100 $\mu$M) or $N^GN'^G$-dimethyl-L-arginine (symmetric dimethylarginine; SDMA; 100 $\mu$M).

### *Effect of NO synthase inhibitors on the metabolism of [$^{14}$C]-L-NMMA by SGHEC-7 cells*

SGHEC-7 cells seeded in 6 well plates ($5 \times 10^5$ cells per well) were washed with Krebs (37°C) and incubated in 300 $\mu$l of Krebs solution containing [$^{14}$C]-L-NMMA (0.4 $\mu Ci\,ml^{-1}$) alone or [$^{14}$C]-L-NMMA (0.4 $\mu Ci\,ml^{-1}$) and $N^G$-nitro-L-arginine (L-NOARG; 1 mM), $N^G$-nitro-L-arginine methylester (L-NAME; 1 $\mu$M–1 mM), L-canavanine (1 mM), ADMA (1 mM) or SDMA (1 mM). After 1 h the cells were washed with 2 ml cold Krebs and extracted as above.

### *Incubation of human saphenous vein with [$^{14}$C]-L-NMMA*

Saphenous vein was obtained from patients undergoing cardiac surgery and used immediately. Each segment was weighed and incubated for 2 h at 37°C in 1 ml of Krebs containing 0.1 $\mu$Ci of [$^{14}$C]-L-NMMA. After 2 h the supernatant was centrifuged at 10,000 *g* for 10 min and analysed by h.p.l.c. Vein segments were mechanically homogenized in 4 ml of methanol/water (Ultra Turrax, Janke and Kunkel, Staufen, Germany) and centrifuged at 10,000 *g* as above. The supernatant was aspirated and evaporated to dryness under nitrogen. The residue was dissolved in distilled water for analysis by h.p.l.c.

### *Effect of L-NMMA on ADMA production by endothelial cells*

SGHEC-7 cells were grown to confluence in 9 cm dishes and incubated for 72 h in culture medium containing 1 mM L-NMMA. The medium was aspirated, centrifuged at 10,000 *g* for 10 min and partial purification of dimethylarginines achieved by passage through a Bond Elut SCX column and elution with ammonia/methanol as described previously (Vallance *et al.*, 1992). ADMA levels were measured by h.p.l.c. and cell protein was measured as above.

### *H.p.l.c. analysis*

Separation of amino acids was achieved on an ODS $C_{18}$ h.p.l.c. column using an ion pair based mobile phase containing 0.025 M phosphoric acid buffer (pH 5.0), 0.01 M hexane sulphonic acid and 1% (v/v) acetonitrile. Flow was maintained at 1 $ml\,min^{-1}$ and amino acids detected by u.v. absorbance at 200 nm and/or $^{14}$C scintillation counting using an on-line solid scintillator (Beckman System Gold, Beckman Instruments (UK) Ltd, High Wycombe, Bucks).

### *Materials*

The following reagents were used: medium M199 and RPMI 1640 (Gibco Ltd, Paisley, Scotland), foetal calf serum (Advanced Protein Products, UK), new born calf serum (Imperial Laboratories, Salisbury, Wilts), endothelial cell growth supplement and heparin (Sigma Chemical Co, Poole, Dorset), arginine-free media (Integra Biosciences, Northumberland), bovine serum albumin, L-canavanine, L-NAME and L-NOARG (Sigma), ADMA, SDMA, L-NMMA and [$^{14}$C]-L-NMMA (56 $\mu Ci\,\mu mol^{-1}$; a kind gift from Drs Hodson and Moncada, Wellcome Research Laboraties, Beckenham, Kent), [$^{14}$C]-citrulline (55.6 $\mu Ci\,\mu mol^{-1}$: NEN Research Products, Stevenage, Herts), acetonitrile and hexane sulphonic acid (Romil, Loughborough, Leicestershire). Bio-Rad protein assay was obtained from Bio-Rad Laboratories Inc, Hemel Hempstead, Herts. Bond Elut columns were obtained from Jones Chromatography Ltd, Hengoed, Mid Glamorgan. H.p.l.c. columns were obtained from Phase Separation, Queensferry, Wales.

### *Calculations and statistics*

The area under each radiochromatogram peak was calculated by computerized integration following calibration with synthetic standards. Results are expressed as $mol\,mg^{-1}$ cell protein (cultured cell studies) or $mol\,g^{-1}$ wet tissue (saphenous vein studies) except for those undertaken in 6-well plates which are expressed as mol per million cells. All data are expressed as the mean ± s.e.mean and compared by Student's *t* test for paired or unpaired observations as appropriate, where $P<0.05$ is considered significant.

## Results

The radiochromatogram of [$^{14}$C]-L-NMMA consisted of a single peak (Figure 1) with no evidence of impurities either on the $^{14}$C or u.v. chromatograms.

### *Metabolism of [$^{14}$C]-L-NMMA by endothelial cells*

The radiochromatograms of the cytosolic extract of SGHEC-7 cells contained 2 peaks, one that co-eluted with [$^{14}$C]-L-NMMA and the other that co-eluted with [$^{14}$C]-citrulline (Figure 1). Analysis of the Krebs incubate confirmed a similar pattern (data not shown). The intracellular level of [$^{14}$C]-L-NMMA was $113 \pm 22$ pmol mg$^{-1}$ protein and of [$^{14}$C]-citrulline was $67.6 \pm 6.2$ pmol mg$^{-1}$ protein ($n = 7$; Figure 2). Incubation of SGHEC-7 cells on ice reduced the amount of [$^{14}$C]-citrulline to $2.5 \pm 0.3$ pmol mg$^{-1}$ protein (>95% inhibition; $P<0.001$; $n = 4$; Figure 1) but did not alter the amount of [$^{14}$C]-L-NMMA ($87.8 \pm 9.4$ pmol mg$^{-1}$ protein; $P>0.3$; $n = 4$). Co-incubation with ADMA (100 μM) reduced [$^{14}$C]-citrulline to $26.3 \pm 3.7$ pmol mg$^{-1}$ protein (60% inhibition; $P<0.01$; $n = 3$); co-incubation with SDMA (100 μM) had no effect ($n = 3$; $P>0.3$; Figure 2). Neither ADMA nor SDMA had any significant effect on [$^{14}$C]-L-NMMA levels (Figure 2).

The radiochromatograms of the cytosolic extract of HUVEC were similar to that of SGHEC-7 (Figure 3) although intracellular levels of [$^{14}$C]-L-NMMA were higher ($361.5 \pm 60.6$ pmol mg$^{-1}$ protein; $n = 4$; $P<0.002$) and [$^{14}$C]-citrulline lower ($23.9 \pm 5.1$ pmol mg$^{-1}$ protein; $n = 4$; $P<0.002$). In addition there was a third peak which co-eluted with [$^{14}$C]-arginine ($14.6 \pm 5.2$ pmol mg$^{-1}$ protein; $n = 4$). Incubation with [$^{14}$C]-citrulline confirmed that HUVEC ($n = 3$) but not SGHEC-7 ($n = 4$) metabolized [$^{14}$C]-citrulline to [$^{14}$C]-arginine (Figure 3).

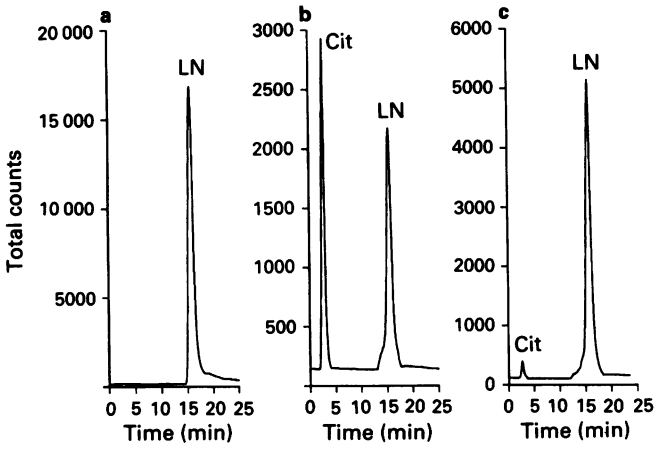


**Figure 1** Representative radiochromatograms of: (a) [$^{14}$C]-L-NMMA (LN) standard (0.01 μCi); (b) SGHEC-7 cytosolic extract incubated for 1 h in [$^{14}$C]-L-NMMA (0.5 μCi ml$^{-1}$) at 37°C; L-NMMA (LN), citrulline (Cit). (c) SGHEC-7 cytosolic extract incubated for 1 h in [$^{14}$C]-L-NMMA (0.5 μCi ml$^{-1}$) at 4°C; L-NMMA (LN), citrulline (Cit). For other abbreviations, see text.

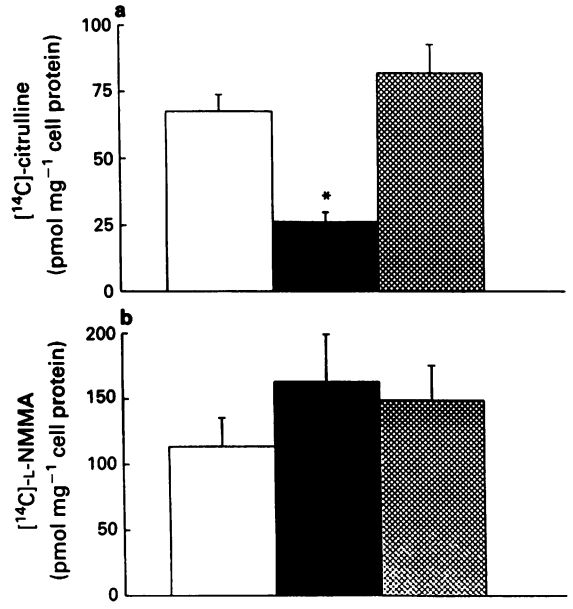


**Figure 2** Intracellular levels of [$^{14}$C]-citrulline (a) and [$^{14}$C]-L-NMMA (b) following incubation of SGHEC-7 cells with [$^{14}$C]-L-NMMA (0.5 μCi ml$^{-1}$) alone (open columns; $n = 7$), or [$^{14}$C]-L-NMMA with ADMA (100 μM; solid columns; $n = 3$) or SDMA (100 μM; cross hatched columns; $n = 3$). Error bars show s.e.mean and data were compared by Student's $t$ test (*$P<0.01$ compared with control). For abbreviations, see text.

### *Concentration of [$^{14}$C]-L-NMMA in endothelial cells*

Assuming an endothelial cell volume of 1 pl (Baydoun *et al.*, 1990), the mean intracellular concentration of [$^{14}$C]-L-NMMA in SGHEC-7 cells was approximately 40 μM when the concentration of [$^{14}$C]-L-NMMA in the Krebs incubate was 8.9 μM. The extraction efficiency for intracellular amino acids was of the order of 90% (unpublished observations), indicating that these cells concentrate [$^{14}$C]-L-NMMA approximately 5 fold.

### *Metabolism of [$^{14}$C]-L-NMMA by human saphenous vein*

The radiochromatograms of human saphenous vein incubated with [$^{14}$C]-L-NMMA were composed of 3 peaks co-eluting with [$^{14}$C]-citrulline, [$^{14}$C]-arginine and [$^{14}$C]-L-NMMA (Figure 3; $n = 3$). The quantity of [$^{14}$C]-L-NMMA, [$^{14}$C]-citrulline and [$^{14}$C]-arginine was $2.2 \pm 0.3$, $0.16 \pm 0.05$ and $0.25 \pm 0.07$ pmol g$^{-1}$ tissue respectively. Analysis of the Krebs incubate showed a similar radiochromatogram pattern (data not shown).

### *Effect of NO synthase inhibitors on the metabolism of [$^{14}$C]-L-NMMA*

The intracellular levels of [$^{14}$C]-L-NMMA and [$^{14}$C]-citrulline in SGHEC-7 cells grown in 6 well plates were $41.9 \pm 6.3$ and $42.5 \pm 5.4$ pmol per $10^6$ cells respectively ($n = 16$). The NO synthase inhibitors, L-canavanine (1 mM), L-NAME (1 mM),

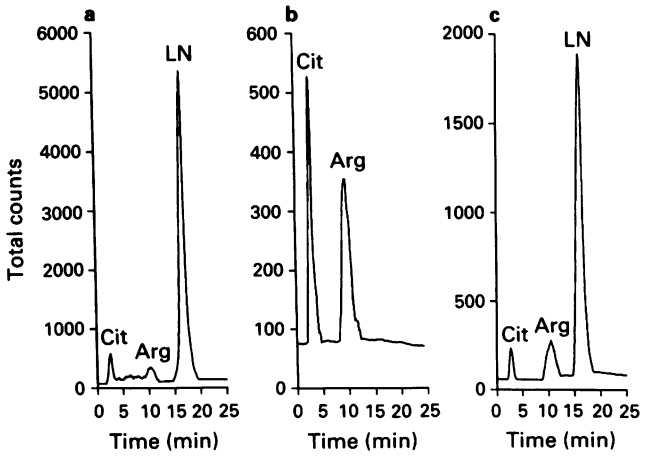


**Figure 3** Representative radiochromatograms of: (a) HUVEC cytosolic extract incubated for 1 h with [$^{14}$C]-L-NMMA (0.5 μCi ml$^{-1}$); L-NMMA (LN), citrulline (Cit), arginine (Arg); (b) HUVEC cytosolic extract incubated for 1 h with [$^{14}$C]-citrulline (0.25 μCi ml$^{-1}$); citrulline (Cit), arginine (Arg); (c) Human saphenous vein extract incubated for 2 h in [$^{14}$C]-L-NMMA (0.1 μCi ml$^{-1}$); L-NMMA (LN), citrulline (Cit), arginine (Arg). For other abbreviations, see text.

L-NOARG (1 mM; Figure 4) and ADMA (1 mM) reduced the intracellular levels of [$^{14}$C]-citrulline to 5.3 ± 1.1 ($P<0.0001$; $n=9$), 10.1 ± 1.5 ($P<0.0002$; $n=9$), 20 ± 2.6 ($P<0.01$; $n=9$) and 1.2 ± 1.2 ($P<0.05$; $n=3$) pmol per $10^6$ cells respectively. Intracellular levels of [$^{14}$C]-L-NMMA were higher following incubation with L-NOARG (72.1 ± 8.8 pmol per $10^6$ cells; $P<0.01$; $n=9$) but unaffected by L-canavanine, L-NAME (Figure 4) or ADMA. SDMA (1 mM) had no effect on the intracellular levels of either amino acid ($n=3$; data not shown). L-NAME caused dose-dependent inhibition of [$^{14}$C]-citrulline synthesis from [$^{14}$C]-L-NMMA (Figure 5): 100 μM L-NAME caused approximately 40% inhibition of [$^{14}$C]-citrulline synthesis.

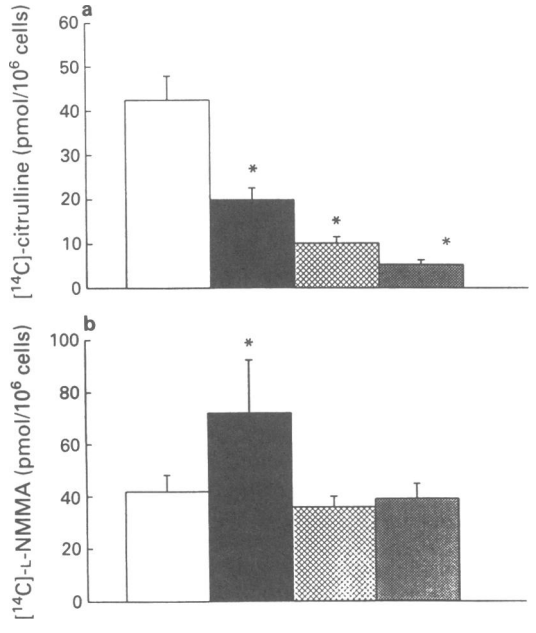


**Figure 4** Intracellular levels of [$^{14}$C]-citrulline (a) and [$^{14}$C]-L-NMMA (b) following incubation of SGHEC-7 cells with [$^{14}$C]-L-NMMA (0.4 μCi ml$^{-1}$) alone (open columns; $n=16$), or [$^{14}$C]-L-NMMA with L-NOARG (1 mM; solid columns; $n=9$), L-NAME (1 mM; cross hatched columns; $n=9$) or L-canavanine (1 mM; stippled columns; $n=9$). Error bars show s.e.mean and data were compared by Student's $t$ test (*$P<0.001$ compared with control). For abbreviations, see text.

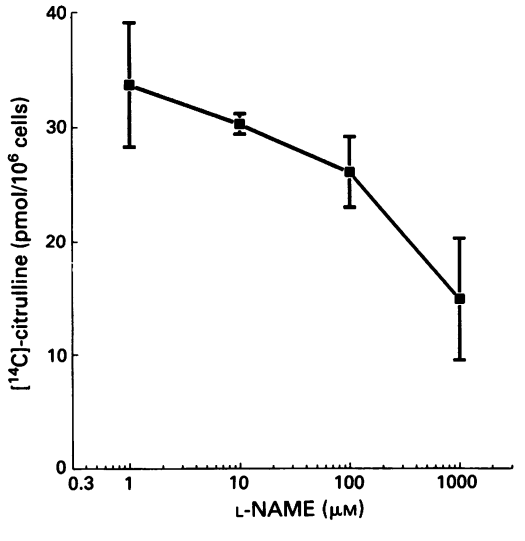


**Figure 5** The effect of L-NAME on the synthesis of [$^{14}$C]-citrulline from [$^{14}$C]-L-NMMA by SGHEC-7 cells ($n=4$). For abbreviations, see text.

### *Effect of L-NMMA on ADMA production by SGHEC-7 cells*

The amount of endogenous ADMA released into the culture medium by SGHEC-7 cells was increased by co-incubation with L-NMMA (1 mM). The amount of ADMA in the culture medium (10 ml) following a 72 h incubation period was 1.98 ± 0.08 nmol mg$^{-1}$ cell protein in the absence of L-NMMA and 2.74 ± 0.36 nmol mg$^{-1}$ cell protein in the presence of L-NMMA ($P<0.05$; $n=4$), an increase of approximately 40%.

## Discussion

Methylarginines that inhibit nitric oxide synthesis are synthesized endogenously *in vivo* in animals (Kakimoto & Akazawa, 1970) and man (Vallance *et al.*, 1992) and *in vitro* by human endothelial cells in culture (Fickling *et al.*, 1993). Metabolism of methylarginines has been reported in the rat (Ogawa *et al.*, 1987) and the rabbit (McDermott, 1976) and indirect evidence suggests that bovine endothelial cells metabolize L-NMMA to citrulline (Hecker *et al.*, 1990a). The results of this study demonstrate the presence of an enzyme with the characteristics of dimethylarginase in human tissues. Human cultured endothelial cells and human blood vessels *in vitro* metabolized [$^{14}$C]-L-NMMA to [$^{14}$C]-citrulline and the activity of this enzyme determined the amount of ADMA released by the cells into the culture medium.

Direct evidence for a pathway for the metabolism of methylarginines is provided by these experiments using [$^{14}$C]-L-NMMA. SGHEC-7 cells, primary cultures of HUVEC and isolated human saphenous veins metabolized [$^{14}$C]-L-NMMA to [$^{14}$C]-citrulline. The metabolism of [$^{14}$C]-L-NMMA occurred at 37°C but not at 4°C and was inhibited by ADMA but not SDMA suggesting that this is an enzymatic process and that there is competition between ADMA and L-NMMA for metabolism. Similar results have been obtained with dimethylarginase isolated from rat kidney which metabolizes L-NMMA ($K_m=0.36$ mM) and ADMA ($K_m=0.18$ mM) but not SDMA (Ogawa *et al.*, 1989).

HUVEC and saphenous vein metabolized [$^{14}$C]-citrulline to [$^{14}$C]-arginine, a property shared by bovine aortic endothelial cells (Hecker *et al.*, 1990b). This pathway is unlikely to involve the urea cycle as [$^{14}$C]-citrulline was not metabolized via [$^{14}$C]-ornithine to [$^{14}$C]-arginine. The enzyme responsible has the characteristics of arginosuccinate synthase (Hecker *et al.*, 1990b) and makes any estimate of the activity of dimethylarginase in these cells unreliable. In contrast, SGHEC-7 cells did not metabolize [$^{14}$C]-citrulline to [$^{14}$C]-arginine and the rate of production of [$^{14}$C]-citrulline from [$^{14}$C]-L-NMMA in these cells was of the order of 40 pmol per $10^6$ cells h$^{-1}$, which represents a 50% conversion of the intracellular [$^{14}$C]-L-NMMA but only a 1% conversion of the total [$^{14}$C]-L-NMMA present. This rate of metabolism is unlikely to deplete the levels of [$^{14}$C]-L-NMMA added to cells and tissues for pharmacological studies *in vitro*, but might affect the distribution, tissue levels and profile of activity of this drug administered to experimental animals or man *in vivo* or influence the levels of endogenous methylarginines within the cells. Indeed, when exogenous L-NMMA was added to endothelial cells the amount of ADMA released into the culture medium increased by about 40% (approximately 0.5 μM to 0.7 μM). The precise concentration of ADMA in the cells is not known, but the studies with [$^{14}$C]-L-NMMA suggest that methylarginines are concentrated within human endothelial cells indicating that ADMA levels inside the cell may be higher than those we have measured in the culture medium.

NO synthase also metabolizes arginine and L-NMMA to citrulline (Feldman *et al.*, 1993); however, the rate of conversion is 20 times slower for L-NMMA than for arginine (Olken & Marletta, 1993) and unlikely to account for the

activity we have seen in endothelial cells; in SGHEC-7 cells the rate of conversion of [$^{14}$C]-L-NMMA to [$^{14}$C]-citrulline was of the same order of magnitude as the rate of conversion of [$^{14}$C]-arginine to [$^{14}$C]-citrulline either in whole cells (about 100 pmol per $10^6$ cells $h^{-1}$; unpublished observations) or in homogenates of SGHEC-7 cells (24 pmol $mg^{-1}$ $h^{-1}$; Radomski *et al.*, 1993). Our results are most consistent with metabolism of [$^{14}$C]-L-NMMA to [$^{14}$C]-citrulline by dimethylarginase in endothelial cells.

Structural analogues of ADMA and L-NMMA inhibited the conversion of [$^{14}$C]-L-NMMA to [$^{14}$C]-citrulline. L-NAME produced a dose-dependent inhibition and at a concentration of 1 mM caused near total abolition of [$^{14}$C]-citrulline production. L-Canavanine and L-NOARG had similar effects. L-NOARG has previously been reported to inhibit the activity of rat isolated dimethylarginase (Ogawa *et al.*, 1989) and L-canavanine appears to inhibit NO synthase and endothelial dimethylarginase in the same concentration-range. Further studies will be required to determine whether the inhibition of metabolism of endogenous ADMA produced by these compounds contributes to their actions as NO synthase inhibitors. Therefore it would be useful to identify compounds that inhibited dimethylarginase but had no direct effect on NO synthase.

ADMA and SDMA are produced by human endothelial cells and ADMA is an inhibitor of NO synthase. Dimethylarginase metabolizes ADMA but not SDMA and the finding that an enzyme similar to or identical with dimethylarginase is present in human endothelial cells provides further evidence for possible physiological or pathophysiological roles for ADMA. Our results demonstrate that alterations in the activity of this enzyme affect the amount of ADMA released by endothelial cells and possibly the intracellular concentrations of this compound. The potential importance of dimethylarginase is supported by the demonstration of dimethylarginase-like activity in human saphenous vein and also the identification of dimethylarginase in the brain, aorta and immune cells of the rat by use of monoclonal antibodies (Kimoto *et al.*, 1993). Manipulation of this enzyme might provide a novel approach to the regulation of nitric oxide synthesis and would help determine the biological significance of endogenous methylated arginines in the regulation of the L-arginine:NO pathway.

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# Modulation of vasodilatation to levcromakalim by adenosine analogues in the rabbit ear: an explanation for hypoxic augmentation

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**1** We have used a rabbit isolated ear, buffered-perfused preparation to investigate the effects of adenosine analogues on the vasodilatation to the potassium channel opener, levcromakalim (the active (−)-enantiomer of cromakalim). We have examined the effects of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), a selective adenosine $A_1$ antagonist, on vasodilatation to levcromakalim under hypoxic conditions and also following inhibition of nitric oxide synthesis.

**2** Levcromakalim relaxed preconstricted preparations with an $EC_{50} = 369 \pm 48$ nM and maximum relaxation of tone ($R_{max}$) = 81.0 ± 3.2%. In the presence of 1 μM $N^6$-cyclohexyladenosine (CHA) a selective adenosine $A_1$ agonist, there was a significant ($P<0.01$) leftward shift in the concentration-response curve with an $EC_{50} = 194 \pm 54$ nM and $R_{max} = 93.2 \pm 2.0$%. Conversely, the presence of CHA did not influence vasodilatation to either pinacidil or sodium nitroprusside.

**3** Hypoxia also significantly ($P<0.001$) increased the vasodilator potency of levcromakalim ($EC_{50} = 134 \pm 22$ nM), and this enhancement was completely reversed ($EC_{50} = 380 \pm 107$ nM, $P<0.01$) by pretreatment of the preparations with 5 μM DPCPX, a selective $A_1$ adenosine antagonist. However, under normoxic conditions DPCPX did not influence vasodilatation to levcromakalin.

**4** Inhibition of nitric oxide synthesis with 100 μM $N^G$-nitro-L-arginine methyl ester (L-NAME) caused a significant ($P<0.001$) leftward shift in the concentration-response curve to levcromakalim ($EC_{50} = 73.0 \pm 7.6$ nM). Pretreatment of preparations with DPCPX partially reversed the increase in potency found in the absence of nitric oxide synthesis ($EC_{50} = 153 \pm 18$ nM, $P<0.001$).

**5** We have shown that an adenosine $A_1$ agonist may increase the potency of levcromakalim indicating that adenosine receptor activation may augment the vasodilator activity of levcromakalim. That responses to levcromakalim but not those to pinacidil were affected by CHA points to further differences in the pharmacology of these potassium channel openers. The reversal by the adenosine $A_1$ antagonist of the hypoxic-potentiation of vasodilatation to levcromakalim, and also augmentation following inhibition of nitric oxide synthesis, suggests that under these conditions there is an endogenous release of adenosine which may enhance responses to levcromakalim. The findings of this study suggest that levcromakalim may selectively dilate vessels where there is elevated adenosine release.

**Keywords:** Levcromakalim; pinacidil; $N^G$-nitro-L-arginine methyl ester; hypoxia; nitric oxide; potassium channel opener-sensitive potassium channels (KCO-channels); adenosine; 8-cyclopentyl-1,3-dipropylxanthine; $N^6$-cyclohexyladenosine

## Introduction

We have previously shown that vasodilatation to the potassium channel opener (KCO) levcromakalim (the active (−)-enantiomer of cromakalim, formerly designated BRL 38227) is augmented under hypoxic conditions. A similar, but even larger, effect was also observed following inhibition of nitric oxide synthesis with $N^G$-nitro-L-arginine methyl ester (L-NAME) (Randall & Griffith, 1993). The activity of KCO-sensitive potassium channels is regulated by purine-derivatives associated with cellular metabolism as intracellular ATP closes these channels and high ADP favours opening (for review, see Nichols & Lederer, 1991). Pharmacologically, these channels may be regulated by KCOs which reduce channel sensitivity towards ATP thereby promoting channel opening (see Edwards & Weston, 1990; Nichols & Lederer, 1991), while the hypoglycaemic sulphonylureas block the channels (Sturgess *et al.*, 1985).

Recent evidence, from patch clamp studies using membrane patches of rat cultured ventricular myocytes (Kirsch *et al.*, 1990) and whole-cell current recordings from porcine isolated coronary vascular smooth muscle cells (Dart & Standen, 1993), has indicated that adenosine $A_1$ receptors may be positively coupled, via a G-protein, to KCO-sensitive channels. The possibility that adenosine is coupled to KCO-sensitive potassium channels receives further functional support from evidence that adenosine $A_1$, but not $A_2$, receptor agonists cause sulphonylurea-sensitive vasorelaxation of porcine coronary vessels (Merkel *et al.*, 1992). Adenosine is an important mediator of blood-flow regulation which corrects an imbalance between energy production and demand (Berne *et al.*, 1983), as the supply-to-demand ratio for oxygen determines the formation of adenosine (Bardenheuer & Schrader, 1986). This has led to the so-called 'adenosine hypothesis' of local blood flow regulation, in which locally produced adenosine leads to vasodilatation and improved blood flow (Berne, 1980). In this context Marshall *et al.* (1993) have recently reported that hypoxia, via adenosine release, leads to skeletal muscle vasodilatation through the activation of sulphonylurea-sensitive potassium channels.

We have previously observed that levcromakalim selectively vasodilates collateral vessels (Randall & Griffith, 1992) and that both hypoxia and metabolic inhibitors selectively increase the vasodilator potency of this agent (Randall &

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Griffith, 1993). Hypoxia is associated with compromised metabolic activity leading to adenosine release (Mian & Marshall, 1991; Marshall *et al.*, 1993). This has led us now to investigate the actions of adenosine analogues on responses to levcromakalim and other vasodilators in order to examine whether locally released adenosine may influence vasodilator responses. In addition to hypoxic augmentation, vasodilatation to levcromakalim is also enhanced following inhibition of nitric oxide synthesis by L-NAME, and this effect is even greater than that seen in hypoxia (Randall & Griffith, 1993). Inhibition of nitric oxide activity has important consequences for vascular resistance and blood flow regulation (Griffith *et al.*, 1987). However, following inhibition of nitric oxide production in the guinea-pig heart, there is a significant compensatory increase in adenosine release, presumably as a consequence of mismatches in flow and demand (Kostic & Schrader, 1992). This compensatory release of adenosine is sufficient to exert a significant protective effect in a rabbit model of coronary occlusion, and limits the level of ischaemic damage (Patel *et al.*, 1993). We have accordingly examined whether endogenous release of adenosine, following inhibition of nitric oxide synthesis, may contribute towards the increase in potency for levcromakalim in the presence of L-NAME.

A preliminary account of part of this work was communicated to the January 1994 meeting of the British Pharmacological Society (Randall *et al.*, 1994).

## Methods

### *Preparation of the rabbit ear vascular bed*

Male New Zealand White rabbits (2–2.5 kg) were killed by cervical dislocation. An ear was removed and the central artery cannulated and perfused with Holman's solution (composition, mM: NaCl 120, KCl 5, $CaCl_2$ 2.5, $NaH_2PO_4$ 1.3, $NaHCO_3$ 25, sucrose 10 and D-glucose 11) at a flow rate of 3.5 ml $min^{-1}$. The physiological buffer also contained 10 μM indomethacin to eliminate prostanoid activity. The buffer was gassed with either 95% $O_2$/5% $CO_2$ (normoxia, $Po_2$ = 500–600 mmHg) or 95% $N_2$/$CO_2$ (hypoxia, $Po_2$ = 20–30 mmHg) and maintained as 35°C.

### *Experimental protocols*

The perfusion pressure of the intact preparation was continuously monitored by means of a pressure transducer placed close to the inflow cannula. The pressure drop across the cannula was determined at the end of each experiment and subtracted from the recorded pressure in order to determine the pressure drop across the vascular bed.

To characterize vasodilator responses, preparations were equilibrated for 1 h. Perfusion pressure was raised pharmacologically with the combination of 5-hydroxytryptamine and histamine in equimolar concentrations (1 μM) to achieve submaximal (ca. 60% of maximal tone, Randall & Griffith, 1991). Cumulative vasodilator concentration-response curves were obtained in different preparations for levcromakalim, pinacidil and sodium nitroprusside by addition of each agent to the perfusion fluid in volumes less than 100 μl.

The effects of the selective adenosine $A_1$ agonist, $N^6$-cyclohexyladenosine (CHA), were investigated by inclusion of this agent at a concentration of 1 μM in the perfusion fluid after steady state preconstriction, but 15 min prior to construction of the concentration-response curves for levcromakalim, pinacidil or sodium nitroprusside.

In subsequent experiments, concentration-response curves for levcromakalim were constructed under hypoxic perfusion and the results were compared with control responses obtained from different preparations perfused with normoxic buffer. The influence of the selective adenosine $A_1$ receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), was examined on the vasodilatation to levcromakalim under both normoxic (control) and hypoxic conditions. In these experiments the preparations were equilibrated for 1 h with the buffer of the appropriate oxygen tension after which time the preparations were preconstricted and then continuously perfused with 5 μM DPCPX. The concentration-response curve to levcromakalim was constructed 15 min after inclusion of DPCPX.

In order to investigate the influence of inhibition of nitric oxide synthesis on vasodilatation to levcromakalim, different preparations were perfused with 100 μM L-NAME which was added to the perfusion fluid 30 min prior to preconstriction. We have previously shown that perfusion with 100 μM L-NAME selectively abolishes endothelium-dependent relaxations to acetylcholine and inhibits basal nitric oxide activity (Randall & Griffith, 1991). Inhibition of basal nitric oxide production leads to augmented constrictor responses and, therefore, in these experiments the equimolar concentrations of the vasoconstrictor agents were reduced to 300 nM to give an equivalent level of tone (Randall & Griffith, 1993). In further experiments, preparations were perfused with 5 μM DPCPX 30 min after inclusion of L-NAME and subsequent preconstriction but 15 min prior to constructing concentration-response curves for levcromakalim.

### *Data and statistical analysis*

All data are given as the mean ± s.e.mean and were compared by either paired or unpaired Student's *t*-tests or analysis of variance, as appropriate. $EC_{50}$ values for vasodilator responses were obtained from individual concentration-response curves as the concentration at which half-maximal reduction of established tone occurred. These values were converted to the logarithmic values ($pD_2$) for statistical analysis.

### *Drugs*

All solutions were prepared on the day of the experiment. $N^G$-nitro-L-arginine methyl ester, 5-hydroxytryptamine as creatinine sulphate complex, histamine dihydrochloride and sodium nitroprusside (all from Sigma Chemical Company, Poole, Dorset), were dissolved in saline. Levcromakalim (a generous gift from Smith Kline Beecham, Surrey, U.K.), pinacidil (a generous gift from Leo, Bucks), $N^6$-cyclohexyladenosine and indomethacin (both from Sigma) and 8-cyclopentyl-1,3-dipropylxanthine (from Research Biochemicals Incorporated, Natick, MA, U.S.A.) were dissolved in 70% (v/v) ethanol. All drugs were then diluted to the required concentrations in the Holman's solution.

## Results

### *Effect of $N^6$-cyclohexyladenosine on vasodilator responses to levcromakalim and pinacidil*

In the 15 control preparations basal perfusion pressure was 32.4 ± 5.5 mmHg and was increased by 113 ± 10 mmHg following addition of the vasoconstrictor agents. The concentration-response curve for the vasodilator effects of levcromakalim (10 nM–10 μM) under control conditions is shown in Figure 1a and is described by an $EC_{50}$ of 369 ± 48 nM and the maximum relaxation of tone ($R_{max}$) was 81.0 ± 3.2% ($n$ = 15) (Table 1).

In subsequent experiments addition of 1 μM CHA to the perfusion fluid did not influence the level of induced tone (Table 1). However, in the presence of CHA levcromakalim was significantly ($P<0.01$) more potent as a vasodilator (Figure 1a) with an $EC_{50}$ value of 194 ± 54 nM and there was a significant ($P<0.05$) increase in the maximum relaxation of tone (93.2 ± 2.0%) (Table 1).

In 11 control preparations, pinacidil (10 nM–30 μM)

**Table 1** Vasodilator properties of levcromakalim and pinacidil in the absence and presence of 1 μM $N^6$-cyclohexyladenosine (CHA)

| | *Levcromakalim* | *Levcromakalim + CHA* | *Pinacidil* | *Pinacidil + CHA* |
|---|---|---|---|---|
| *n* | 15 | 8 | 11 | 7 |
| Basal perfusion pressure (mmHg) | 32.4 ± 5.5 | 17.5 ± 2.2 | 26.8 ± 5.3 | 21.4 ± 3.4 |
| Increase in perfusion pressure (mmHg) | 113 ± 10 | 129 ± 10 | 106 ± 9 | 112 ± 8 |
| Total increase in perfusion pressure in presence of CHA (mmHg) | - | 132 ± 12 | - | 112 ± 5 |
| $EC_{50}$ (nM) | 369 ± 48 | 194 ± 54** | 1,783 ± 336 | 973 ± 150 |
| Maximum relaxation (%) | 81.0 ± 3.2 | 93.2 ± 2.0* | 93.5 ± 2.7 | 95.8 ± 4.4 |

For each vasodilator the statistical differences between the presence and absence of $N^6$-cyclohexyladenosine are indicated by *($P<0.05$) and **($P<0.01$).

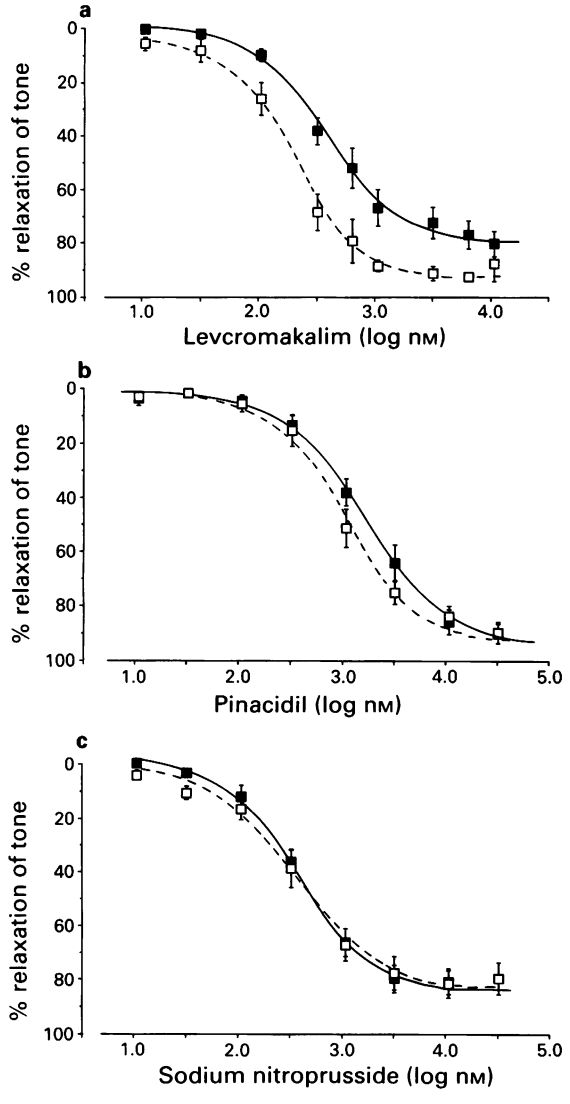


**Figure 1** Concentration-response curves for the relaxation of established tone in rabbit ear isolated perfused preparations by (a) levcromakalim in the absence (■, $n = 15$) and presence (□, $n = 8$) of 1 μM $N^6$-cyclohexyladenosine; (b) pinacidil in the absence (■, $n = 8$) and presence (□, $n = 7$) of 1 μM $N^6$-cyclohexyladenosine; (c) sodium nitroprusside in the absence (■, $n = 6$) and presence (□, $n = 7$) of $N^6$-cyclohexyladenosine. The vertical bars indicate ± s.e.mean.

similarly gave concentration-related relaxations of tone, but was some 5 fold less potent than levcromakalim (Figures 1a,b and Table 1). In 7 different preparations CHA did not influence either the level of established tone (Table 1) or vasodilatation to pinacidil (Figure 1b and Table 1).

### *Effects of $N^6$-cyclohexyladenosine on vasodilatation to sodium nitroprusside*

In 6 control preparations, basal perfusion pressure was 19.8 ± 2.3 mmHg and was increased by 102 ± 16 mmHg following addition of the vasoconstrictors. Sodium nitroprusside (10 nM–10 μM) gave rise to concentration-related relaxations of established tone with an $EC_{50} = 367 \pm 32$ nM and $R_{max} = 84.9 \pm 4.7\%$ (Figure 1c). In another 6 preparations basal perfusion pressure was 18.6 ± 3.7 mmHg, while after addition of the vasoconstrictors perfusion pressure was increased by 124 ± 12 mmHg. Addition of 1 μM CHA had no effect on vascular tone (124 ± 14 mmHg). In the presence of CHA the vasorelaxant potency of sodium nitroprusside was 335 ± 70 nM and the $R_{max}$ was 82.9 ± 5.1% (Figure 1c), these parameters were not significantly different from those in the absence of CHA ($EC_{50} = 367 \pm 32$ nM, $R_{max} = 84.9 \pm 4.7\%$).

### *Effects of hypoxia on vasodilatation to levcromakalim in the absence and presence of 5 μM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX)*

In 12 hypoxic preparations basal perfusion pressure was similar to that under normoxic conditions (Table 2). Similarly the oxygen tension did not influence the increase in perfusion pressure induced by the combination of vasoconstrictors.

Figure 2a and Table 2 indicate that levcromakalim was approximately 3 times ($P<0.001$) more potent as a vasodilator under hypoxic compared to normoxic conditions. However, Figure 2a and Table 2 show that in preparations which are pretreated with 5 μM DPCPX the hypoxic augmentation of vasodilator responses to levcromakalim is abolished completely. DPCPX had no effects on vascular tone in the preconstricted preparations in any of the groups to which it was added (Table 2). In normoxic preparations pretreatment with DPCPX did not influence vasodilatation to levcromakalim (Figure 2b and Table 2).

### *Effects of 5 μM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) on vasodilatation to levcromakalim in the presence of L-NAME*

Pretreatment with L-NAME did not influence perfusion pressure (28.0 ± 3.6 mmHg v. 32.4 ± 4.5 mmHg, $n = 9$). In the presence of L-NAME there was a significant ($P<0.001$) five fold leftward shift in the concentration-response curve for the relaxation of tone by levcromakalim (Figure 3 and Table 3). In 7 different preparations this shift was partially ($P<0.001$) attenuated by pretreatment of the preparations with 5 μM DPCPX such that the $EC_{50}$ was intermediate between control and that obtained in the presence of DPCPX alone. In these preparations addition of DPCPX did not significantly alter the level of established tone (146 ± 20 v. 130 ± 21 mmHg).

**Table 2** Vasodilator properties of levcromakalim under normoxic and hypoxic conditions in the absence and presence of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX)

| | *Normoxia* | *Normoxia + DPCPX* | *Hypoxia* | *Hypoxia + DPCPX* |
|---|---|---|---|---|
| *n* | 15 | 7 | 12 | 6 |
| Basal perfusion pressure (mmHg) | 32.4 ± 5.5 | 23.6 ± 4.6 | 20.3 ± 3.0 | 21.7 ± 1.7 |
| Increase in perfusion pressure (mmHg) | 113 ± 10 | 112 ± 14 | 111 ± 6 | 119 ± 12 |
| Total increase in perfusion pressure in presence of DPCPX (mmHg) | - | 111 ± 17 | - | 120 ± 8 |
| $EC_{50}$ (nM) | 369 ± 48 | 310 ± 74 | 134 ± 22*** | 380 ± 107†† |
| Maximum relaxation (%) | 81.0 ± 3.2 | 76.7 ± 6.6 | 90.1 ± 2.5 | 93.2 ± 2.9 |

The statistical differences between the vasodilator responses under normoxic conditions are shown by ***($P<0.001$) and the difference between the absence and presence of DPCPX under hypoxic conditions are shown by ††($P<0.01$). The control data for vasodilatation to levcromakalim under normoxic conditions is taken from Table 1 and is included for comparison.

**Table 3** Vasodilator properties of levcromakalim in the presence and absence of 100 μM $N^G$-nitro-L-arginine methyl ester (L-NAME) with or without 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) pretreatment

| | *Levcromakalim* | *Levcromakalim + L-NAME* | *Levcromakalim + L-NAME + DPCPX* |
|---|---|---|---|
| *n* | 15 | 9 | 7 |
| Basal perfusion pressure (mmHg) | 32.4 ± 5.5 | 28.0 ± 3.6 | 26.7 ± 2.6 |
| Increase in perfusion pressure (mmHg) | 113 ± 10 | 124 ± 7 | 146 ± 20 |
| Total increase in perfusion pressure in presence of DPCPX (mmHg) | - | - | 130 ± 21 |
| $EC_{50}$ (nM) | 369 ± 48 | 73.0 ± 7.6*** | 153 ± 18**(†††) |
| Maximum relaxation (%) | 81.0 ± 3.2 | 88.6 ± 3.3 | 87.8 ± 3.8 |

Statistical differences for the vasodilator potency between the absence and presence of L-NAME are indicated by **($P<0.01$) and ***($P<0.001$) while †††($P<0.001$) indicates statistical differences between the absence and presence of DPCPX in the L-NAME pretreated preparations.

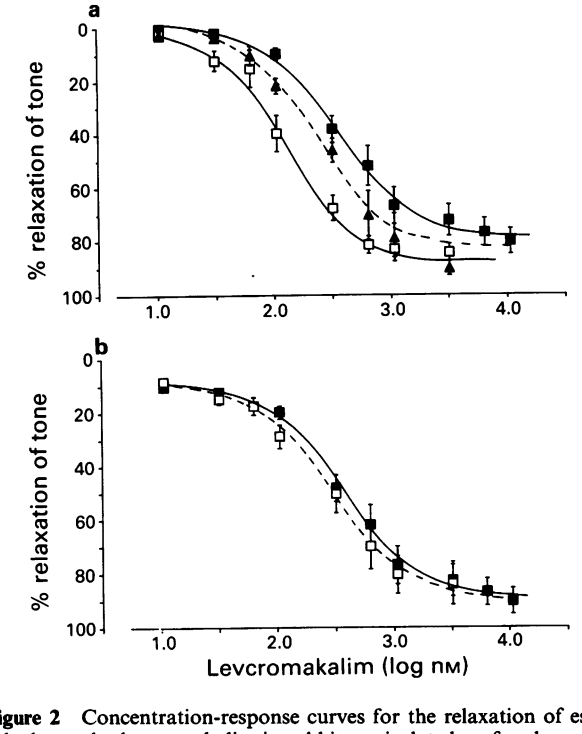


**Figure 2** Concentration-response curves for the relaxation of established tone by levcromakalim in rabbit ear isolated perfused preparations; (a) (■, $n = 15$) indicates control data obtained under normoxic conditions and is taken from Figure 1a for the purposes of comparison, (□, $n = 12$) show the data obtained under hypoxic perfusion and (▲, $n = 6$) show the data obtained under hypoxic perfusion in the presence of 5 μM 8-cyclopentyl-1,3-dipropylxanthine; (b) (■, $n = 15$) indicates data obtained under normoxic conditions that is taken from Figure 1a for the purposes of comparison; (□, $n = 7$) show the data obtained under normoxic perfusion in the presence of 5 μM 8-cyclopentyl-1,3-dipropylxanthine. Ther vertical bars indicate ± s.e.mean.

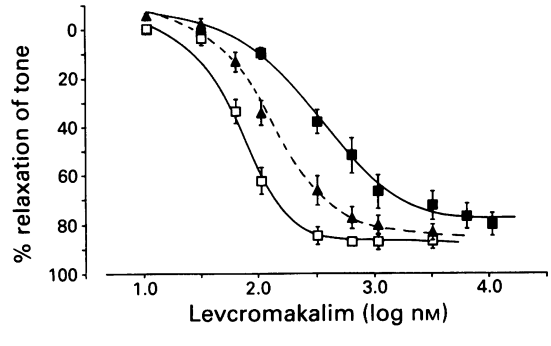


**Figure 3** Concentration-response curves for the relaxation of established tone by levcromakalim in rabbit ear isolated perfused preparations; (■, $n = 15$) indicate control data obtained in the absence of $N^G$-nitro-L-arginine methyl ester (L-NAME) and is taken from Figure 1a for the purposes of comparison, (□, $n = 9$) show the data obtained in the presence of 100 μM L-NAME and (▲, $n = 7$) show the data obtained in the presence of both 100 μM L-NAME and 5 μM 8-cyclopentyl-1,3-dipropylxanthine. The vertical bars indicate ± s.e.mean.

## Discussion

The results of the present study clearly point to an interaction between the KCO, levcromakalim, and adenosine. This interaction provides an explanation as to the augmentation of vasodilatation to levcromakalim in both hypoxia and following nitric oxide synthase inhibition. The findings corroborate our previous findings concerning the different pharmacology of levcromakalim and pinacidil (Randall & Griffith, 1993).

That the potency of levcromakalim is augmented by the adenosine agonist CHA accords with reports indicating that the adenosine $A_1$ receptor may be coupled, via a G-protein,

to KCO-sensitive potassium channels (Kirsch *et al.*, 1990; Dart & Standen, 1993). Not only are our data compatible with adenosine $A_1$ receptors interacting with KCO-sensitive potassium channels, but more importantly, they demonstrate that adenosine may modulate the activity of KCOs. Since CHA did not have any direct vascular actions at the reasonably high concentrations used, then an independent, direct action on the rabbit ear vasculature may be excluded. Furthermore, the lack of interaction between CHA and sodium nitroprusside and pinacidil confirms that the effects observed with levcromakalim are not due to a non-specific interaction between the different vasodilators. That CHA did not augment vasodilatation to pinacidil, a structurally different KCO, may point to differences in the pharmacology of these agents. This would appear to accord with other evidence in the literature pointing to differences in their pharmacology (see Cook & Quast, 1990). For example there are haemodynamic differences between cromakalim and pinacidil (Longman *et al.*, 1988), while McPherson & Angus (1990) also identified differences in the pharmacology of cromakalim and pinacidil, in that glibenclamide, phentolamine and alinidine non-competively inhibited the actions of cromakalim on the canine coronary artery while having competitive actions against pinacidil. More recently, Lawson *et al.* (1992) have demonstrated that endothelin-1 discriminates between the actions of levcromakalim and pinacidil, and proposed that these agents interact with different sites on the potassium channel. In our previous study we also proposed that there were differences in their comparative pharmacology, since we observed that the potency of levcromakalim, but not that of pinacidil, was augmented by both hypoxia and L-NAME (Randall & Griffith, 1993). In view of the lack of effect of L-NAME and hypoxia on responses to pinacidil (Randall & Griffith, 1993) we examined only the influence of the adenosine antagonist on vasodilatation to levcromakalim under these conditions.

Our previous finding that vasodilatation to levcromakalim was augmented by hypoxia was of interest in view of the specific potentiation of responses by CHA, a mimetic of adenosine. We therefore repeated these experiments with the inclusion of the adenosine $A_1$ antagonist, DPCPX in the perfusate. Under normoxic control conditions DPCPX did not influence vascular tone but more importantly did not alter vasodilatation to levcromakalim. The lack of effect on vascular tone indicates that this antagonist is acting specifically and that any basally released adenosine does not contribute significantly towards vascular regulation under any of the conditions used. However, under hypoxic perfusion DPCPX caused a rightward shift of the concentration-response curve for relaxation by levcromakalim, thereby abolishing the hypoxic augmentation. DPCPX did not influence vasodilatation to levcromakalim under normoxic conditions and this excludes a direct antagonist action against levcromakalim. Since we have demonstrated functionally that the adenosine-mimetic may interact with KCO-sensitive potassium channels, then the most likely explanation for these results is that adenosine activity is increased sufficiently to enhance KCO activity. Although the most probable change in adenosine activity in hypoxia is increased release, other potential mechanisms may operate and they include receptor upregulation and reduced reuptake. The present experiments do not discriminate between these alternatives.

Vasorelaxant responses to levcromakalim are also augmented by inhibition of nitric oxide synthases by L-NAME, an effect that is completely reversed by exogenous L-arginine (Randall & Griffith, 1993). Interestingly the enhancement following inhibition of nitric oxide synthase is more pronounced than that in hypoxia. Recent studies have shown that inhibition of nitric oxide production leads to mismatches of perfusion and demand which promotes a compensatory release of adenosine (Griffith *et al.*, 1987; Kostic & Schrader, 1992). That DPCPX partially reversed the augmentation of vasodilatation to levcromakalim in the presence of L-NAME indicates that following inhibition of nitric oxide synthesis in the rabbit ear there is an increase in adenosine release. The shift of the concentration-response curve produced by DPCPX was comparable to that produced by the adenosine antagonist under hypoxic conditions and would indicate that the release of adenosine following blockade of nitric oxide synthesis occurs at a comparable level to that found in hypoxia. The high concentration of DPCPX used only partially reversed the augmentation due to inhibition of nitric oxide synthesis and this suggests that mechanisms other than the interaction between adenosine and the KCO may also be in operation.

Additional mechanisms for the potentiation of vasodilatation to levcromakalim in the absence of nitric oxide production are not apparent from the present data. Nitric oxide, in some (Tare *et al.*, 1990; Garland & McPherson, 1992), but not all (Komori *et al.*, 1988), vascular preparations causes hyperpolarization. Associated with this is the cyclic GMP-dependent activation of calcium-sensitive potassium channels leading to hyperpolarization and relaxation (Fujino *et al.*, 1991). If nitric oxide exerts a hyperpolarizing effect in the rabbit ear, then inhibitors of this input may lead to vascular smooth muscle depolarization, which might enable a potassium channel opener to have a greater hyperpolarizing effect leading to augmented vasodilatation. This action of nitric oxide may account for its synergism with cromakalim reported by others (Rae & Corrêa, 1992). In the present context, loss of nitric oxide activity following treatment with L-NAME may therefore potentially enable levcromakalim to have greater impact. However, since vasodilatation to pinacidil is unaffected by L-NAME (Randall & Griffith, 1993) then the above mechanisms would appear doubtful.

Potentiation of responses to levcromakalim has been reported in other circumstances. In a recent report by Pavlovic *et al.* (1993) the relaxant action of levcromakalim on rat tracheal smooth muscle was selectively potentiated by destruction of the airway epithelium leading to loss of the epithelium-derived inhibitory factor.

Despite our observations of augmented vasodilatation to levcromakalim in the presence of L-NAME, others, in different preparations, have not observed such a change in activity (Gardiner *et al.*, 1991). Differences betwen preparations may be accounted for by differences in adenosine release and reuptake and also differences in adenosine receptor populations between vascular beds.

The results of the present study clearly indicate that the increase in vasodilator potency of levcromakalim in hypoxia, and to a lesser extent in the presence of L-NAME, can be explained by local increases in adenosine activity leading to potentiated responses. The apparent enhancement of the vasodilator activity of levcromakalim by adenosine may perhaps explain the selectivity of cromakalim for chronically ischaemic tissues (Angersbach & Nicholson, 1988), and the ability of levcromakalim to improve substantially collateral flow after acute arterial occlusion (Randall & Griffith, 1992). In conclusion, we have reported an important and significant interaction between adenosine, an endogenous mediator associated with hypoperfusion and hypoxia, and levcromakalim. This interaction may enable levcromakalim to exert selective vasodilator effects on ischaemic tissues.

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# $\beta_1$- and $\beta_2$-adrenoceptor antagonist activities of ICI-215001, a putative $\beta_3$-adrenoceptor agonist

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**1** The present study was undertaken to characterize the $\beta_3$-adrenoceptor agonist activity of ICI-215001 and to determine whether it exhibits additional activities on $\beta_1$- and $\beta_2$-adrenoceptors in isolated spontaneously beating atrium, trachea and ileum of guinea-pig.

**2** In guinea-pig atrium, isoprenaline, a non-selective β-adrenoceptor agonist, caused concentration-dependent, positive chronotropic effects that were inhibited by atenolol, a selective $\beta_1$-antagonist. ICI-215001 also competitively antagonized the increase in heart rate caused by isoprenaline.

**3** ICI-215001 exhibited low intrinsic activity at increasing the beating rate of atrium and no activity on resting or induced tone of tracheal strips.

**4** In strips of guinea-pig trachea, contracted submaximally with carbachol, isoprenaline, caused concentration-dependent relaxations. Both ICI-118551, a selective $\beta_2$-adrenoceptor antagonist, and ICI-215001 competitively inhibited the relaxations caused by isoprenaline.

**5** In isolated strips of guinea-pig ileum longitudinal smooth muscle contracted with histamine, isoprenaline and ICI-215001 caused relaxations which were inhibited by alprenolol, a β-adrenoceptor antagonist with modest affinity for $\beta_3$-adrenoceptors, but were resistant to ICI-118551 and atenolol.

**6** These results indicate that ICI-215001 exhibits $\beta_3$-adrenoceptor agonist activity as demonstrated by relaxations mediated via atypical β-adrenoceptors in the longitudinal smooth muscle of guinea-pig ileum. Further, the studies demonstrate that ICI-215001 can act as an antagonist at $\beta_1$- and $\beta_2$-adrenoceptors in situations where its intrinsic agonist activity is low.

**Keywords:** Atypical β-adrenoceptors; guinea-pig atrium; guinea-pig ileum; guinea-pig trachea; ICI-215001; relaxation

## Introduction

The subclassification of β-adrenoceptors into the two subgroups of $\beta_1$ and $\beta_2$ was based on the selectivity of action of different β-agonists as cardiac stimulants and bronchodilators, respectively (Lands *et al.*, 1967). Quantitative pharmacological experiments have shown the existence of adrenoceptors distinct from the defined α- and β-subtypes (Bond *et al.*, 1986; 1988). This 'atypical' β adrenoceptor is resistant to blockade by α-adrenoceptor antagonists and β-adrenoceptor antagonists (Bond *et al.*, 1988). Furthermore a human gene has been isolated from brown adipocytes which encodes for a β-adrenoceptor distinct from $\beta_1$- or $\beta_2$-receptors, and was referred to as 'the $\beta_3$-adrenergic receptor' (Emorine *et al.*, 1989; Krief *et al.*, 1993). The $\beta_3$-adrenoceptors have gained attention as potential therapeutic targets for specific agonists that might provide antiobesity, thermoregulatory or antidiabetic properties (Arch *et al.*, 1984).

Furthermore, others have reported a propranolol-resistant component to the relaxations of various sections of gastrointestinal tract induced by isoprenaline and other β-adrenoceptor agonists, which resembles the one described in brown adipocytes, implying that an 'atypical' β-adrenoceptor might be responsible for these effects (Bond *et al.*, 1988; Blue *et al.*, 1990; Taneja & Clarke, 1991). From the above account it is clear that the 'atypical' β-adrenoceptor is a pharmacologically defined entity and little information exists with regard to its functional characterization and the interaction of selective $\beta_3$-adrenoceptor agonists with other β-adrenoceptor types.

It has recently been reported that ICI-215001 and its prodrug form, ICI-D7114, {4-[2-[(2-hydroxy-3-phenoxypropyl) amino] ethoxy] phenoxyacetamides} have potent activity at 'atypical' β-adrenoceptors in brown adipocytes leading to an increased whole body temperature (Holloway *et al.*, 1991; Champigny *et al.*, 1992). It was of interest to characterize further $\beta_3$-adrenoceptor agonist activity of ICI-215001 and to evaluate whether it exhibits additional activities at $\beta_1$- and $\beta_2$-adrenoceptor subtypes. This task has been approached by examining its direct agonist activity in guinea-pig isolated ileum as well as by comparing its antagonist activity with competitive antagonists selective for $\beta_1$-adrenoceptors (atenolol) and $\beta_2$-adrenoceptors (ICI-118551) in isolated spontaneously beating atrium and tracheal preparations of guinea-pig (Lands *et al.*, 1986; Bilski *et al.*, 1983).

## Methods

### *Guinea atrium*

Male Hartley guinea-pigs (~300 g) were killed by exsanguination after asphyxia with carbon dioxide. The pericardium was carefully removed from the heart and the right atrium was dissected. A suture was tied to the upper and lower tip of the atrium. The spontaneously beating atrium was suspended between a fixed end and the distal end was connected to a force transducer for measurement of beating rate. Beating rates were determined by tachograph which integrated the beating rate to a linear scale on the recorder. The atria were placed in an organ bath filled with physiological salt solution containing (mM): NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, disodium EDTA 0.026 and glucose 5.5. The solutions were kept at 37°C and were continuously gassed with 95% $O_2$: 5% $CO_2$ to maintain the pH at 7.4. The resting tension was set at 1 g during a 1 h equilibration period. Cumulative concentration-response curves for the positive chronotropic effect of isoprenaline and ICI-215001 were determined. The beating rate was assessed 1 min after the addition of each successive concentration of β-adrenoceptor agonists. For assessment of antagonist activity, the responses of the atrium to isoprena-

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line were determined in the presence of ICI-215001 or atenolol (0.1–10 μM). Antagonists were added 30 min before addition of agonists.

*Guinea-pig trachea*

Tracheal strips (~5 mm long) were prepared and suspended between a fixed base and strain gauge for measurement of isometric circumferential force. The strips were placed in organ baths filled with physiological salt solution which were kept at 37°C and were continuously gassed with 95% $O_2$: 5% $CO_2$. The length of the smooth muscle was increased stepwise over 90 min to adjust basal tension to 2 g. This tension was found to be optimal for contractions of guinea-pig trachea by testing the contractions to potassium (80 mM). Once basal tension was established, the length of the strips was not altered thereafter. The tracheal strips were contracted with carbachol and then exposed to increasing half-log cumulative concentrations of agonists.

*Guinea-pig ileum*

After careful flushing of the luminal contents, the outer layer of ileum containing longitudinal smooth muscle was carefully removed with a cotton swab. Each strip (~3 cm long) was tied with a nylon suture at each end, and was mounted on a force transducer under an initial resting tension of 0.5 g, which was found to be optimal for contractions by testing repeated contractions to potassium (80 mM). To measure relaxations, strips were contracted with histamine and were then exposed to increasing cumulative concentration of agonists.

In guinea-pig trachea and ileum studies, phentolamine (10 μM) and indomethacin (3 μM) were added to block α-adrenoceptors and to inhibit rhythmic motility, respectively. In ileal preparations, atropine (0.1 μM) was added to block muscarinic receptors. The inhibitors had no effect on the resting or induced tone of tissues.

*Drugs*

The pharmacological agents used were the following: atenolol, (−)-alprenolol, atropine, carbachol, histamine, indomethacin, (−)-isoprenaline, phenylephrine, phentolamine, salbutamol, sodium nitroprusside (Sigma Chemical, St. Louis, MO, U.S.A.), ICI-215001 and ICI-118551 {erythro-1-(7-methylindan-4-yloxy)-3-isopropylamino-butan-2-ol} (imperial Chemical Industries, Macclesfield, England). Unless otherwise specified, drugs were dissolved in distilled water. ICI-215001 was solubilized in dimethyl sulphoxide and further dilutions were made in water. Indomethacin was prepared in 2% $Na_2CO_3$.

*Data analysis*

Changes in sinus rate are expressed as a percentage of the maximum increase in beating rate caused by isoprenaline (10 μM). Relaxations are expressed as percentage decrease in tension from the level of induced tone. The half-maximal inhibitory concentration ($IC_{50}$) was determined graphically as the concentration causing 50% of the maximal relaxation. Antagonist potencies were evaluated by calculating their $pA_2$ values. Concentration-response curves for agonists were made in the presence of three different concentrations of antagonist. Schild plots were constructed from the individual experiments and the $pA_2$ values were calculated. (Arunlakshana & Schild, 1959). Antagonism was considered to be competitive if the slope of the regression line did not significantly differ from unity. The values are expressed as means ± s.e. Statistical evaluation of the data was made using repeated measures of analysis of variance or Student's *t* test for paired comparisons of mean values. Values with *P* less than 0.05 were regarded as significant. In all experiments, *n* equals the number of guinea-pigs from which the tissues were taken.

## Results

*Guinea-pig atrium*

Isoprenaline (0.1 nM–10 μM), a non-selective β-adrenoceptor agonist, had a concentration-dependent positive chronotropic effect on the atrium ($EC_{50}$: 7.1 ± 1.2 nM, $n = 12$). Atenolol (0.1–10 μM) shifted the isoprenaline concentration-response curves, resulting in Schild plots with slopes not significantly different from unity ($pA_2$: 6.1 ± 0.2; with isoprenaline as the agonist). ICI-215001 (0.1, 1 and 10 μM) also competitively antagonized the increase in beating rate caused by isoprenaline, yielding a Schild plot with a slope not significantly different from unity and a $pA_2$ value of 6.7 ± 0.2, $n = 7$ (Figure 1). In the guinea-pig atrium, ICI-215001 (0.1, 1, 10 μM) had weak agonist activity (6.1 ± 2.6, 11 ± 2.6 and 11 ± 6.4% maximal isoprenaline, respectively, $n = 7$).

*Guinea-pig trachea*

In strips of guinea-pig trachea contracted submaximally with carbachol (1 μM), isoprenaline (0.1 nM–1 μM) caused concentration-dependent relaxations ($IC_{50}$: 6.1 ± 2.5 nM, $n = 10$). These relaxations were competitively antagonized by ICI-118551 ($pA_2$: 7.4 ± 0.8). In the guinea-pig trachea, ICI-215001 (0.1, 1, 10 μM) produced competitive antagonism of isoprenaline-induced relaxations yielding a Schild plot with a slope not significantly different from unity and a $pA_2$ value of 7.3 ± 0.4, $n = 7$ (Figure 2). ICI-215001 (0.1–10 μM) had no effect on the resting or induced tone of tracheal strips.

*Guinea-pig ileum*

In strips of guinea-pig ileum longitudinal smooth muscle contracted with histamine (10 μM), isoprenaline (0.01–100 μM), caused concentration-dependent relaxations ($IC_{50}$: 31 ± 0.8 nM, $n = 7$). These relaxations were significantly inhibited by treatment of strips with (−)-alprenolol (1, 10 μM) (Figure 3). The inhibition was more marked with the higher concentration of (−)-alprenolol (10 μM). In contrast, isoprenaline-induced relaxations were resistant to treatment of strips with atenolol (10 μM) or ICI-118551 (10 μM). In guinea-pig ileum longitudinal muscle, ICI-215001 (0.01–10 μM) caused concentration-dependent relaxations ($IC_{50}$: 316 ± 0.6 nM, $n = 8$) (Figure 4). These relaxations were markedly inhibited by (−)-alprenolol (1, 10 μM) (Figure 4). By contrast, (−)-alprenolol (10 μM) did not have any significant effect on the

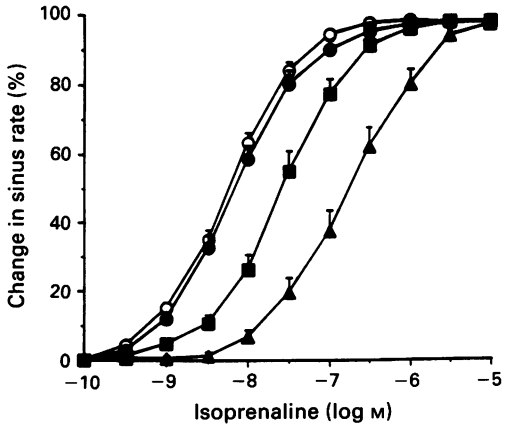


**Figure 1** In control (○) spontaneously beating guinea-pig atrium, isoprenaline caused a concentration-dependent positive chronotropic effect. ICI-215001 in concentrations 0.1 μM (●), 1 μM (■) and 10 μM (▲) competitively antagonized the increase in sinus rate caused by isoprenaline, resulting in a parallel right-ward shift of the concentration-response curves. Values are expressed as maximal response to isoprenaline (10 μM), means ± s.e., $n = 12$.

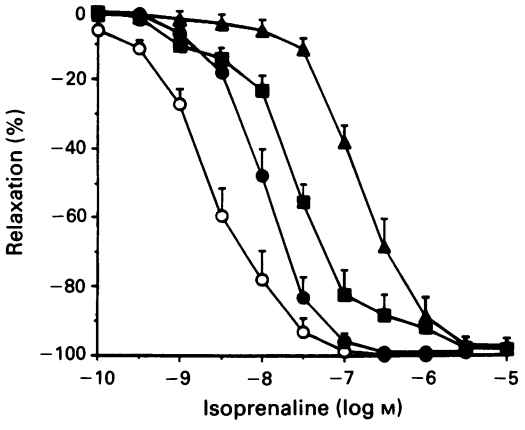


**Figure 2** In control (○) guinea-pig tracheal strips contracted with carbachol (1 μM), isoprenaline caused concentration-dependent relaxations. These relaxations were competitively antagonized by ICI-215001 in concentrations 0.1 μM (●), 1 μM (■) and 10 μM (▲). $n = 7$.

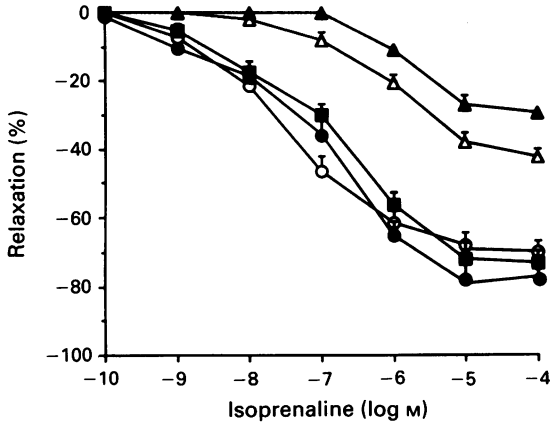


**Figure 3** In control (○) isolated longitudinal muscle of guinea-pig ileum contracted with histamine (10 μM), isoprenaline caused concentration-dependent relaxations. These relaxations were significantly ($P < 0.05$) inhibited by alprenolol in concentrations 1 μM (▲) and 10 μM (△) but not by atenolol 10 μM (■) or ICI-118551 10 μM (●). $n = 7$.

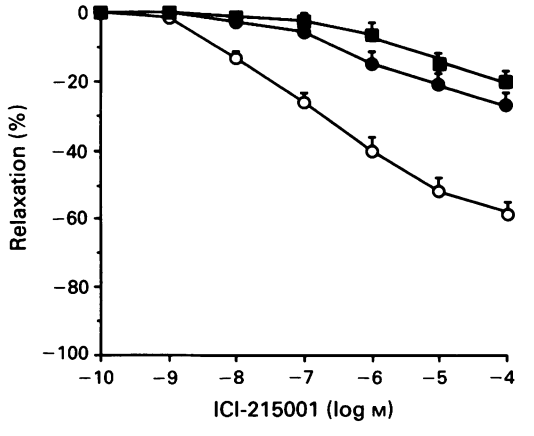


**Figure 4** In control (○) isolated longitudinal muscle of guinea-pig ileum contracted with histamine (10 μM), ICI-215001 caused concentration-dependent relaxations, that were significantly ($P < 0.05$) inhibited by alprenolol at concentrations of 1 μM (●) and 10 μM (■). $n = 8$.

relaxations caused by sodium nitroprusside (1 μM, in absence and presence of alprenolol: 99 ± 0.9 vs 97 ± 2.1%, respectively, $n = 6$). In guinea-pig ileum longitudinal smooth muscle, salbutamol, a selective $\beta_2$-adrenoceptor agonist (0.1 nM–100 μM), did not cause any relaxations (data not shown, $n = 4$).

## Discussion

In agreement with previous reports, isoprenaline-induced positive chronotropic effects were competitively antagonized by the selective $\beta_1$-adrenoceptor antagonist, atenolol, indicating that they are mediated by stimulation of $\beta_1$-adrenoceptors in the atrium (Lands *et al.*, 1967). An interesting finding in the present study is that ICI-215001 competitively antagonized the increase in beating rate caused by isoprenaline suggesting that ICI-215001 possesses $\beta_1$-adrenoceptor antagonist activity. Similarly, others have also shown that ICI-D7114, the prodrug form of ICI-215001, caused inhibition of responses to isoprenaline in guinea-pig atrium (Growcott *et al.*, 1993). In this regard, the affinity of ICI-215001 for $\beta_1$-adrenoceptors was compaable to that of atenolol. ICI-215001 exhibited low intrinsic activity on $\beta_1$-adrenoceptors as demonstrated by its weak effect on the beating rate of the atrium. In contrast to ICI-215001, another $\beta_3$-agonist, BRL-37344, has been reported to cause $\beta_1$-adrenoceptor-mediated positive chronotropic and inotropic responses in dog atria (Takayama *et al.*, 1993).

The inhibition by the selective $\beta_2$-antagonist, ICI-118551, of the relaxations of trachea caused by isoprenaline demonstrates that the relaxations were mediated by stimulation of $\beta_2$-adrenoceptors. The competitive antagonism by ICI-215001 of the relaxations caused by isoprenaline in trachea indicates that ICI-215001 exhibits affinity for $\beta_2$-adrenoceptors. In this regard, ICI-215001 and ICI-118551 showed similar affinity for $\beta_2$-adrenoceptors. Further, the absence of any effect of ICI-215001 in resting or contracted tracheal strips indicates its lack of direct intrinsic activity on $\beta_2$-adrenoceptors.

In an attempt to characterize further the 'atypical' β-adrenoceptors, we used the guinea-pig ileum which has been previously described as revealing the 'atypical' β-adrenoceptor (Bond *et al.*, 1988). Prior studies have suggested that $\beta_2$-adrenoceptors are located on circular smooth muscle or epithelial cells, whereas 'atypical' β-adrenoceptors are probably distributed on the longitudinal smooth muscle of the ileum (Van Der Vliet *et al.*, 1990). In the present study, efforts were made to confirm whether 'atypical' β-adrenoceptors mediate relaxations of the longitudinal muscle of the ileum. Indeed isoprenaline-induced relaxations were resistant to the selective $\beta_1$- and $\beta_2$-antagonists, implying that the conventional $\beta_1$- and $\beta_2$-adrenoceptors play no role in the inhibitory response produced by isoprenaline in this preparation. In addition, the lack of effect of salbutamol rules out $\beta_2$-adrenoceptor-mediated responses. Like isoprenaline, ICI-215001 also caused relaxation of the ileum suggesting that the relaxations are probably mediated by activation of 'atypical' β-adrenoceptors. This suggestion is strengthened by the blockade of isoprenaline- and ICI-215001-induced relaxations by (−)-alprenolol, a compound which exhibits moderate affinity for 'atypical' β-adrenoceptors (Blue *et al.*, 1990). The lack of effect of (−)-alprenolol on the relaxations caused by nitroprusside excludes a non-specific effect. These findings are in agreement with previous reports which have shown that relaxations induced by electrical stimulation of sympathetic neurones were blocked by (−)-alprenolol, indicating that the responses may be mediated via activation of $\beta_3$-adrenoceptors (Blue *et al.*, 1990; Taneja & Clarke, 1991). Furthermore, the current study has demonstrated that the 'atypical' β-adrenoceptors are distributed predominantly on the longitudinal smooth muscle cells which lie adjacent to the myenteric plexus. Whether the atypical β-adrenoceptors in the longitudinal muscle are the same as those $\beta_3$-receptors described in brown adipocytes is unknown. Because the 'atypical' β-

adrenoceptors have been shown to receive adrenergic innervation, this raises the possibility that these receptors may be a target site for modulating gut motility (Taneja & Clarke, 1991).

In summary, the present study has further characterized atypical β-adrenoceptors on the longitudinal smooth muscle of guinea-pig ileum in terms of the lack of affinity of $\beta_1$- and $\beta_2$-adrenoceptor antagonists. In addition to its agonist activity at atypical β-adrenoceptors, ICI-215001 also exhibits antagonist activity at $\beta_1$- and $\beta_2$-adrenoceptors.

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# Ontogenic increase in $PGE_2$ and $PGF_{2\alpha}$ receptor density in brain microvessels of pigs

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**1** The hypothesis that the relative vasoconstrictor ineffectiveness of prostaglandin $E_2$ ($PGE_2$) and $PGF_{2\alpha}$ on cerebral vessels of newborn pigs might be due to fewer receptors for these prostanoids was tested by comparing receptors for $PGE_2$ (EP) and $PGF_{2\alpha}$ (FP) in cerebral microvessels from newborn and adult pigs.

**2** Specific binding of [$^3$H]-$PGE_2$ and [$^3$H]-$PGF_{2\alpha}$ to membranes prepared from brain microvessels showed that EP and FP receptor density ($B_{max}$) in tissues from newborn animals was less than 50% of that determined in tissues from adults. By contrast, estimates of affinity ($K_D$) were unchanged.

**3** Specifically bound [$^3$H]-$PGE_2$ to brain microvessels from both the newborn and adult was displaced by AH 6809 ($EP_1$-selective antagonist) by 80–90%, and only by approximately 30–35% by both 11-deoxy $PGE_1$ ($EP_2/EP_3$ agonist) and M&B 28,767 ($EP_3$ agonist); butaprost ($EP_2$ agonist) was completely ineffective.

**4** $PGE_2$, 17-phenyl trinor $PGE_2$ ($EP_1$ agonist), $PGF_{2\alpha}$ and fenprostalene ($PGF_{2\alpha}$ analogue) caused significantly less increase in inositol 1,4,5-triphosphate ($IP_3$) in brain microvessels from the newborn than in those from adult pigs. The stimulation of $IP_3$ by $PGE_2$ and 17-phenyl trinor $PGE_2$ was almost completely inhibited by the $EP_1$ antagonist, AH 6809.

**5** $PGE_2$, 11-deoxy $PGE_1$ and M&B 28,767 produced small reduction of adenosine 3':5'-cyclic monophosphate (cyclic AMP) production in adult vessels but no effect in newborn tissues.

**6** The lower density of EP and FP receptors in microvessels of newborn pigs compared to adults may explain the reduced ability of $PGE_2$ and $PGF_{2\alpha}$ to stimulate production of $IP_3$ in tissues from newborn animals. This in turn, may provide an explanation for previous observations demonstrating that these prostanoids elicit contraction of adult cerebral microvessels, but exert minimal effects on these vessels in newborn animals.

**Keywords:** $PGE_2$ receptors; $PGF_{2\alpha}$ receptors; prostaglandin $E_2$; prostaglandin $F_{2\alpha}$; inositol 1,4,5-triphosphate; adenosine 3',5'-cyclic monophosphate; microvessels; ontogeny of prostanoid receptors

## Introduction

Prostaglandin $E_2$ ($PGE_2$) and $PGF_{2\alpha}$ are major prostanoids in the brain and the cerebral vasculature (Gaudet *et al.*, 1980; White & Hagen, 1982; Chemtob *et al.*, 1990a,c). Concentrations of these prostanoids in the blood and brain are much higher in the perinatal period than in adult life (Mitchell *et al.*, 1978; Jones *et al.*, 1993). $PGE_2$ and $PGF_{2\alpha}$ have been implicated in various cerebral functions including the autoregulation of blood flow (Chemtob *et al.*, 1990b). However, there are major differences in the vasoactive responses to $PGE_2$ and $PGF_{2\alpha}$ during development. Indeed $PGE_2$ and $PGF_{2\alpha}$ are potent cerebral vasoconstrictors in adult animals and man (White & Hagen, 1982; Hayashi *et al.*, 1985; Hadházy *et al.*, 1988) but exert minimal constrictor effects on the cerebral vasculature of the newborn (Hayashi *et al.*, 1985; Leffler & Busija, 1987; Chemtob *et al.*, 1989). The mechanisms underlying these age-dependent differences in the actions of $PGE_2$ and $PGF_{2\alpha}$ on the cerebral vasculature are not known.

Prostanoids exert their effects through specific receptors, defined as EP receptors for $PGE_2$ and FP receptors for $PGF_{2\alpha}$ (Kennedy *et al.*, 1982; Sasaki *et al.*, 1985; Coleman, 1987; Santoian *et al.*, 1989; Abran *et al.*, 1994). EP receptors are further classified into $EP_1$, $EP_2$ and $EP_3$ subtypes (Coleman *et al.*, 1987; Coleman, 1987; Halushka *et al.*, 1989; Eglen & Whiting, 1989). Activation of FP and $EP_1$ receptors increases inositol 1,4,5-trisphosphate ($IP_3$) production (Suba & Roth, 1987; Halushka *et al.*, 1989) whereas $EP_2$ receptors cause an increase and $EP_3$ receptors lead to a decrease in adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation (Jumblatt & Paterson, 1991; Sugimoto *et al.*, 1992). An increase in $IP_3$ has been shown to be associated with vascular contraction (Suba & Roth, 1987; Heaslip & Sickels, 1989), whereas a rise in cyclic AMP is often observed with vasodilatation (Lincoln & Cornwell, 1991). Activation of EP and FP receptors by $PGE_2$ and $PGF_{2\alpha}$ on cerebral vessels elicits significant contraction in the adult but minimal effects in the newborn (Hayashi *et al.*, 1985; Chemtob *et al.*, 1989). Differences in cerebral vascular FP and EP receptors and their subtypes and/or in receptor-second messenger coupling between the newborn and adult may explain the differences in the vasomotor effects of $PGE_2$ and $PGF_{2\alpha}$ with age. In order to test this hypothesis, we measured $PGF_{2\alpha}$ and $PGE_2$ receptors and their subtypes as well as the effects of $PGE_2$ and $PGF_{2\alpha}$ and their analogues on $IP_3$ and cyclic AMP production in brain microvessels from newborn and adult pigs.

## Methods

### *Animals*

Animals were used in accordance with a protocol of the Animal Care Committee of Ste. Justine Hospital. Newborn pigs (1–3 days old) were obtained from Fermes Ménard Inc., Quebec, Canada. Newborn animals were killed with an intracardiac injection of pentobarbitone (100 mg kg$^{-1}$) under halothane anaesthesia and brains were removed. Brains from adult pigs (5–7 months old) were collected from a local

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abattoir (Bienvenue-Olympia, St. Valérien, Quebec, Canada) immediately after they had been killed and transported to the laboratory on dry ice.

### *Preparation of brain microvessels*

Brain microvessels were prepared according to the method described by Nathanson (1980). Briefly, brains were homogenized in 5 mM Tris-HCl buffer (pH 7.4) containing 1.1 mM ASA, 0.5 mM EGTA, 1 mM benzamidine, 0.1 mM PMSF and 100 $\mu g\ ml^{-1}$ soybean trypsin inhibitor. The homogenate was filtered through a nylon mesh filter (200 $\mu m$) and rinsed with the above buffer. Microvessels were collected from the nylon mesh, resuspended in the above buffer, rehomogenized and filtered as above; vessels $\geqslant 200\ \mu m$ have been shown to exert a major contribution in the control of cerebral blood flow primarily at the upper limit of autoregulation (Baumbach & Heistad, 1985). The purified microvessels were homogenized with a hand pestle and then centrifuged at 1,000 $g$ for 15 min. The supernatant was recentrifuged at 100,000 $g$ for 45 min and the pellet was stored at − 80°C until used for the experiments. The purity of the microvessel preparation was confirmed by microscopy and the higher activity of gamma-glutamyl transpeptidase (measured by the method of Sasz, 1969), in cerebral microvessels compared to brain tissue (brain parenchyma: 0.29–0.36 mu $mg^{-1}$ protein; brain microvessels: 5.6–6.1 mu $mg^{-1}$ protein) (Goldstein *et al.*, 1975).

### *Receptor assays*

Binding of [$^3$H]-$PGE_2$ and [$^3$H]-$PGF_{2\alpha}$ was performed as previously described (Li *et al.*, 1994). To determine [$^3$H]-$PGE_2$ and [$^3$H]-$PGF_{2\alpha}$ binding as a function of membrane protein, aliquots of microvessel preparations containing 100–400 $\mu g$ protein were incubated at 37°C for 30 min in 100 $\mu l$ of 10 mM sodium phosphate buffer (pH 7.4) and 5 nM [$^3$H]-$PGE_2$ or [$^3$H]-$PGF_{2\alpha}$ in the absence or the presence of excess (25 $\mu M$) unlabelled $PGE_2$ or $PGF_{2\alpha}$. For time-course of association and dissociation, membranes were incubated with 5 nM [$^3$H]-$PGE_2$ or [$^3$H]-$PGF_{2\alpha}$ for up to 30 min in the absence or the presence of 25 $\mu M$ unlabelled $PGE_2$ or $PGF_{2\alpha}$. At this point, 25 $\mu M$ $PGE_2$ or $PGF_{2\alpha}$ was added to the tubes which had been incubated with 5 nM [$^3$H]-$PGE_2$ or [$^3$H]-$PGF_{2\alpha}$ alone and dissociation was determined at different times.

Saturation experiments were performed by incubating microvessel membranes with increasing concentrations of [$^3$H]-$PGE_2$ or [$^3$H]-$PGF_{2\alpha}$ in the absence or the presence of 25 $\mu M$ unlabelled $PGE_2$ or $PGF_{2\alpha}$. To demonstrate the specificity and subtypes of $PGE_2$ or $PGF_{2\alpha}$ receptors, we determined the displacement of [$^3$H]-$PGE_2$ binding by increasing concentrations of unlabelled $PGE_2$, 16,16-dimethyl $PGE_2$ ($PGE_2$ analogue non-selective for $PGE_2$ receptor subtypes), U46619 (stable thromboxane analogue), AH 6809 ($EP_1$ antagonist), 11-deoxy $PGE_1$ ($EP_2$ and $EP_3$ agonist), butaprost ($EP_2$-selective agonist) and M&B 28,767 ($EP_3$-selective agonist) (Coleman *et al.*, 1987; Coleman, 1987; Lawrence *et al.*, 1992) and that of [$^3$H]-$PGF_{2\alpha}$ binding by $PGF_{2\alpha}$ and its analogue, fenprostalene, $PGE_2$ and U46619. Receptor densities ($B_{max}$) and dissociation constants ($K_d$) were determined from the saturation isotherms by Scatchard analysis using the Ligand programme (Munson & Rodbard, 1980), and fitting of the saturation and displacement curves were obtained by use of the Ligand and ReceptorFit (Lundon Software, Chagrin Falls, OH, U.S.A.) programmes. Receptor density was also determined from the displacement curves using the formula: $B_{max} = Bo \times (IC_{50}/L)$ (Deblasi *et al.*, 1989).

### *Cyclic AMP and $IP_3$ assays*

Cyclic AMP and $IP_3$ were assayed as previously described (Li *et al.*, 1994). For cyclic AMP stimulation experiments, microvessel membranes (100 $\mu g$ protein) were incubated at 37°C for 10 min in an assay mixture (100 $\mu l$) containing 10 mM Tris-HCl buffer (pH 8.0), 1 mM adenosine triphosphate, 7.5 mM $MgCl_2$, 15 mM creatine phosphate, 185 u $ml^{-1}$ creatine phosphokinase, 200 $\mu g\ ml^{-1}$ ASA, 0.5 mM EGTA, 0.5 mM isobutyl methylxanthine, 1 mM dithiothreitol, 1 mM benzamidine, 0.1 mM PMSF and 100 $\mu g\ ml^{-1}$ soybean trypsin inhibitor in the absence or the presence of test agents. The

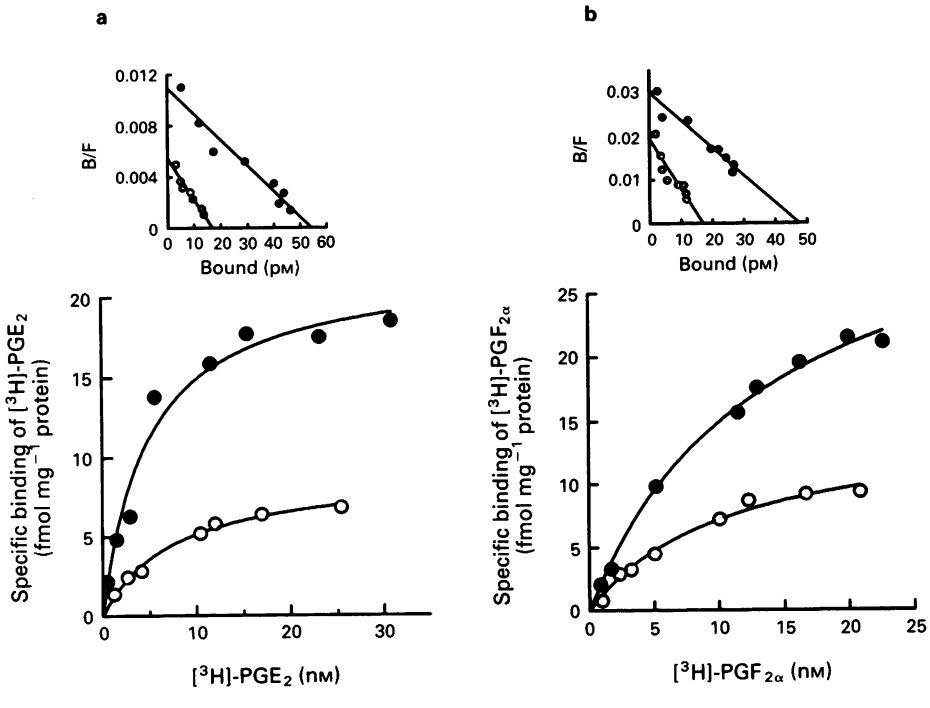


**Figure 1** Representative saturation curves and Scatchard plots of (a) [$^3$H]-prostaglandin $E_2$ ([$^3$H]-$PGE_2$) and (b) [$^3$H]-$PGF_{2\alpha}$ specific binding to brain microvessel membranes from newborn (○) and adult pigs (●). Microvessel membranes were incubated with 0 to 25 nM [$^3$H]-$PGE_2$ or [$^3$H]-$PGF_{2\alpha}$ at 37°C for 30 min in the absence or the presence of 25 $\mu M$ unlabelled $PGE_2$ or $PGF_{2\alpha}$, respectively. B/F: bound/free.

reaction was terminated with 200 μl acidic ethanol (1 N HCl: ethanol, 1:100) and allowed to stand at room temperature for 5 min before being centrifuged at 1,000 *g* for 10 min. The supernatant was collected. The pellet was washed and centrifuged again. Both supernatants were pooled, vacuum dried and stored at − 80°C until assayed for cyclic AMP with a commercial kit (Amersham, Mississauga, Ontario, Canada). The recovery of cyclic AMP was >90%.

For $IP_3$ stimulation experiments, microvessel membrane preparations (200 μg protein) were incubated at 37°C for 10 min in 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid) buffer (pH 7.4) containing (mM): NaCl 108, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.18, LiCl 10.0, disodium edetate 0.045, dithiothreitol 1.0, benzamidine 1.0 in the absence or the presence of test agents. The reaction was terminated by the addition of 0.2 volume ice-cold 20% (v/v) $HClO_4$ and kept on ice for 20 min. Proteins were sedimented by centrifugation at 2,000 *g* for 15 min at 4°C. The supernatant was titrated to pH 7.5 with KOH (1.5 M) and centrifuged again. The supernatant was evaporated under vacuum and stored at − 80°C until assayed for $IP_3$ with a commercial radioimmunoassay kit (Amersham, Mississauga, Ontario, Canada). The recovery of $IP_3$ was >95%; no correction was made for $IP_3$ and cyclic AMP recovery. Net increase in $IP_3$ and cyclic AMP in pmol $mg^{-1}$ protein $min^{-1}$ was calculated by correcting for basal $IP_3$ and cyclic AMP synthesis. Proteins were measured by the dye-binding technique (Bradford, 1976) with bovine serum albumin used as the standard.

### *Chemicals*

The following agents were generous gifts: AH 6809 (6-isopropoxy-9 oxoxanthen-2-carboxylic acid) from Glaxo, U.K., butaprost from Miles, U.S.A. and M&B 28,767 (15S-hydroxy-9-oxo-16-phenoxy-ω-tetranorprost-13E-enoic acid) from Rhone-Poulenc Rorer, U.K. The following products were purchased: [$^3$H]-$PGE_2$ (191 Ci $mmol^{-1}$), [$^3$H]-$PGF_{2\alpha}$ (219 Ci $mmol^{-1}$) (Amersham, Mississauga, Ontario, Canada); soybean trypsin inhibitor (type II-S), phenylmethylsulphonylfluoride (PMSF), acetylsalicylic acid (ASA), benzamidine and ethylene-*bis*(oxyethylenenitrilo)tetraacetic acid (EGTA) (Sigma Chemicals, St. Louis, Missouri, U.S.A.); U46619 (7-(6-(3-hydroxy-1-octenyl)-2-oxabicyclo-hept-5-yl)-5-heptenoic acid), $PGE_2$, $PGF_{2\alpha}$, 16,16-dimethyl $PGE_2$, 17-phenyl trinor $PGE_2$ and 11-deoxy $PGE_1$ (Cayman Chemicals, Ann Arbor, Michigan, U.S.A.); fenprostalene (Syntex, Mississauga, Ontario, Canada); all other high purity chemicals (Fisher, Montreal, Quebec, Canada).

Stock solution of prostanoids (1 mM) were prepared in ethanol and stored at − 20°C. Upon use, the solutions were evaporated under 100% nitrogen and diluted with the appropriate buffer for the experiments in which they were used.

### *Statistics*

Data from adult and newborn animals were compared by Student's unpaired *t* test and significance was set at $P<0.05$. Throughout this paper data are presented as mean ± s.e. mean.

## Results

### *Prostanoid receptors*

The specific binding of [$^3$H]-$PGE_2$ and [$^3$H]-$PGF_{2\alpha}$ to brain microvessel membrane preparations increased linearly as a function of membrane protein concentration (100–400 μg); experiments were performed in the presence of 250 μg of protein which yielded reproducible radioactive counts even at low concentrations of the ligand. The specific binding of [$^3$H]-$PGE_2$ and [$^3$H]-$PGF_{2\alpha}$ reached equilibrium within 10 min and was stable for approximately 40 min. The dissociation of bound [$^3$H]-$PGE_2$ and [$^3$H]-$PGF_{2\alpha}$ was complete within 10 min after the addition of excess of $PGE_2$ and $PGF_{2\alpha}$, respectively.

The specific binding of [$^3$H]-$PGE_2$ and [$^3$H]-$PGF_{2\alpha}$ was

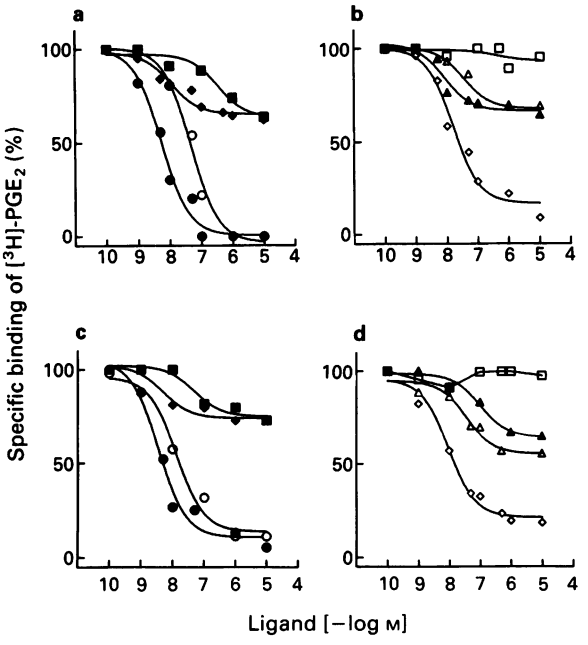


**Figure 2** Competitive displacement of specific binding of [$^3$H]-prostaglandin $E_2$ ([$^3$H]-$PGE_2$) to brain microvessel membranes from newborn (a and b) and adult (c and d) pigs by prostanoids and analogues. Microvessel membranes were incubated with 8 nM [$^3$H]-$PGE_2$ at 37°C for 30 min in the absence or the presence of unlabelled $PGE_2$ (●), 16,16-dimethyl $PGE_2$ (○), $PGF_{2\alpha}$ (◆), AH 6800 (◇), M&B 28,767 (▲), 11-deoxy $PGE_1$ (△), U46619 (■) and butaprost (□). Displacement by 25 μM unlabelled $PGE_2$, which was maximal, was treated as 100% specific binding. Each point is the mean of three separate experiments, each performed in duplicate.

**Table 1** Specific binding of [$^3$H]-prostaglandin $E_2$ ([$^3$H]-$PGE_2$) and [$^3$H]-$PGF_{2\alpha}$ to brain microvessels from newborn and adult pigs

| *Ligand* | *Derived by* | *Variables* | *Newborn* | *Adult* |
|---|---|---|---|---|
| $PGE_2$ | Scatchard | $B_{max}$ (fmol $mg^{-1}$ protein) | 11.7 ± 1.8* | 25.5 ± 1.0 |
| $PGE_2$ | Displacement | $B_{max}$ (fmol $mg^{-1}$ protein) | 12.2 ± 1.6* | 27.8 ± 2.9 |
| $PGE_2$ | Scatchard | $K_d$ (nM) | 6.0 ± 1.2 | 6.8 ± 0.4 |
| $PGE_2$ | Displacement | $IC_{50}$ (nM) | 6.3 ± 2.8 | 7.2 ± 1.8 |
| $PGF_{2\alpha}$ | Scatchard | $B_{max}$ (fmol $mg^{-1}$ protein) | 11.4 ± 1.6* | 30.5 ± 3.1 |
| $PGF_{2\alpha}$ | Displacement | $B_{max}$ (fmol $mg^{-1}$ protein) | 13.9 ± 1.4* | 27.9 ± 2.5 |
| $PGF_{2\alpha}$ | Scatchard | $K_d$ (nM) | 10.1 ± 1.5 | 13.0 ± 1.8 |
| $PGF_{2\alpha}$ | Displacement | $IC_{50}$ (nM) | 9.3 ± 0.8 | 9.8 ± 1.4 |

Values are the mean ± s.e.mean of four experiments, each performed in duplicate. Variables were derived by Scatchard analysis or from displacement curves. *Significantly ($P<0.01$) different from the corresponding value for the adult.

saturable (Figure 1). The maximum number of binding sites for [$^3$H]-$PGE_2$ and [$^3$H]-$PGF_{2\alpha}$ in microvessels from the adult pigs was significantly ($P<0.01$) greater than that in preparations from newborn animals; there was no significant different in $K_d$ values (Table 1).

The binding of [$^3$H]-$PGE_2$ to preparations from both newborn and adult pigs was inhibited by $PGE_2$ and its analogue, 16,16-dimethyl $PGE_2$ but only slightly by $PGF_{2\alpha}$ and U46619 (Figure 2). The $EP_1$ receptor antagonist, AH 6809, displaced bound [$^3$H]-$PGE_2$ by 82–91% on newborn ($IC_{50}$ = 11.4 ± 2.5 nM) and adult ($IC_{50}$ = 8.9 ± 1.3 nM) microvessel membranes. The $EP_2$ and $EP_3$ receptor agonist, 11-deoxy $PGE_1$ ($IC_{50}$: newborn: 34.7 ± 2.8 nM, adult: 31.7 ± 6.7 nM), as well as $EP_3$ receptor agonist, MB 28,767 ($IC_{50}$: newborn: 32.8 ± 7.4 nM, adult: 44.0 ± 9.2 nM), displaced bound [$^3$H]-$PGE_2$ by 31–36%; the $EP_2$ receptor agonist, butaprost, was completely ineffective. The determination of receptor density from the displacement curves revealed that $EP_1$ receptors (displacement by the $EP_1$ antagonist, AH 6809) comprised 81.2% of the total number of $PGE_2$ receptors in the newborn and 82.4% of that in adult pig microvessels. The total number of receptors calculated from displacement curves was very similar to $B_{max}$ values determined from the Scatchard plots (Table 1).

On microvessel preparations from both newborn and adult pigs, the specific binding of [$^3$H]-$PGF_{2\alpha}$ was competitively inhibited by $PGF_{2\alpha}$ and its analogue, fenprostalene, but only slightly by $PGE_2$ and U46619 (Figure 3); $B_{max}$ values derived from displacement curves were simiar to the $B_{max}$ calculated from the Scatchard plots (Table 1).

### *$IP_3$ and cyclic AMP production*

$PGE_2$, 17-phenyl trinor $PGE_2$ ($EP_1$ agonist), $PGF_{2\alpha}$ and its analogue, fenprostalene, caused a significantly ($P<0.01$) greater stimulation of $IP_3$ in brain microvessels from the adult than from the newborn pigs (Table 2). The $IP_3$ stimulation by $PGE_2$ and 17-phenyl trinor $PGE_2$ was almost completely blocked by AH 6809 ($EP_1$ receptor antagonist).

In adult brain microvessels, $PGE_2$, 11-deoxy $PGE_1$ and M&B 28,767 inhibited cyclic AMP production albeit to a small extent; but in newborn vessels these drugs did not significantly affect cyclic AMP production (Table 3). As expected, $PGF_{2\alpha}$ had no effect on cyclic AMP synthesis. NaF caused a similar stimulation of cyclic AMP production in the newborn and adult brain microvessels.

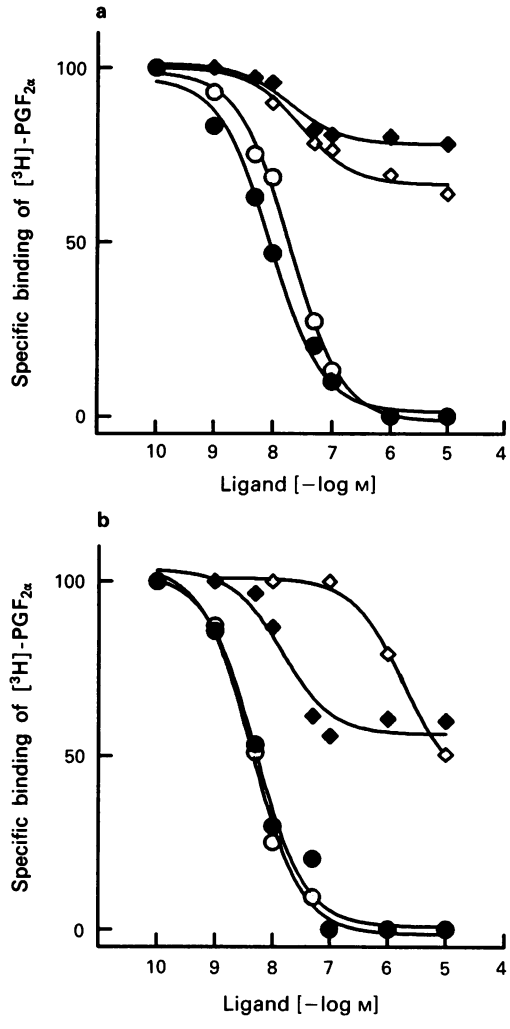


**Figure 3** Competitive displacement of specific binding of [$^3$H]-prostaglandin $F_{2\alpha}$ ([$^3$H]-$PGF_{2\alpha}$) to brain microvessel membranes from newborn (a) and adult (b) pigs by prostanoids and analogues. Microvessel membranes were incubated with 8 nM [$^3$H]-$PGF_{2\alpha}$ at 37°C for 30 min in the absence or the presence of unlabelled $PGF_{2\alpha}$ (●), fenprostalene (○), $PGE_2$ (◆) and U46619 (◇). Displacement by 25 μM unlabelled $PGF_{2\alpha}$, which was maximal, was treated as 100% specific binding. Each point is the mean of three separate experiments, each performed in duplicate.

**Table 2** Effect of prostaglandin $E_2$ ($PGE_2$) and its analogue, 17-phenyl trinor $PGE_2$, in the absence and the presence of $EP_1$ receptor antagonist, AH 6809, and of $PGF_{2\alpha}$ and its analogue, fenprostalene, on net inositol 1,4,5-triphosphate ($IP_3$) production by brain microvessels from newborn and adult pigs

| *Test agents* | *Newborn* Net $IP_3$ production (pmol $mg^{-1}$ protein $min^{-1}$) | *Adult* |
|---|---|---|
| $PGE_2$ (0.1 μM) | 3.0 ± 0.9* | 13.7 ± 1.3 |
| $PGE_2$ (0.1 μM) + AH 6809 (10 μM) | 0.4 ± 0.1** | 2.2 ± 0.6** |
| $PGE_2$ (1 μM) | 3.8 ± 0.9* | 16.2 ± 0.9 |
| $PGE_2$ (1 μM) + AH 6809 (10 μM) | 1.1 ± 0.8** | 2.0 ± 0.3** |
| 17-Phenyl trinor $PGE_2$ (0.1 μM) | 4.2 ± 2.0* | 13.0 ± 2.1 |
| 17-Phenyl trinor $PGE_2$ (0.1 μM) + AH 6809 (10 μM) | 1.6 ± 0.8** | 1.1 ± 0.2** |
| 17-Phenyl trinor $PGE_2$ (1 μM) | 5.8 ± 0.9* | 15.2 ± 0.2 |
| 17-Phenyl trinor $PGE_2$ (1 μM) + AH 6809 (10 μM) | 1.6 ± 0.8** | 1.8 ± 0.4** |
| $PGF_{2\alpha}$ (0.1 μM) | 4.9 ± 1.2* | 25.9 ± 2.2 |
| $PGF_{2\alpha}$ (1 μM) | 6.0 ± 2.4* | 33.3 ± 4.6 |
| Fenprostalene (0.1 μM) | 5.6 ± 1.1* | 14.5 ± 1.7 |
| Fenprostalene (1 μM) | 7.8 ± 1.7* | 24.6 ± 3.7 |
| Noradrenaline (1 μM) | 5.0 ± 0.5* | 29.9 ± 4.7 |

Values are mean ± s.e.mean of four separate experiments, each performed in duplicate. The net $IP_3$ production was $IP_3$ after the agent minus basal $IP_3$ synthesis, which was (pmol $mg^{-1}$ protein $min^{-1}$) 1.7 ± 0.7 by microvessels from newborn and 3.4 ± 0.2 by tissues from adult pigs. *Significantly ($P<0.01$) different from corresponding values for the adult; **Significantly ($P<0.01$) different from the value immediately above.

**Table 3** Effect of different agents on net adenosine 3':5'-cyclic monophosphate (cyclic AMP) production by brain microvessels from newborn and adult pigs

| *Agents* | *Newborn* Net cyclic AMP synthesis (pmol $mg^{-1}$ protein $min^{-1}$) | *Adult* |
|---|---|---|
| $PGE_2$ (1 μM) | − 1.9 ± 2.3 | − 3.8 ± 0.3* |
| 11-deoxy $PGE_1$ (1 μM) | − 2.9 ± 2.0 | − 3.6 ± 0.8* |
| M&B 28,767 (1 μM) | − 2.1 ± 2.0 | − 4.6 ± 0.6* |
| $PGF_{2\alpha}$ (1 μM) | 0.6 ± 0.1 | 0.8 ± 0.2 |
| NaF (1 mM) | 29.2 ± 3.9* | 26.3 ± 1.3* |

Values are the mean ± s.e.mean of four separate experiments, each performed in duplicate. Net cyclic AMP production was cyclic AMP after the agent minus basal cyclic AMP synthesis, which was (pmol $mg^{-1}$ protein $min^{-1}$) 12.2 ± 0.9 by microvessels from newborn and 14.7 ± 0.2 by those from adult pigs. *Significantly ($P<0.05$) different from zero.

## Discussion

There are several reports suggesting that $PGE_2$ and $PGF_{2\alpha}$ exert vasomotor effects on the cerebral vasculature (White & Hagen, 1982; Leffler & Busija, 1987; Hadházy *et al.*, 1988; Chemtob *et al.*, 1989). Evidence also exists that vascular smooth muscle contraction to $PGE_2$ and $PGF_{2\alpha}$ is mediated through their own specific receptors (Stinger *et al.*, 1982; Santoian *et al.*, 1989; Mihara *et al.*, 1988; Abran *et al.*, 1994). However, activation of EP and FP receptors by $PGE_2$ and $PGF_{2\alpha}$ on cerebral vessels elicits significant contraction in the adult but minimal effects in the newborn (Hayashi *et al.*, 1985; Chemtob *et al.*, 1989). To our knowledge, the present study constitutes the first report on the characterization of $PGE_2$ receptors and their subtypes and $PGF_{2\alpha}$ receptors in brain microvessels of the newborn and adult pigs. The main findings of this study are that there are two- to three-fold fewer $PGE_2$ and $PGF_{2\alpha}$ receptors in brain microvessels from newborn than from adult animals (Table 1), and that both $PGE_2$ and $PGF_{2\alpha}$ cause a significantly ($P<0.01$) smaller increase in $IP_3$ production in microvessels from newborn than from adult pigs (Table 2). These findings might explain the age-dependent differences in the effects of $PGE_2$ and $PGF_{2\alpha}$ on cerebral vasculature (Hayashi *et al.*, 1985; Chemtob *et al.*, 1989) and most importantly the narrow range of cerebral blood flow (CBF) autoregulation in the newborn (Hernandez *et al.*, 1980; Chemtob *et al.*, 1990a).

The affinity values for the $PGE_2$ and $PGF_{2\alpha}$ receptors observed in this study are consistent with those previously reported in the literature (Malet *et al.*, 1982; Bhattacherjee *et al.*, 1990; Watabe *et al.*, 1993; Li *et al.*, 1994). Moreover for the same types of prostaglandin receptors the affinity values were similar on newborn and adult tissues (Table 1); our experimental conditions thus seemed adequate.

The observations of the present study suggest that $PGE_2$ receptors in brain microvessels of both the newborn and adult pigs are mainly $EP_1$ and to a lesser extent $EP_3$ subtypes. Because the affinities of $EP_1$ and $EP_3$ for $PGE_2$ are somewhat comparable (Watabe *et al.*, 1993; Li *et al.*, 1994) and the majority of the receptors were of the $EP_1$ subtype, two binding sites could not be revealed by the saturation isotherms and displacement of [$^3$H]-$PGE_2$ by $PGE_2$ (Figure 2). On the other hand the presence of $EP_1$ and $EP_3$ was clearly detected with selective ligands for these receptors (Figure 2).

The displacement of bound [$^3$H]-$PGE_2$ by AH 6809, a selective $EP_1$ antagonist, was markedly greater than by 11-deoxy $PGE_1$, a $EP_2/EP_3$ agonist, by butaprost, a selective $EP_2$ agonist and by M&B 28,767, a selective $EP_3$ agonist (Coleman *et al.*, 1987; Coleman, 1987; Lawrence *et al.*, 1992). Approximately 80% of $PGE_2$ receptors were of the $EP_1$ subtype as determined from the displacement curves with AH 6809 (Figure 2). The $EP_1$ receptor agonist, 17-phenyl trinor $PGE_2$, was as effective as $PGE_2$ in stimulating $IP_3$ production and the effect of both these agents was antagonized by the $EP_1$ receptor antagonist AH 6809 (Table 2). However, [$^3$H]-$PGE_2$ binding was partially displaced by the $EP_3$ receptor agonist, MB 28,767 (Figure 2); based on calculations from the displacement curves approximately 30% of $PGE_2$ receptors were of the $EP_3$ subtype. The presence of $EP_3$ receptors is also suggested by the observation that $PGE_2$ (non-selective for EP receptors), 11-deoxy $PGE_1$ ($EP_2$ and $EP_3$ agonist) and M&B 28,767 ($EP_3$ agonist) all caused nearly identical inhibition of cyclic AMP formation (Table 3), suggesting that $PGE_2$ and its analogues act on the same receptor linked to adenylate cyclase, namely $EP_3$ receptor. Since the $EP_2$-selective agonist, butaprost, was completely ineffective in inhibiting [$^3$H]-$PGE_2$ bound to brain microvessels from both the newborn and adult animals, it would seem that pig brain microvessels do not contain $EP_2$ receptors. Therefore, we believe that approximately 80% of EP receptors are of the $EP_1$ subtype and the remainder are of the $EP_3$ subtype.

The present data also suggest that the age-dependent differences in the actions of $PGE_2$ and cerebral vasculature might be due to greater $EP_1$-mediated $IP_3$ production in the adult than in the newborn tissues (Table 2). $EP_3$-mediated cyclic AMP inhibition might be of little significance, because it was of small magnitude in brain microvessels from both adult and newborn animals (Table 3).

The predominance of $EP_1$-mediated actions of $PGE_2$ in brain microvessels is in contrast to our previous results in brain synaptosomes (Li *et al.*, 1994), which contain only $EP_3$ receptors in the newborn and $EP_2$ and $EP_3$ receptors in the adult; no $EP_1$ receptors were detected in brain synaptosomes. The reason for this difference in EP receptor subtypes in the brain synaptosomes and microvessels is not clear and might be related to their specific functions in the brain.

Our present findings might be of physiological and pathological significance to cerebral blood flow (CBF) regulation in the newborn and adult. The newborn exhibits a narrow CBF autoregulatory range (Hernandez *et al.*, 1980; Chemtob *et al.*, 1990a). To maintain a constant CBF during increases in systemic blood pressure, appropriate vasoconstriction is required. In the adult, $PGE_2$ via $EP_1$ and $PGF_{2\alpha}$ via FP receptors activate phospholipase C which results in $IP_3$ formation (Heaslip & Sickels, 1989; Suba & Roth, 1990), leading to increases in intracellular calcium (Berridge & Irvine, 1989) and subsequent vasoconstriction; this vasoconstriction, which has been shown in cerebral vessels (White & Hagen, 1982; Hayashi *et al.*, 1985; Sasaki *et al.*, 1985; Chemtob *et al.*, 1989), may significantly prevent CBF from increasing when systemic blood pressure rises (Rapela & Green, 1964). In contrast in the newborn, due to the relative deficiency in $PGE_2$ and $PGF_{2\alpha}$ receptors on the cerebral vasculature, an increase in $PGE_2$ and $PGF_{2\alpha}$ levels as blood pressure is raised from its basal value (Chemtob *et al.*, 1990a) might not be able to produce sufficient vasoconstriction to prevent an increase in CBF (Chemtob *et al.*, 1989).

In conclusion, our results indicate that there are fewer $PGE_2$ and $PGF_{2\alpha}$ receptors in brain microvessels of the newborn than in the adult. These findings might provide an explanation for the age-dependent differences in the actions of $PGE_2$ and $PGF_{2\alpha}$ on cerebral vasculature and haemo-

dynamics, and consequently on the range of CBF autoregulation.

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# Regulation of baseline vascular resistance in the canine diaphragm by nitric oxide

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**1** The role played by nitric oxide (NO) in the regulation of blood flow to the canine isolated hemidiaphragm was evaluated by determining (a) the effects of the L-arginine analogues $N^G$-nitro-L-arginine methyl ester (L-NAME), $N^G$-nitro-L-arginine (L-NOARG), and argininosuccinic acid (ArgSA) on baseline vascular resistance and of the latter two agents on endothelium-dependent (acetylcholine, ACh) and endothelium independent (sodium nitroprusside, SNP) vasodilatation; (b) the effects of L- and D-arginine on baseline vascular resistance; and (c) the effects of L-glutamine, an inhibitor of intracellular recycling of L-citrulline to L-arginine, on baseline resistance and on the response to ACh and SNP.

**2** L-NAME, L-NOARG and ArgSA ($6 \times 10^{-4}$ M final concentration) increased baseline diaphragmatic vascular resistance to a similar extent ($28.6 \pm 4.2\%$, $26.7 \pm 4.3\%$ and $32.8 \pm 4.6\%$ respectively). L-NOARG and ArgSA reversed the vasodilator effect of ACh but not of SNP.

**3** L- and D-arginine had no effect on vascular resistance.

**4** L-Glutamine ($10^{-3}$ M) increased baseline vascular resistance by $10 \pm 1.9\%$ ($P<0.05$) but did not alter responses to either ACh or SNP.

**5** Basal NO release plays a role in the regulation of baseline diaphragmatic vascular resistance. L-Arginine analogues tested potently and specifically inhibited this process. Moreover, extracellular L-arginine appears to have no effect on baseline diaphragmatic vascular resistance.

**Keywords:** EDRF; nitroarginine; argininosuccinic acid; L-arginine; L-glutamine; blood flow; respiratory muscles

## Introduction

Endothelial release of vasodilator substances, including nitric oxide (NO) has been shown to play a pivotal role in the regulation of blood flow in a number of vascular beds. The importance of the L-arginine-NO pathway in the regulation of resting vascular tone and as a mediator of endothelium-dependent vasodilatation has been evaluated by observing the effects of inhibiting nitric oxide synthase activity with L-arginine analogues. The effect of these false substrates, however, has varied among experimental preparations. While in the majority of studies, an increase in baseline vascular resistance and inhibition of endothelium-dependent dilatation have been observed, a few investigators have also described no inhibition of endothelium-dependent dilatation or non-selective inhibition of agonist-induced dilatation following the infusion of L-arginine analogues (Mugge *et al.*, 1991; Thomas *et al.*, 1989). Such discrepant results obtained in apparently similar experimental preparations indicate that conclusions drawn from these studies, must be restricted to the vascular bed under study. In addition, L-arginine analogues have been shown to differ in their potency (Vargas *et al.*, 1991), specificity (Thomas & Ramwell, 1988), reversibility by L-arginine (Thomas & Ramwell, 1992; Bogle *et al.*, 1992), method of endothelial cell entry (Bogle *et al.*, 1992) and mechanism of action (Gross *et al.*, 1990). Such pharmacological differences may, therefore, contribute to the conflicts which currently complicate interpretation of this literature.

The source of L-arginine on which the endothelial cell depends for substrate in the synthesis of NO also varies. In some isolated vessel preparations (Gold *et al.*, 1989; Kaley *et al.*, 1992) and in the pial circulation *in vivo* (Morikawa *et al.*, 1992) availability of extracellular L-arginine appears to limit basal NO formation. In other preparations (Fortes *et al.*, 1990; Gardiner *et al.*, 1990; Lahera *et al.*, 1990; Thomas *et al.*, 1989) administration of exogenous L-arginine has no effect on vascular tone. These vessels presumably depend on intracellular synthesis of L-arginine and on the recycling of L-citrulline formed in the process of NO synthesis (Hecker *et al.*, 1990; Sessa *et al.*, 1990).

Failure to maintain substrate supply to the diaphragm in proportion to its metabolic requirements results in muscle failure (Macklem & Roussos, 1977; Supinski *et al.*, 1988). Study of the mechanisms by which diaphragm blood flow is regulated has, therefore, been of high priority among physicians seeking to develop rational treatment strategies for patients with respiratory failure (Aubier *et al.*, 1989). Since, for the reasons discussed above, the results obtained from previous studies cannot be extrapolated to the diaphragm, further progress in this field requires that the role of the nitric oxide pathway in this circulation be specifically determined.

We have, therefore, evaluated the role of NO in the regulation of blood flow in the canine diaphragm by determining (a) the effects of three L-arginine analogues, $N^G$-nitro-L-arginine methyl ester (L-NAME), $N^G$-nitro-L-arginine (L-NOARG) and argininosuccinic acid (ArgSA) on baseline vascular resistance and of the latter two agents on endothelium-dependent (acetylcholine, ACh) and -independent sodium nitroprusside, SNP) vasodilatation; (b) the effects of L- and D-arginine on baseline vascular tone resistance; and (c) the effects of L-glutamine, an inhibitor of L-arginine synthesis from L-citrulline (Sessa *et al.*, 1990) on baseline resistance and on ACh and SNP-induced vasodilatation.

## Methods

### *Animal preparation*

Studies were performed on mongrel dogs (weight = $27.8 \pm 1.27$ kg s.d.). The animals were anaesthetized with sodium thiopentone (10 mg kg$^{-1}$) followed by α-chloralose

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(60–80 mg $kg^{-1}$). Supplemental doses of α-chloralose were given as needed to eliminate the jaw tone but maintain knee reflexes. All animals were treated with indomethacin 5 mg $kg^{-1}$ to block prostaglandin synthesis. The animals were supine, intubated with cuffed endotracheal tubes and mechanically ventilated (tidal volume 15 ml $kg^{-1}$, frequency adjusted to maintain the arterial $PCO_2$ from 38 to 42 mmHg). Supplemental oxygen was supplied through the inspiratory line and arterial $PO_2$ maintained above 100 mmHg. Positive end-expiratory pressure (5 $cmH_2O$) was applied at the expiratory line. A catheter was placed in the aorta through the right carotid artery to monitor arterial pressure ($P_{art}$) and another catheter was placed in the right femoral vein to administer supplemental anaesthetic. Core body temperature was kept constant at approximately 37°C by a heating pad placed under the animal.

### *Isolated left hemidiaphragm*

In all animals, we used the *in situ*, vascularly isolated left hemidiaphragm preparation previously described in detail (Hussain *et al.*, 1989). Briefly, through an abdominal incision, silk sutures were placed 1 cm apart in the left hemidiaphragm to be used for future reference in adjusting the muscle's resting length. The intercostal vessels and the left internal mammary artery were ligated and the lower left ribs were removed. The diaphragm was divided into two halves and the ribs and cartilages of the lower costal margin were freed and suspended vertically from metal bars. Resting diaphragm length was adjusted to that *in situ* by use of the reference sutures. The stomach, liver, spleen, and left kidney were retracted to expose the left phrenic artery. This artery was ligated proximally and a polyethylene catheter (PE 160, 1.14 mm i.d., 1.57 mm o.d., 3 cm long) was introduced into the distal portion. A side port in the catheter was used for measurement of phrenic perfusion pressure ($P_{phr}$). The catheter was connected to an electromagnetic flow probe (Carolina Medical Electronics, 1.91 mm i.d.) in order to measure phrenic arterial flow ($Q_{phr}$). The pressure-flow relationship of the catheter-probe system was linear over the range of flows in the study with conductance = 2.6 ml $min^{-1}$ $mmHg^{-1}$. The other side of the probe was connected to a Y shaped connector, one arm of which was connected to a catheter in the left femoral artery, thereby allowing the diaphragm to be perfused with blood diverted from this source. The other arm of the Y was connected via a constant perfusion pump to a catheter in the right femoral artery. The diaphragm could, thereby, be pump perfused using a constant flow pump (Cole-Parmer Masterflex No. 7523-00, head No. 7016-20, No. 16 tubing) with blood from the right femoral artery by clamping the left femoral arterial catheter. Drugs were administered to the phrenic circulation through a side port in the Y connector using a Harvard infusion pump. All animals were heparinized after catheterizing the phrenic artery.

The temperature of the diaphragm was monitored continuously with a temperature probe fixed to the surface of the isolated hemidiaphragm (Mon-a-therm, Model 6000, Zimmer of Canada Ltd., Mississauga, Ont, Canada) and maintained between 37.5 and 38.5°C with a heat lamp if required. At the end of the experiment the animals were exsanguinated and the diaphragm was dissected free and weighed.

### *Experimental protocols*

*Baseline vascular resistance* Studies were performed on separate groups of animals, each group (6 animals in each group) receiving a different inhibitor of NO release. In each experiment, the left diaphragm was autoperfused from the left femoral artery for 30 min. Following this stabilization period, pump perfusion was begun, as described above, at the flow rate identical to that recorded during the period of autoperfusion. The Harvard pump was then used to infuse saline into the phrenic circulation at a rate equal to 1/100$^{th}$ of the phrenic arterial blood flow. After 20 min of infusion, control measurements of $P_{art}$ and $P_{phr}$ were made. The saline syringe was then removed from the Harvard pump and replaced with one containing one of the following solutions: $N^G$-nitro-L-arginine (L-NOARG) (LNA), $6 \times 10^{-2}$ M; $N^G$-nitro-L-arginine methyl ester (L-NAME), $6 \times 10^{-2}$ M; argininosuccinic acid (ArgSA), $6 \times 10^{-2}$ M; L-glutamine, $10^{-1}$ M. The test agent was infused for 20 min, at which time measurements of $P_{art}$ and $P_{phr}$ were repeated. All agents, except L-NOARG, were dissolved in normal saline with correction of pH to 7.4. L-NOARG solution of $6 \times 10^{-2}$ M was prepared by dissolving L-NOARG in 0.5 ml of 1 N HCl and 19.5 ml of normal saline. We then added enough NaOH to correct the pH to 7.4. Infusion of the L-NOARG vehicle elicited no changes in diaphragmatic vascular resistance.

*Response to ACh and SNP* In 3 animals, the dose-response curves for ACh and SNP were determined. Increasing concentrations of these agents were infused into the phrenic circulation, each at 1/100$^{th}$ of the total phrenic arterial flow rate. The final concentrations found to produce 80% of maximal vasodilatation averaged $10^{-5}$ M for ACh and 2 μg $ml^{-1}$ for SNP. These concentrations were then used in the following studies of the effects of the test agents on the responses to these vasodilators.

Studies were performed on separate groups of animals (6 per group), each receiving a different inhibitor of NO release. Following an initial 30 min stabilization period of autoperfusion, the diaphragm was pump-perfused at the same rate as that recorded during autoperfusion. A dual syringe pump was loaded with two syringes containing saline. Saline from both syringes was then infused into the phrenic circulation at a rate equal to 1/100$^{th}$ of the phrenic arterial flow per syringe. After 20 min, baseline measurements of $P_{art}$ and $P_{phr}$ were performed. One of the saline syringes was then removed from the pump and replaced with one containing either ACh ($10^{-3}$ M) or SNP (200 μg $ml^{-1}$), and infusion was continued at the same rate. After 20 min of simultaneous infusion of saline and either ACh or SNP, by which time a steady state vascular resistance had been achieved in all animals, measurements of $P_{art}$ and $P_{phr}$ were repeated. The second saline syringe was then removed and replaced with a syringe containing one of the following solutions: L-NOARG, $6 \times 10^{-2}$ M; ArgSA, $6 \times 10^{-2}$ M, L-glutamine, $10^{-1}$ M. After 20 min of simultaneous infusion of either ACh or SNP and the test agent, measurements of $P_{art}$ and $P_{phr}$ were repeated.

*L-Arginine and D-arginine* In 2 separate groups of animals (6 per group) the left hemi-diaphragm was autoperfused for 30 min and then pump perfused at the natural flow rate as described above. The Harvard pump was subsequently used to infuse saline into the phrenic circulation at a rate equal to 1/100$^{th}$ of the phrenic arterial blood flow. After 20 min of infusion, control measurements of $P_{art}$ and $P_{phr}$ were made. The saline syringe was then removed from the Harvard pump and replaced with one containing either L-arginine or D-arginine (pH = 7.4) and infusion was continued at the same rate. The initial concentration in the syringe was $0.6 \times 10^{-1}$ M to yield a final concentration of $0.6 \times 10^{-3}$ M in the phrenic arterial blood. This syringe was replaced sequentially with syringes containing solutions which yielded final concentrations of $3 \times 10^{-3}$ M and $6 \times 10^{-3}$ M. The test agent was infused for 10 min at each concentration, at which time repeat measurements of $P_{art}$ and $P_{phr}$ were performed.

### *Data analysis*

Results are expressed as mean ± s.e.mean. Two mean comparisons were performed with two tailed paired *t* tests. Comparisons of multiple means were performed using analysis of variance (ANOVA) corrected for multiple measures when appropriate and analysed *post-hoc* using the Neuman-Keuls

procedure. Differences were deemed significant when $P<0.05$.

## Results

### *Effect on baseline vascular resistance*

Baseline $\dot{Q}_{phr}$ before L-NOARG, L-NAME, ArgSa and L-glutamine infusion averaged 20.8, 26.4, 29.2 and 28.5 $ml^{-1}$ 100 g $min^{-1}$, respectively. Figure 1 illustrates the mean values of $P_{phr}$ measured at baseline and after 20 min infusion of these agents. L-NAME ($6 \times 10^{-4}$ M), L-NOARG ($6 \times 10^{-4}$ M) and ArgSA ($6 \times 10^{-4}$ M) significantly ($P<0.01$) increased phrenic vascular resistance ($28.6 \pm 4.2\%$, $26.7 \pm 4.3\%$ and $32.8 \pm 4.6\%$ respectively compared to baseline), whereas L-glutamine ($10^{-3}$ M) increased vascular resistance by a statistically significant ($P<0.05$) but much smaller amount ($10 \pm 1.9\%$, Figure 1). The increase in phrenic vascular resistance in response to all of these agents reached a steady state level within 10 min of the infusion with no change thereafter. In addition, the pressor response to L-NOARG, L-NAME and L-glutamine was well maintained and persisted for over 60 min after the cessation of infusion. By comparison, phrenic vascular resistance recovered completely after 20 min of the cessation of ArgSA infusion. Figure 2 illustrates an example of the changes in phrenic perfusion pressure in response to $6 \times 10^{-4}$ M L-NAME infusion. The increase in phrenic vascular resistance plateaued within 10 min of L-NAME infusion. Infusion of normal saline into the phrenic artery over 20 min period elicited no significant alterations in phrenic vascular resistance.

### *Effect on response to ACh and SNP*

Figure 3 illustrates the effects of L-NOARG (a), ArgSA (b), and L-glutamine (c), on the vasodilator response to ACh and SNP. Infusion of L-NOARG and ArgSA completely reversed the vasodilatation induced by ACh while having no significant effect on the SNP response. L-Glutamine had no effect on the magnitude of vasodilatation produced by either ACh or SNP.

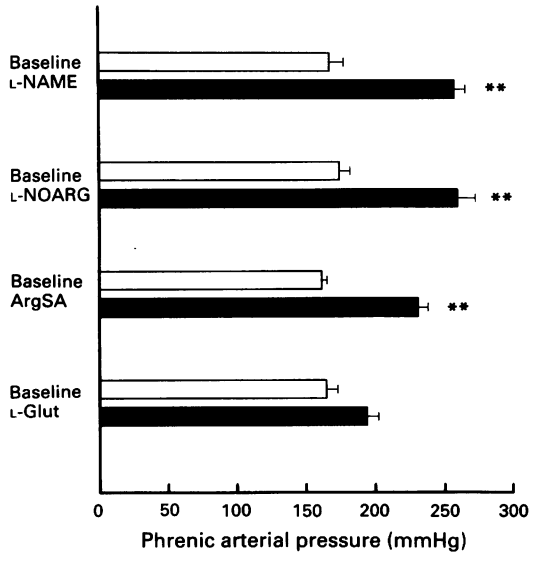


**Figure 1** Change in phrenic arterial perfusion pressure following infusion of $N^G$-nitro-L-arginine methyl ester (L-NAME, $6 \times 10^{-4}$ M), $N^G$-nitro-L-arginine (L-NOARG, $6 \times 10^{-4}$ M), argininosuccinic acid (ArgSA, $6 \times 10^{-4}$ M) and L-glutamine (L-Glut, $10^{-3}$ M). $*P<0.05$; $**P<0.01$ for the change compared to baseline perfusion pressure.

### *Effects of L-arginine and D-arginine*

Figure 4 presents the values of $P_{phr}$ during infusion of L-arginine and D-arginine. Neither isomer had any effect on diaphragmatic vascular resistance at any of the concentrations tested.

## Discussion

The main findings of this study are: (1) infusion of the L-arginine analogues L-NOARG, L-NAME and ArgSA increased the baseline vascular resistance of the phrenic circula-

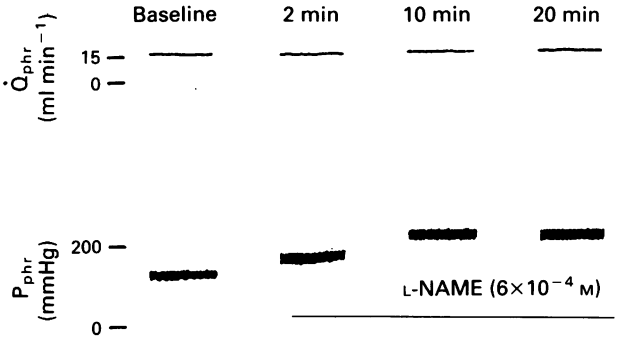


**Figure 2** A representative tracing of the changes in phrenic perfusion pressure in response to $6 \times 10^{-4}$ M $N^G$-nitro-L-arginine methyl ester (L-NAME).

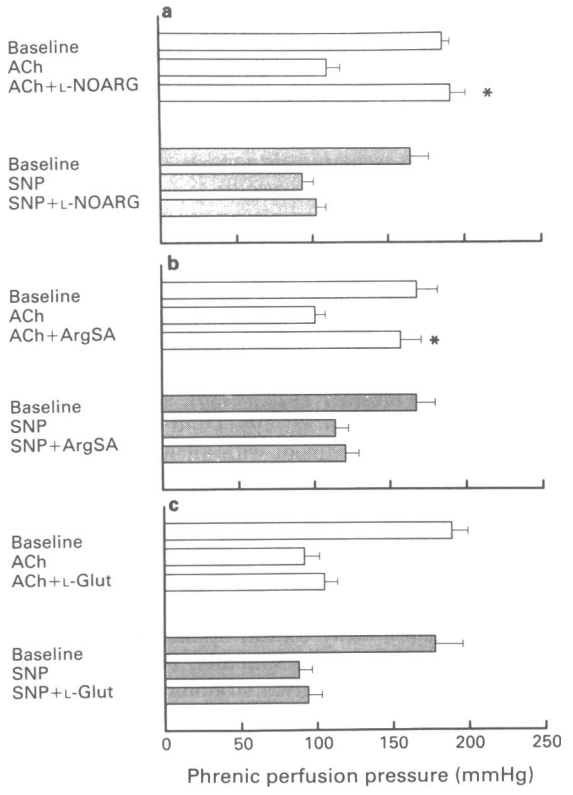


**Figure 3** Effects of $N^G$-nitro-L-arginine (L-NOARG, $6 \times 10^{-4}$ M), argininosuccinic acid (ArgSA, $6 \times 10^{-4}$ M) and L-glutamine (L-Glut, $10^{-3}$ M) on the changes in phrenic arterial perfusion pressure during acetylcholine (ACh, $10^{-5}$ M) and sodium nitroprusside (SNP, 2 $\mu l\ ml^{-1}$) infusion. $*P<0.05$ compared with response to vasodilator alone.

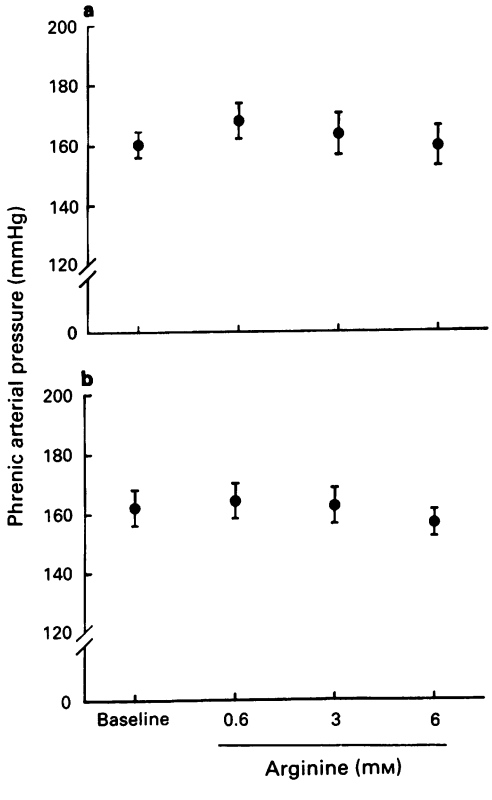


**Figure 4** Effects of (a) L-arginine and (b) D-arginine on baseline phrenic arterial perfusion pressure.

tion *in vivo*; (2) infusion of L-NOARG or ArgSA attenuated the vasodilator effect of ACh infusion while having no effect on vasodilatation in response to SNP; (4) L-arginine and D-arginine at physiological pH have no effect on baseline diaphragmatic vascular resistance; (5) L-glutamine ($10^{-3}$ M) increased baseline vascular resistance but had no effect on either ACh or SNP induced vasodilatation.

### *Basal NO release*

Basal release of EDRF has been detected by bioassay from isolated vessels (Rubanyi *et al.*, 1985; Kelm & Schrader, 1990). *In vivo* studies have demonstrated increased baseline arterial pressure following systemic infusion of L-arginine analogues in rats (Gardiner *et al.*, 1989), guinea-pigs (Aisaka *et al.*, 1989), cats (Bellan *et al.*, 1991) and dogs (Klabunde *et al.*, 1990). Regionally, treatment with analogues of L-arginine have been found to increase vascular resistance in rabbit isolated hearts (Lamontagne *et al.*, 1991), rabbit hindlimb (Mugge *et al.*, 1991), dog hindlimb (White *et al.*, 1993) and human forearm (Vallance *et al.*, 1989). In rat cremaster muscle, arteriolar vasoconstriction has been demonstrated following light-dye injury to the endothelium (Koller & Kaley, 1990) and following topical application of L-arginine analogues (Kaley *et al.*, 1992). Similarly, Persson *et al.* (1990) have shown $N^G$-monomethyl-L-arginine (L-NMMA) to reduce microvascular diameters in rabbit tenuissimus muscle. Consistent with this evidence, we have found the baseline resistance of the diaphragmatic vasculature to increase significantly following the administration of L-arginine analogues. This effect could not have been due to suppression of vasodilator prostanoid synthesis (Koller *et al.*, 1990) since all animals had been pretreated with indomethacin. These results, therefore, suggest a role for modulation of basal endothelial release of NO in the control of resting smooth muscle tone in this vascular bed as well.

### *Endothelium-dependent and -independent vasodilatation*

The results of studies of the role of NO in agonist-induced vasodilator responses have been less consistent than those concerning its role in regulation of baseline vascular tone. Some studies have shown that the *in vivo* responses to endothelium-dependent agents are selectively inhibited by treatment with L-arginine analogues, while responses to endothelium-independent agents are not significantly decreased (Aisaka *et al.*, 1989; Gold *et al.*, 1989; Gardiner *et al.*, 1989; Bellan *et al.*, 1991; 1993; Kaley *et al.*, 1992; White *et al.*, 1993). Other studies, however, have shown either no decrease in responses to endothelium-dependent vasodilators (Mugge *et al.*, 1991, Ross *et al.*, 1991) or a non-selective inhibition of both endothelium-dependent and -independent vasodilatation (Thomas *et al.*, 1989; Klabunde *et al.*, 1990). The degree to which L-arginine analogues are able to inhibit the vasodilatation induced by ACh has also been variable, prompting some authors to propose additional mechanisms of action for this vasodilator in their preparations (Gold *et al.*, 1989; Bellan *et al.*, 1991; Ross *et al.*, 1991). The dose and duration of treatment have also been reported to alter the relative effect of L-arginine analogues on baseline vascular tone in comparison to their effects on the response to ACh (Bellan *et al.*, 1991). Furthermore, the relative potencies of two of these agents L-NOARG and L-NMMA, in the *in vivo* canine femoral circulation has been shown to differ from that predicted from *in vitro* studies (Kirkeboen *et al.*, 1992). Differences in species, vessel type, agent used, dose and duration of treatment may all, therefore, contribute to these apparent discrepancies.

Three L-arginine analogues were used in the current study. We found that exposure of the diaphragmatic vasculature to equimolar concentrations of these three analogues increased baseline vascular resistance to a similar extent. In addition, L-NOARG and ArgSA, in the concentration used, attenuated the vasodilatation induced by ACh but did not increase phrenic vascular resistance beyond the baseline values. These results indicate that L-NOARG and ArgSA did not completely reverse ACh-induced vasodilatation. Moreover, these findings suggest that in the indomethacin pretreated canine diaphragmatic circulation, the vasodilatation induced by ACh infusion is mediated partly by augmentation of NO release and that these L-arginine analogues are specific inhibitors of this effect. We speculate also that part of the ACh-induced phrenic vasodilatation is mediated by NO-independent mechanisms such as endothelium-derived relaxing factor (EDHF, Komori & Vanhoutte, 1990). In rat isolated vessels, ACh induces endothelium-dependent hyperpolarization which partially mediate the vasodilator effect of ACh (Van de Voorde *et al.*, 1992).

### *L-Arginine*

Endothelium-dependent relaxing factor/NO is synthesized from the guanido nitrogen of L-arginine and molecular oxygen by the NADPH and calmodulin-dependent action of NO synthase (NOS) (Busse & Mulsch, 1990; Forstermann *et al.*, 1991). Citrulline is a coproduct of this reaction. FMN, FAD, haeme and tetrahydrobiopterin are co-factors (Nathan, 1992). Substrate levels adequate to support ongoing NO synthesis are maintained by uptake of extracellular L-arginine and through the synthesis of L-arginine by a process linked to the release of NO (Mitchell *et al.*, 1990b). Endothelial cells can generate L-arginine from at least two sources. These include the peptidyl L-arginine pool which is available through the action of peptidyl arginine deimidase (Thomas & Ramwell, 1988) and recycling of accumulated L-citrulline to L-arginine through the intermediate formation and subsequent cleavage of argininosuccinate (Hecker *et al.*, 1990). Intracellular levels of L-arginine, therefore, normally remain high (0.1–1 mM, approximately 30 times the apparent $K_m$ for

purified NOS, (Pollock *et al.*, 1991) even during prolonged stimulation of NO release (Mitchell *et al.*, 1990a).

In isolated vessel preparations, the effect of administration of exogenous L-arginine on vascular smooth muscle tone has been variable. In preparations in which vasodilatation was elicited, intracellular L-arginine had been depleted either because of prolonged (>2 h) preparation time (Shini & Vanhoutte, 1991; Sun *et al.*, 1992) or by continuous stimulation of NO synthesis in arginine-free media (Gold *et al.*, 1989). In fresh preparations, no effect of L-arginine is observed (Shini & Vanhoutte, 1991; Sun *et al.*, 1992).

In the majority of blood perfused tissue preparations (Thomas *et al.*, 1989; Fortes *et al.*, 1990; Gardiner *et al.*, 1990; Lahera *et al.*, 1990), infusion of L-arginine had no effect on vascular resistance. Nevertheless, in the pial circulation, exogenous L-arginine, but not D-arginine administration resulted in vasodilatation which was reversible by the topical administration of L-NAME (Morikawa *et al.*, 1992). In the human forearm, furthermore, Panza *et al.* (1993) found that infusion of L-arginine but not D-arginine, enhanced the vasodilator response to acetylcholine, although such treatment had no effect on baseline vascular resistance. The role of extracellular L-arginine availability as a rate limiting factor in endothelial NO synthesis under physiological conditions, therefore, varies depending upon the tissue and the species under study. Our current finding that neither of the arginine isomers significantly altered vascular resistance indicates that L-arginine availability does not normally limit basal NO release in the canine diaphragmatic circulation.

### *L-Glutamine*

In cultured endothelial cells, Sessa *et al.* (1990) found that L-glutamine resulted in significant (46.5%) inhibition of EDRF release as detected by bioassay. The effect was observed over a concentration range of 0.1–2.0 mM, with maximum effect at concentrations of 0.2 mM and above. L-glutamine exerted this action through inhibition of intracellular conversion of L-citrulline to L-arginine. It did not interfere with the conversion of L-Arg-L-Phe or L-Argsucc to L-arginine not did it inhibit the uptake of L-arginine into the endothelial cells. The inhibitory action of L-glutamine on L-citrulline conversion is most likely effected by allosteric interaction with argininosuccinate synthetase or by competitive interference due to the structural similarity between these two amino acids.

In the current study we found that L-glutamine, at a final concentration in the perfusing blood of 1 mM, produced a small but significant increase in diaphragmatic vascular resistance. The exact mechanism behind the increase in phrenic vascular resistance after L-glutamine infusion is not clear. Based on the experiments of Sessa *et al.*, one may speculate that L-glutamine inhibits basal NO release by preventing the conversion of L-citrulline to L-arginine. However, L-arginine requirements for basal NO synthesis can also be provided through an influx of intracellular L-arginine. Our experiments also indicated that L-glutamine infusion did not attenuate the vasodilator response to stimulation of NO release by ACh. These results suggest that during ACh infusion, intracellular L-arginine concentration may not be sufficiently reduced by L-glutamine to influence NOS activity. Alternatively, other sources of L-arginine may become more important or else conversion of L-citrulline to L-arginine by an as yet unknown transamination reaction may be activated during stimulation of NO production.

In summary, modulation of basal NO release by the endothelial cell plays an important role in the regulation of baseline diaphragmatic vascular resistance. Extracellular L-arginine availability does not appear to be rate limiting in this process. Intracellular recycling of L-citrulline, however, is likely to be an important source of substrate for this reaction under basal conditions. The L-arginine analogues, L-NOARG and ArgSA are potent and specific inhibitors of basal NO release in the canine phrenic circulation.

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# Effects of $P_1$ and $P_{2Y}$ purinoceptor antagonists on endothelium-dependent and -independent relaxations of rat mesenteric artery to GTP and guanosine

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**1** Guanosine 5'-triphosphate (GTP) and guanosine can relax both endothelium-intact and -denuded arterial preparations. In the present work the $P_1$ and $P_{2Y}$ purinoceptor antagonists, 8-phenyltheophylline and reactive blue 2, respectively, were used to study the mechanisms of relaxation responses induced by GTP, guanosine, adenosine 5'-triphosphate (ATP) and adenosine in noradrenaline-precontracted rat mesenteric artery rings.

**2** GTP (10 μM–1 mM) dose-dependently relaxed endothelium-intact mesenteric artery rings and also induced moderate relaxation responses in endothelium-denuded preparations. Pretreatment of the rings with 8-phenyltheophylline (10 μM) or reactive blue 2 (10 μM) did not attenuate the relaxant effect of GTP.

**3** Guanosine (10 μM–1 mM) relaxed both endothelium-intact and -denuded artery rings in a dose-dependent manner. The presence of 8-phenyltheophylline or reactive blue 2 had no effects on guanosine-induced relaxations.

**4** ATP-induced (0.1 μM–0.1 mM) relaxation of endothelium-intact artery rings was attenuated by reactive blue 2 while 8-phenyltheophylline was ineffective. ATP also relaxed endothelium-denuded artery rings and this relaxation was inhibited by 8-phenyltheophylline, but not by reactive blue 2.

**5** Adenosine-induced (10 μM–1 mM) relaxation of endothelium-intact and -denuded artery rings was attenuated by the presence of 8-phenyltheophylline, but not of reactive blue 2.

**6** In conclusion, the endothelium-dependent and -independent relaxations of rat mesenteric arteries to GTP and guanosine are not mediated via $P_1$ and $P_{2Y}$ purinoceptors. Therefore, these results support our previous suggestion on the presence of a novel guanine nucleotide-specific receptor, a putative $P_G$ receptor, on both endothelial and smooth muscle cells, which may participate in the regulation of arterial tone.

**Keywords:** Endothelium; vascular tone; guanine nucleotides; adenine nucleotides; purinoceptors; purinoceptor antagonists

## Introduction

Extracellular adenosine and adenine nucleotides modulate vascular tone and platelet function by interacting with specific receptors on the cell surface, ATP and ADP acting at $P_2$ purinoceptors and adenosine at $P_1$ purinoceptors (Burnstock, 1978; Burnstock & Kennedy, 1985; Gordon, 1986; Olsson & Pearson, 1990). ATP and ADP can be liberated into the extracellular space as a consequence of vessel wall damage and local platelet aggregation (Gordon, 1986), after which they are rapidly sequentially dephosphorylated to adenosine by ectonucleotidases of endothelium and circulating blood cells (Coade & Pearson, 1989). The endothelium-independent effect of ATP can be either vasodilatation or vasoconstriction mainly depending on the subclass of the $P_2$ purinoceptor in question (Kennedy *et al.*, 1985; Houston *et al.*, 1987; Pearson, 1988). The endothelium-dependent effect of ATP is vasodilatation due to its action at $P_{2Y}$ purinoceptors which leads to release of endothelium-derived relaxing factor (EDRF-NO) and/or prostacyclin (De Mey & Vanhoutte, 1981; Gordon & Martin, 1983; Needham *et al.*, 1987; Boeynaems & Pearson, 1990). It is suggested that the vasodilator action of adenosine is mediated via the $A_2$ subclass of $P_1$ purinoceptors and stimulation of adenylate cyclase in smooth muscle (Collis & Brown, 1983; Ramagopal *et al.*, 1988). The location of adenosine receptors has not been well-defined and both endothelium-dependent (Gordon & Martin, 1983; Rubanyi & Vanhoutte, 1985) and -independent (Kennedy *et al.*, 1985; White & Angus, 1987) responses have been described.

Naturally occuring xanthines, such as theophylline and caffeine, are $P_1$ receptor antagonists (Olsson & Pearson, 1990). Alkylxanthine derivatives, such as 8-phenyltheophylline, are nonselective, but equally potent antagonists of $A_1$ and $A_2$ purinoceptors, and can be used as tools to study adenosine-mediated mechanisms (Bruns *et al.*, 1983).

The anthraquinone sulphonic acid derivative, reactive blue 2, which can be regarded as an ATP analogue, has been used as a $P_{2Y}$ antagonist, which over a narrow concentration-range selectively antagonizes $P_{2Y}$-mediated dilator responses to ATP in various blood vessels (Burnstock & Warland, 1987; Hopwood & Burnstock, 1987; Houston *et al.*, 1987; Reilly *et al.*, 1987).

Although the vasoactive effects of extracellular adenine nucleotides are well characterized, there is little information about the effects of extracellular guanine nucleotides on vascular tone. We have recently reported that exogenous GTP and guanosine (i) induce smooth muscle relaxation (ii) promote accumulation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) without affecting the levels of adenosine 3':5'-cyclic monophosphate (cyclic AMP) and (iii) augment the effects of nitrovasodilators in both endothelium-intact and -denuded rat mesenteric artery rings (Vuorinen *et al.*, 1991; 1992). The endothelium-dependent response to GTP of endothelium-intact rings can be explained by the subsequent release of EDRF-NO, whereas that induced by guanosine is

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only partially mediated via autacoid release from the endothelium. Moreover, the endothelium-independent responses to GTP and guanosine are direct, unknown effects of these compounds on smooth muscle and guanylyl cyclase (Vuorinen *et al.*, 1992). The present work was carried out to examine the effects of the $P_1$ and $P_{2Y}$ purinoceptor antagonists, 8-phenyltheophylline and reactive blue 2, respectively, on endothelium-dependent and -independent relaxations to exogenous GTP and guanosine to obtain more evidence for the existence of novel purinoceptors specific for guanine nucleotides. The known purinoceptor agonists, ATP and adenosine, were used as reference compounds.

## Methods

### *Relaxation of rat mesenteric arteries*

Non-fasted male Sprague-Dawley rats weighing about 300 g were decapitated. The mesenteric artery was excised and cut into 3 mm long rings. Six rings were usually obtained from one artery. When endothelium-dependent effects were studied, the endothelium was left intact but for studies of endothelium-denuded rings the endothelium was removed by rubbing it gently with a jagged injection needle. The rings were placed between two stainless steel hooks and mounted in an organ bath chamber containing Krebs bicarbonate buffer solution (pH 7.4) of the following composition (in mM): NaCl 119.0, $NaHCO_3$, 25.0, glucose 11.1, $CaCl_2$ 1.6, KCl 4.7, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2. The preparations were maintained at 37°C and aerated with 95% $O_2$ and 5% $CO_2$ and before the experiments they were equilibrated for 1 h with a resting tension of 1.5 g. The force of contraction was measured with an isometric force-displacement transducer (Grass FT03) and registered on a Grass Polygraph (Model 7 E Polygraph; Grass Instrument Co., Quincy, MA, USA).

Successful removal or the integrity of the endothelium was confirmed by adding acetylcholine (1 μM, final concentration) to 0.5 μM noradrenaline-precontracted vascular rings. If any relaxation of the denuded preparation was observed, the endothelium was further rubbed. For studies with endothelium-intact vascular rings, the relaxation with acetylcholine had to be nearly 100%. The absence or presence of the endothelium when applying the present methods has been confirmed in our previous study by electron microscopy, showing undamaged underlying smooth muscle cells in denuded preparations and a well preserved endothelial cell monolayer in intact rings (Arvola *et al.*, 1992).

After rinsing four times with Krebs buffer the rings were stabilized for an additional 30 min. Thereafter cumulative vascular relaxation responses to GTP (10 μM–1 mM), guanosine (10 μM–1 mM), ATP (0.1 μM–1 mM) or adenosine (10 μM–1 mM) were elicited. The rings were precontracted with 0.5 μM noradrenaline, and after the contraction had fully developed increasing concentrations of relaxing agents were added cumulatively. The relaxation time for each dose was 5 min but for the highest dose it was 10 min. The rings were then washed four times with Krebs buffer and allowed to return to baseline tension. After 30 min 10 μM 8-phenyltheophylline or 10 μM reactive blue 2 was added, and 15 min later the rings were again contracted with noradrenaline and the cumulative relaxations were repeated in the presence of antagonist. Only one of the above purines was used to study the relaxation responses in each vascular ring and the concentrations and incubation periods were chosen on the basis of our own pilot studies.

### *Drugs*

Acetylcholine chloride, GTP, ATP, guanosine, adenosine, 8-phenyltheophylline and reactive blue 2 were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). (-)-Noradrenaline-L-hydrogen tartrate was from Fluka Chemie AG (Buchs, Switzerland). The test agents were prepared in Krebs buffer on the day of use.

### *Statistical analysis*

The results are expressed as mean ± s.e.mean. Statistical analysis was carried out by one-way analysis of variance (ANOVA) for repeated measurements. The data were analysed with BMDP Statistical Software (Los Angeles, CA., U.S.A.). $P$ values of less than 0.05 were considered to be statistically significant.

## Results

### *The effects of 8-phenyltheophylline and reactive blue 2 on endothelium-dependent and -independent relaxations to GTP and guanosine*

GTP (10 μM–1 mM) dose-dependently relaxed noradrenaline-precontracted endothelium-intact and -denuded artery rings. The relaxation at 1 mM GTP was 99 ± 1% in intact and 35 ± 1% in denuded rings (Figure 1). Pretreatment of the rings with 10 μM 8-phenyltheophylline or 10 μM reactive blue 2 for 15 min before inducing contraction did not attenuate the relaxant effect of GTP (Figure 1).

Guanosine (10 μM–1 mM) relaxed both endothelium-intact and -denuded artery rings in a concentration-dependent manner. The relaxation to 1 mM guanosine was 73 ± 3% in

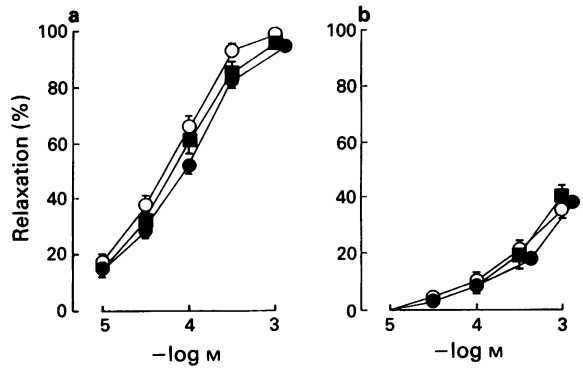


**Figure 1** Concentration-response curves for GTP in the absence (○) and presence of 10 μM 8-phenyltheophylline (■) and 10 μM reactive blue 2 (●) in endothelium intact (a) and endothelium-denuded (b) rat mesenteric artery rings. Values are the mean ± s.e.mean ($n$ = 6–12 rings of 4–6 rats).

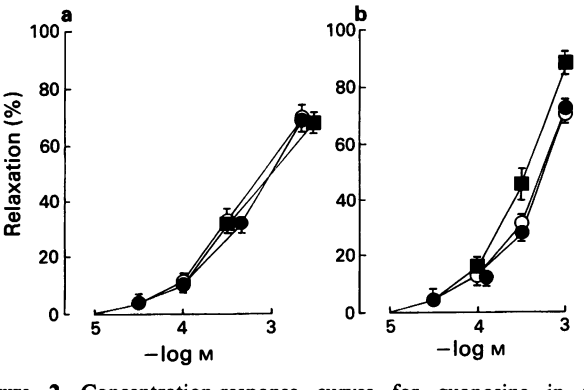


**Figure 2** Concentration-response curves for guanosine in the absence (○) and presence of 10 μM 8-phenyltheophylline (■) and 10 μM reactive blue 2 (●) in endothelium-intact (a) and endothelium denuded (b) rat mesenteric artery rings. Values are the mean ± s.e.mean ($n$ = 6–12 rings of 4–6 rats).

intact and 70 ± 3% in denuded rings (Figure 2). The presence of 10 μM 8-phenyltheophylline or 10 μM reactive blue 2 had no significant effects on guanosine-induced relaxations. 8-Phenyltheophylline even tended to augment the relaxation in denuded rings (Figure 2b).

### *The effects of 8-phenyltheophylline and reactive blue 2 on endothelium-dependent and -independent relaxations to ATP and adenosine*

ATP (0.1 μM–0.1 mM) efficiently relaxed endothelium-intact artery rings in a dose-dependent manner, the relaxation at 0.1 mM of ATP being 97 ± 1% (Figure 3a). The presence of 10 μM reactive blue 2 significantly ($P<0.01$) attenuated ATP-induced relaxation, but 10 μM 8-phenyltheophylline had no effect (Figure 3a). ATP also relaxed endothelium-denuded rings at higher concentrations (10 μM–1 mM), the relaxation at 1 mM ATP being 89 ± 3% (Figure 3b). Pretreatment with 10 μM reactive blue 2 did not affect ATP-induced relaxation of denuded rings, but 10 μM 8-phenyltheophylline had an inhibitory effect ($P<0.01$) (Figure 3b).

Adenosine (10 μM–1 mM) relaxed both endothelium-intact and -denuded artery rings in a concentration-dependent manner, the relaxations being slightly more pronounced in intact rings. The relaxation induced by 1 mM adenosine was 88 ± 3% in intact and 81 ± 3% in denuded rings (Figure 4). The presence of 10 μM 8-phenyltheophylline attenuated ($P<0.01$) adenosine-induced relaxations in both types of vascular preparations, while reactive blue 2 had no significant effects (Figure 4).

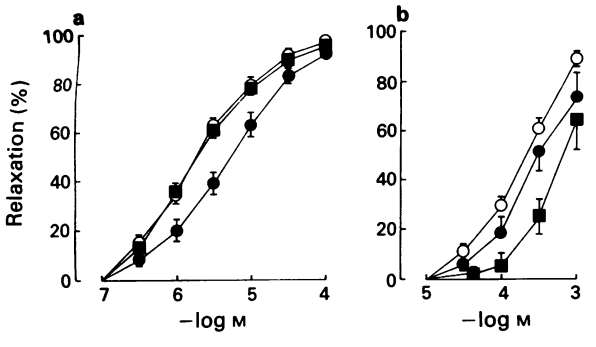


**Figure 3** Concentration-response curves for ATP in the absence (○) and presence of 10 μM 8-phenyltheophylline (■) and 10 μM reactive blue 2 (●) in endothelium-intact (a) and endothelium-denuded (b) rat mesenteric artery rings. Values are the mean ± s.e. mean ($n = 6–12$ rings of 4–6 rats). $P<0.01$ (ANOVA) in intact rings when comparing the relaxation to ATP in the absence and presence of reactive blue 2; $P<0.01$ (ANOVA) in denuded rings between the responses to ATP in the absence and presence of 8-phenyltheophylline.

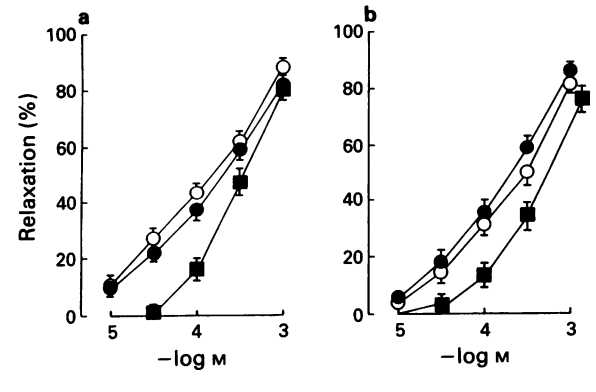


**Figure 4** Concentration-response curves for adenosine in the absence (●) and presence of 10 μM 8-phenyltheophylline (■) and 10 μM reactive blue 2 (●) in endothelium-intact (a) and endothelium-denuded (b) rat mesenteric artery rings. Values are the mean ± s.e.mean ($n = 6–12$ rings of 4–6 rats). $P<0.01$ (ANOVA) in both intact and denuded rings when comparing the relaxations to adenosine in the absence and presence of 8-phenyltheophylline.

## Discussion

In the present study 8-phenyltheophylline and reactive blue 2 were used as tools to examine whether GTP- and guanosine-induced relaxations of rat mesenteric arteries are mediated via $P_1$ and $P_{2Y}$ purinoceptor activation. The well characterized purinoceptor agonists, ATP and adenosine, were used as control relaxants to confirm the efficacy of the antagonists in this experimental model. However, the results obtained with these purinoceptor antagonists as investigative tools must be interpreted with some caution. Xanthines are not particularly potent and selective for $A_1$ and $A_2$ subtypes of $P_1$ receptors, although the C-8 substitution with phenyl increases the potency but not the selectivity of the compound (Collis *et al.*, 1985). Reactive blue 2 is a specific and selective $P_{2Y}$ antagonist but only over a narrow concentration-range (Olsson & Pearson, 1990), and when used at higher concentrations it has nonspecific effects, for example, an attenuating influence on $A_2$ receptor-mediated vasodilatation (Hopwood & Burnstock, 1987).

8-Phenyltheophylline was able to attenuate the adenosine-induced relaxations both in endothelium-intact and -denuded arterial rings. Thus, the present results agree with previous findings that adenosine is a direct vasodilator inducing vascular relaxation via activation of $P_1$ purinoceptors on smooth muscle, and that the presence of an intact endothelium is not required for its relaxant effect on arteries (Furchgott, 1984). 8-Phenyltheophylline also inhibited the relaxation of denuded preparations to ATP, suggesting that this endothelium-independent response of rat mesenteric arteries is due to the production of adenosine from ATP by ectonucleotidases during the incubation period. A similar mechanism has previously been reported to be operative in pulmonary vessels and thoracic aorta of the rat (Liu *et al.*, 1988; Rose'Meyer & Hope, 1990).

In a variety of arteries, including the rat mesenteric bed (Ralevic & Burnstock, 1988), dog coronary artery (Houston *et al.*, 1987), pig aorta (Martin *et al.*, 1985) and rat femoral artery (Kennedy *et al.*, 1985) the $P_{2Y}$ purinoceptor-mediated relaxation induced by ATP and its analogues has been shown to be endothelium-dependent and presumably to occur via the subsequent release of EDRF-NO. In the present study, reactive blue 2 attenuated the ATP-induced relaxation of endothelium-intact rat mesenteric rings thus indicating the inhibition of $P_{2Y}$ receptor activation and the subsequent release of EDRF-NO. Previously we have shown that the ATP-induced relaxation of rat mesenteric rings correlates with an increase in smooth muscle cyclic GMP concentration (Vuorinen *et al.*, 1992).

The applied $P_1$ and $P_{2Y}$ antagonists, 8-phenyltheophylline and reactive blue 2, respectively, which inhibited the ATP- and adenosine-induced relaxations in an expected manner, did not affect the endothelium-dependent and -independent relaxations of rat mesenteric arteries to GTP and guanosine. We have earlier shown that exogenous GTP and guanosine relax precontracted endothelium-intact and -denuded rat mesenteric artery rings by increasing cyclic GMP accumulation without affecting cyclic AMP concentration in smooth muscle (Vuorinen *et al.*, 1992). Moreover, the GTP-induced relaxation of endothelium-intact rings was attenuated by $N^G$-nitro L-arginine methyl ester (L-NAME), an effect which could be reversed by L-arginine, but the guanosine-induced relaxation of endothelium-intact rings was only slightly inhibited by L-NAME (Vuorinen *et al.*, 1992). Thus, the response to GTP of endothelium-intact rings could mainly be explained by the release of EDRF-NO, but that of guanosine was only partly due to EDRF-NO. The endothelium-

independent response of GTP and guanosine is a direct, unknown effect on smooth muscle and guanylyl cyclase.

We have recently reported that exogenous GTP can elevate cyclic GMP concentration in human ADP-stimulated platelet rich plasma and in thrombin-stimulated washed platelets (Laustiola *et al.*, 1991; Vuorinen & Laustiola, 1992). This increase in cyclic GMP was accompanied by decreased platelet aggregation. The stimulation of platelets leads to secretion of nucleotides from granules, where ATP and ADP are stored at concentrations up to one molar (Gordon, 1986). The concentration of guanine nucleotides, mainly GTP and GDP, in granules is about 30% of that of adenine nucleotides (Holmsen, 1985). Therefore, during platelet stimulation and secretion, local GTP concentrations can increase enough to produce an inhibition of platelet function. Taken together, the results on platelets and smooth muscle suggest that GTP may have a local antithrombotic effect via elevation of cyclic GMP. Thus, GTP could regulate platelet-vessel wall interaction even when endothelial function is impaired.

In conclusion, the endothelium-dependent effect of GTP and the putative EDRF-NO-release are not mediated via $P_1$ or $P_{2Y}$ activation, and the endothelium-independent relaxation to GTP and the response to guanosine are not due to activation of adenosine-specific $P_1$ purinoceptors. Therefore, the present results support our earlier suggestions on the presence of a cell membrane site of action, of a novel guanine nucleotide specific receptor (a putative $P_G$ receptor) which can mediate the activation of soluble guanylyl cyclase and participate in the regulation of arterial tone.

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# The effects of the endothelin $ET_A$ receptor antagonist, FR 139317, on infarct size in a rabbit model of acute myocardial ischaemia and reperfusion

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**1** The effects were investigated of the $ET_A$ receptor antagonist, FR 139317, on endothelin-1 (ET-1)-induced coronary vasoconstriction in the isolated perfused heart of the rabbit. In addition, this study examined whether FR 139317 reduced infarct size in a rabbit model of coronary artery occlusion and reperfusion.

**2** In the rabbit isolated perfused heart, ET-1 (1–100 pmol) elicited a dose-dependent increase in coronary perfusion pressure (CPP). For example, 30 pmol ET-1 caused CPP to rise by 22 ± 8 mmHg and 100 pmol ET-1 by 47 ± 10 mmHg ($n = 8$). Infusion of FR 139317 (1 μM) significantly attenuated the increase in CPP caused by ET-1 (30 pmol: 3 ± 1 mmHg, 100 pmol: 8 ± 2 mmHg; $n = 8$).

**3** In the anaesthetized rabbit, infarct size (expressed as a percentage of the area at risk) after 45 or 60 min of coronary artery occlusion followed by 2 h of reperfusion was 47 ± 6% ($n = 6$) and 55 ± 7% ($n = 5$), respectively. A continuous infusion of FR 139317 (0.2 mg $kg^{-1}$ $min^{-1}$ preceded by a loading dose of 1.0 mg $kg^{-1}$, i.v.; $n = 5–6$) had no effect on the extent of the myocardial infarct size (45 min: 47 ± 6%; 60 min: 49 ± 7%). Even a three-times higher dose (0.6 mg $kg^{-1}$ $min^{-1}$ preceded by a loading dose of 3 mg $kg^{-1}$, i.v.; $n = 4$) of FR 139317 had no effect on myocardial infarct size (48 ± 5%) after 45 min occlusion of the antero-lateral branch of the left coronary artery (LAL) and 2 h reperfusion.

**4** In a separate group of experiments, the LAL was occluded for 60 min and subsequently reperfused for 6 h. FR 139317 (0.6 mg $kg^{-1}$ $min^{-1}$ preceded by a loading dose of 3 mg $kg^{-1}$, i.v.; $n = 4$) had no significant effect on infarct size even in this long reperfusion model (control: 48 ± 3%, FR 139317: 61 ± 6%).

**5** Thus, the vasoconstrictor effects elicited by ET-1 in the coronary vasculature of the rabbit are primarily mediated via the $ET_A$ receptor, for they were inhibited by the $ET_A$ receptor antagonist, FR 139317. However, an enhanced formation of endogenous ET-1 does not play a major role in ischaemia/reperfusion injury of the rabbit heart, for FR 139317 had no effect on infarct size.

**Keywords:** Endothelin-1; endothelin $ET_A$ receptor; FR 139317; myocardial infarction

## Introduction

Endothelin-1 (ET-1) is a potent coronary vasoconstrictor in a variety of species including rat (Baydoun *et al.*, 1989; Neubauer *et al.*, 1990a), rabbit (Hirata *et al.*, 1990), pig (Ezra *et al.*, 1989), dog (Clozel & Clozel, 1989; Larkin *et al.*, 1989) and man (Chester *et al.*, 1989; Clarke *et al.*, 1989). Moreover, ischaemia/reperfusion of the isolated perfused heart of the rat enhances the coronary vasoconstriction elicited by ET-1 (Neubauer *et al.*, 1990b; 1991; Stewart & Baffour, 1990; Brunner *et al.*, 1992; Grover *et al.*, 1992; Watts *et al.*, 1992; McMurdo *et al.*, 1993b). Enhanced plasma levels of ET-1 in man are associated with a variety of cardiovascular disorders including acute myocardial infarction (Miyauchi *et al.*, 1989; Salminen *et al.*, 1989), angina pectoris (Nakao *et al.*, 1989), coronary artery vasospasm (Matsuyama *et al.*, 1990) and congestive heart failure (Grenier *et al.*, 1990). The hypothesis that endogenous ET-1 plays a role in the extension of ischaemia/reperfusion injury of the heart is supported by the findings that (i) intravenous administration of a monoclonal antibody against ET-1 reduces infarct size in a model of coronary artery occlusion and reperfusion in the anaesthetized rat (Watanabe *et al.*, 1991) or anaesthetized rabbit (Kusumoto *et al.*, 1993) and (ii) infusion of the endothelin-converting enzyme inhibitor, phosphoramidon, results in a substantial reduction in infarct size in the anaesthetized rat (Grover *et al.*, 1992).

Two endothelin receptors have been cloned and expressed, namely $ET_A$ (Arai *et al.*, 1990) and $ET_B$ (Sakurai *et al.*, 1990). The vasoconstrictor effects of ET-1 are mainly due to activation of the $ET_A$ receptor although $ET_B$ receptors also mediate some vasoconstriction (Douglas *et al.*, 1992; Bigaud & Pelton, 1992; Moreland *et al.*, 1992). The discovery of distinct endothelin receptors has prompted the development of selective endothelin receptor antagonists, such as FR 139317 (Sogabe *et al.*, 1992). FR 139317 is a linear tripeptide, which binds selectively to $ET_A$ receptors and inhibits ET-1 binding to porcine and human aortic microsomes, antagonizes ET-1-induced contractions of rabbit isolated aorta and attenuates the pressor response to ET-1 in conscious rats (Sogabe *et al.*, 1992; 1993) and anaesthetized rabbits (McMurdo *et al.*, 1993a).

Here, we determine which endothelin receptor-subtype mediates ET-1-induced coronary vasoconstriction in the isolated perfused heart of the rabbit and go on to investigate whether the $ET_A$ receptor antagonist, FR 139317, reduces infarct size in a rabbit model of coronary artery occlusion and reperfusion.

## Methods

### *Rabbit isolated perfused hearts*

Male New Zealand White rabbits (2–3 kg) were premedicated with Hypnorm (0.1 ml $kg^{-1}$, i.m.; containing fentanyl citrate at 0.315 mg $ml^{-1}$ and fluanisone at 10 mg $ml^{-1}$). General anaesthesia was then induced with

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sodium pentobarbitone (30 mg $kg^{-1}$, i.v.) and anaesthesia was maintained with supplementary doses of sodium pentobarbitone as required. The trachea was cannulated and the rabbits were ventilated with room air from a Harvard ventilator at a rate of 36–40 strokes per min and a tidal volume of 18–20 ml. Heparin (100 iu $kg^{-1}$) was given into the marginal ear vein, the thorax was opened and the heart was rapidly excised and immersed in ice cold saline. The aorta was dissected free and cannulated with a glass cannula and the heart was transferred to the perfusion apparatus. The coronary circulation was perfused at a constant flow (25 ml $min^{-1}$) with Krebs solution containing (in mM): NaCl 118, $NaHCO_3$ 25, KCl 3, $MgSO_4$ 1.2, $NaH_2PO_4$ 1.2, glucose 11 and $CaCl_2$ 1.4. The buffer was bubbled with 95% $O_2$, 5% $CO_2$ and kept at 37°C. Air temperature around the hearts was maintained by means of a heated (37°C) water-jacket. In this constant flow system, changes in coronary perfusion pressure (CPP) reflect changes in coronary vascular resistance. CPP was recorded with a Transamerica type 4-422-0001 pressure transducer. In addition, developed tension (DT) and heart rate (HR) were measured by means of a hook attached to the apex of the heart which was connected by a thread to an isometric transducer (Grass Instruments, model FT03). The output of each transducer was displayed on a polygraph recorder (Grass Instruments, model 7D). Drugs were administered via an injection port 3 cm distal to the aortic cannula. The hearts were subjected to a resting tension of 2 g and allowed to equilibrate for 20 min in order to establish stable baseline parameters.

ET-1 (1, 3, 10, 30 or 100 pmol) bolus injections were given 10 min after the start of a continuous infusion of vehicle (20% dimethylsulphoxide; DMSO) or FR 139317 (1 μM).

### *Myocardial ischaemia and reperfusion in the anaesthetized rabbit*

*Surgical procedure* Rabbits were anaesthetized and ventilated as described above. Body temperature was monitored and maintained at 38 ± 1°C by means of a rectal probe thermometer attached to a homeothermic blanket control unit (Harvard, Edenbridge, Kent). The left femoral vein was cannulated for drug administration. The left femoral artery was cannulated for the measurement of blood pressure from which mean arterial pressure (MAP) and HR were derived. Haemodynamic parameters were continuously recorded on a polygraph recorder (Grass Instruments, 7D). Lead II electrocardiograms (ECGs) were recorded from subdermal platinum electrodes. ST-segment changes were calculated as absolute differences with respect to the J-point of the QRS complex (of at least 10 cardiac cycles) and expressed as Δ mV. Another catheter was placed in the left ventricle via the right common carotid artery for injection of Evans blue dye solution. A 2–3 cm left intercostal thoracotomy (4th intercostal space) was performed to expose the heart and a snare occluder was placed around the first antero-lateral branch of the left coronary artery (LAL) 1 cm distal from its origin. In contrast to other species, the rabbit LAL supplies most of the left ventricular myocardium including most of the septum and apex (Flores *et al.*, 1984). Care was taken not to include veins draining blood from this area whenever possible.

*Measurement of infarct size* After the reperfusion period, the LAL was re-occluded and Evans blue dye solution (4 ml of 2% w/v) was injected into the left ventricle to distinguish between perfused and non-perfused (myocardium at risk) sections of the heart. The Evans blue solution stains the perfused myocardium, while the occluded vascular bed remains uncoloured. The dose of Evans blue dye used in this study is well within the range reported for nearly exclusive binding to plasma albumin or other proteins in the rabbit (Lindner & Heinle, 1982). After injection of Evans blue dye, the rabbits were killed with an overdose of anaesthetic. The heart was excised and sectioned into 4–5 mm thick slices. After removing the right ventricular wall, the area at risk and non-ischaemic myocardium were separated by following the line of demarcation between blue stained and unstained tissue. To distinguish between ischaemic and infarcted tissue, the area at risk was chopped and incubated in *p*-nitroblue tetrazolium (NBT, 0.5 mg $ml^{-1}$ for 20 min) at 37°C. In the presence of intact dehydrogenase enzyme systems (normal myocardium). NBT forms blue coloured precipitates, while areas of necrosis lack dehydrogenase activity and, therefore, fail to stain (Nachlas & Shnitka, 1963). Tissue was divided into stained (blue) or unstained (red) groups. All tissues from the left ventricle were weighed. Area at risk was expressed as a percentage of the left ventricle, and the infarct size was calculated as a percentage of the area at risk.

*Experimental design* All rabbits were allowed to stabilize for 30 min before being assigned to one of eight groups: (1) 30 min LAL occlusion and 2 h reperfusion treated with vehicle ($n = 4$); (2) 45 min LAL occlusion and 2 h reperfusion treated with vehicle ($n = 5$); (3) 45 min LAL occlusion and 2 h reperfusion treated with a low dose of FR 139317 (0.2 mg $kg^{-1}$ $min^{-1}$ preceded by a loading dose of 1.0 mg $kg^{-1}$, i.v., bolus; $n = 5$); (4) 45 min LAL occlusion and 2 h reperfusion treated with a high dose of FR 139317 (0.6 mg $kg^{-1}$ $min^{-1}$ preceded by a loading dose of 3.0 mg $kg^{-1}$, i.v., bolus, $n = 4$); (5) 60 min LAL occlusion and 2 h reperfusion treated with vehicle ($n = 5$); (6) 60 min LAL occlusion and 2 h reperfusion treated with a low dose of FR 139317 (0.2 mg $kg^{-1}$ $min^{-1}$ preceded by a loading dose of 1.0 mg $kg^{-1}$, i.v., bolus, $n = 6$); (7) 60 min LAL occlusion and 6 h reperfusion treated with vehicle ($n = 5$); (8) 60 min LAL occlusion and 6 h reperfusion treated with a high dose of FR 139317 (0.6 mg $kg^{-1}$ $min^{-1}$ preceded by a loading dose of 3.0 mg $kg^{-1}$, i.v., bolus, $n = 4$).

Vehicle or FR 139317 infusion was started 10 min prior to LAL occlusion and continued throughout the reperfusion period.

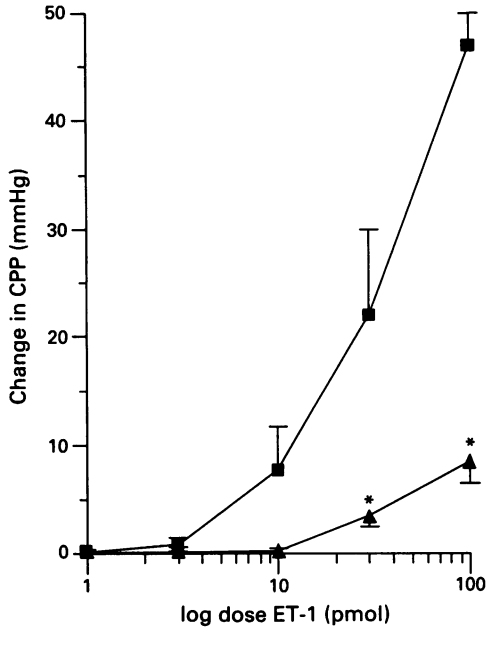


**Figure 1** Changes in coronary perfusion pressure (CPP) induced by endothelin-1 (ET-1, 1-100 pmol, bolus injection) in rabbit isolated hearts treated with either vehicle (■, $n = 8$) or with FR 139317 (▲, 1 μM continuous infusion, $n = 8$). Data are mean ± s.e.mean of $n$ observations. $^*P < 0.05$ when compared to vehicle control.

### *Materials*

Hypnorm was purchased from Janssen Pharmaceutical Co., (Oxford, UK), sodium pentobarbitone (Sagatal) from May & Baker (Dagenham, UK) and heparin from Evans Med., (Middlesex, UK). The Krebs buffer salts, DMSO, bovine serum albumin, Evans blue dye and NBT were obtained from Sigma Chem. Co., (Poole, UK). ET-1 was supplied by Scientific Marketing Associates (London, UK) and was reconstituted in 0.1% acetic acid and diluted in 0.9% w/v saline containing 1% w/v bovine serum albumin and 0.06% sodium bicarbonate. FR 139317 (**R**)-2[(**R**)-2-[(S)-2-1-(hexahydro-1H-azepinyl)]carbonyl]amino-4-methylpentanoyl] amino-3[3-(1-methyl-1H-indolyl]proprionyl] amino-3-(2-pyridyl) proprionic acid was provided by Dr Annette M. Doherty of the Medicinal Chemistry Department at Parke-Davis Pharmaceutical Research Division of Warner-Lambert Co. (Ann Arbor, U.S.A.). FR 139317 was dissolved in 20% DMSO or in saline (6 h reperfusion experiments). Aliquots of ET-1 and FR 139317 were stored frozen (− 20°C) until use.

### *Statistical comparisons*

All values in the figures, tables and text are expressed as mean ± s.e.mean of $n$ observations. Statistical evaluation of the data was by Student's $t$ test for unpaired determinations or by ANOVA. A $P$ value of less than 0.05 was considered significant.

## Results

### *Effects of FR 139137 on the coronary vasoconstriction elicited by ET-1 in the isolated perfused heart of the rabbit*

After the 20 min equilibration period, the mean resting CPP was 29 ± 1 mmHg, the DT was 10 ± 1 g and HR was 158 ± 4 beats $min^{-1}$ ($n = 16$). Infusion of vehicle (20% DMSO) or FR 139317 (1 μM) had no effect on these parameters.

ET-1 induced a dose-dependent increase in CPP. For example, 30 pmol ET-1 caused CPP to rise by 22 ± 8 mmHg and 100 pmol ET-1 by 47 ± 10 mmHg ($n = 8$), respectively (Figure 1). The coronary vasoconstriction elicited by ET-1 was significantly attenuated in the presence of a continuous infusion of FR 139317 (1 μM). For example, 30 pmol and 100 pmol ET-1 elicited rises in CPP of only 3 ± 1 mmHg and 8 ± 2 mmHg, respectively ($n = 8$, Figure 1). ET-1 had no effect on HR or DT in the absence or presence of FR 139317 (data not shown).

### *Effects of FR 139317 on infarct size in a rabbit model of acute myocardial ischaemia and reperfusion*

Of the 48 rabbits which underwent LAL occlusion, 10 died within the experimental period due to ventricular fibrillation or cardiac failure and these were excluded from the study. Nine of these died within 5–20 min of the ischaemic period (7 rabbits receiving vehicle and 2 rabbits receiving FR 139317 at 0.2 mg $kg^{-1}$ $min^{-1}$ preceded by a loading dose of 1.0 mg $kg^{-1}$, i.v.). One rabbit died during reperfusion (receiving FR 139317 at 0.6 mg $kg^{-1}$ $min^{-1}$ preceded by a loading dose of 3 mg $kg^{-1}$, i.v.).

*Haemodynamic data* mean resting values for MAP was 61 ± 2 mmHg and for HR was 237 ± 4 beats $min^{-1}$ ($n = 38$). Infusion of FR 139317 at the highest dose used (0.6 mg $kg^{-1}$ $min^{-1}$ preceded by a loading dose of 3 mg $kg^{-1}$, i.v.) elicited a transient rise (16 ± 4 mmHg) in blood pressure which returned to control levels within 20 min. LAL occlusion (45 or 60 min) and reperfusion (2 or 6 h) had no significant effect on MAP or HR in any of the groups studied (Table 1).

Area at risk and infarct size: The area of the left ventricle at risk (ischaemic myocardium) was similar in all groups studied (Table 2). In hearts subjected to 30 min LAL occlusion plus 2 h of reperfusion, infarct size (expressed as a percentage of area at risk) was 10 ± 1% ($n = 4$). Due to the small infarct size obtained, this model was not used to investigate the effects of FR 139317.

Infarct size after 45 or 60 min of LAL occlusion followed by 2 h of reperfusion in vehicle-treated rabbits was 47 ± 6% ($n = 6$) and 55 ± 7% ($n = 5$), respectively (Table 2). A continuous infusion of FR 139317 (0.2 mg $kg^{-1}$ $min^{-1}$ preceded by a loading dose of 1.0 mg $kg^{-1}$, i.v.; $n = 5–6$) had no effect on the extent of the myocardial infarct (45 min: 47 ± 6%; 60 min; 49 ± 7%, Table 2). Even a three-times higher dose (0.6 mg $kg^{-1}$ $min^{-1}$ preceded by a loading dose of 3 mg $kg^{-1}$, i.v.; $n = 4$) of FR 139317 had no effect on myocardial infarct size (48 ± 5%) after 45 min LAL occlusion and 2 h reperfusion (Table 2).

When the LAL was occluded for 60 min and subsequently reperfused for 6 h, infarct size in vehicle-treated animals was 48 ± 3% ($n = 5$). FR 139317 (0.6 mg $kg^{-1}$ $min^{-1}$ preceded by

**Table 1** Mean arterial pressure (MAP; mmHg) and heart rate (HR; beats $min^{-1}$) in rabbits subjected to: (1) 30 min coronary artery (LAL) occlusion and 2 h reperfusion; (2), (3), (4) 45 min LAL occlusion and 2 h reperfusion; (5), (6) 60 min LAL occlusion and 2 h reperfusion; or (7), (8) 60 min LAL occlusion and 6 h reperfusion. Rabbits received either vehicle, FR 139317 at 0.2 mg $kg^{-1}$ $min^{-1}$ or FR 139317 at 0.6 mg $kg^{-1}$ $min^{-1}$.

| *Group* | *Treatment* (mg $kg^{-1}$ $min^{-1}$) | | *Basal* | *End of occlusion* | *End of reperfusion* |
|---|---|---|---|---|---|
| (1) | Vehicle | MAP | 61 ± 5 | 57 ± 5 | 63 ± 6 |
| | | HR | 247 ± 14 | 240 ± 14 | 235 ± 12 |
| (2) | Vehicle | MAP | 60 ± 3 | 60 ± 3 | 58 ± 5 |
| | | HR | 245 ± 12 | 229 ± 9 | 239 ± 8 |
| (3) | FR 139317 (0.2) | MAP | 62 ± 3 | 59 ± 2 | 53 ± 4 |
| | | HR | 244 ± 8 | 232 ± 8 | 244 ± 6 |
| (4) | FR 139317 (0.6) | MAP | 58 ± 6 | 60 ± 4 | 64 ± 7 |
| | | HR | 263 ± 24 | 248 ± 22 | 267 ± 11 |
| (5) | Vehicle | MAP | 55 ± 5 | 55 ± 4 | 53 ± 5 |
| | | HR | 215 ± 7 | 213 ± 5 | 209 ± 2 |
| (6) | FR 139317 (0.2) | MAP | 68 ± 6 | 64 ± 5 | 61 ± 5 |
| | | HR | 227 ± 6 | 236 ± 7 | 253 ± 6 |
| (7) | Vehicle | MAP | 64 ± 2 | 70 ± 2 | 72 ± 4 |
| | | HR | 230 ± 6 | 219 ± 9 | 213 ± 10 |
| (8) | FR 139317 (0.6) | MAP | 76 ± 5 | 73 ± 6 | 71 ± 8 |
| | | HR | 235 ± 11 | 226 ± 13 | 232 ± 11 |

Values are given as mean ± s.e.mean of 4–6 observations for each group.

**Table 2** Area at risk (expressed as a percentage of left ventricle) and infarct size (expressed as a percentage of area at risk) in rabbits subjected to coronary artery (LAL) occlusion (30, 45 or 60 min) and reperfusion (2 or 6 h)

| Group | Treatment ($mg\ kg^{-1}\ min^{-1}$) | Occlusion (min) | Reperfusion (h) | Area at risk (%) | Infarct (%) | n |
|---|---|---|---|---|---|---|
| (1) | Vehicle | 30 | 2 | 30 ± 4 | 10 ± 1 | 4 |
| (2) | Vehicle | 45 | 2 | 42 ± 4 | 47 ± 6 | 5 |
| (3) | FR 139317 (0.2) | 45 | 2 | 44 ± 2 | 47 ± 6 | 5 |
| (4) | FR 139317 (0.6) | 45 | 2 | 46 ± 8 | 48 ± 5 | 4 |
| (5) | Vehicle | 60 | 2 | 34 ± 3 | 55 ± 7 | 5 |
| (6) | FR 139317 (0.2) | 60 | 2 | 33 ± 3 | 49 ± 7 | 6 |
| (7) | Vehicle | 60 | 6 | 35 ± 4 | 48 ± 3 | 5 |
| (8) | FR 139317 (0.6) | 60 | 6 | 39 ± 6 | 61 ± 6 | 4 |

Rabbits received either vehicle, FR 139317 at $0.2\ mg\ kg^{-1}\ min^{-1}$ preceded by a loading dose of $1.0\ mg\ kg^{-1}$ or FR 139317 at $0.6\ mg\ kg^{-1}\ min^{-1}$ preceded by a loading dose of $3.0\ mg\ kg^{-1}$. Values are given as mean ± s.e.mean of $n$ observations.

**Table 3** Peak elevation in ST-segment in rabbits subjected to: (1) 30 min coronary artery (LAL) occlusion and 2 h reperfusion; (2), (3), (4) 45 min LAL occlusion and 2 h reperfusion; (5), (6) 60 min LAL occlusion and 2 h reperfusion; or (7), (8) 60 min LAL occlusion and 6 h reperfusion

| Group | Treatment ($mg\ kg^{-1}\ min^{-1}$) | ST elevation (mV) |
|---|---|---|
| (1) | Vehicle | 0.28 ± 0.01 |
| (2) | Vehicle | 0.29 ± 0.03 |
| (3) | FR 139317 (0.2) | 0.24 ± 0.07 |
| (4) | FR 139317 (0.6) | 0.26 ± 0.02 |
| (5) | Vehicle | 0.24 ± 0.02 |
| (6) | FR 139317 (0.2) | 0.26 ± 0.02 |
| (7) | Vehicle | 0.36 ± 0.02 |
| (8) | FR 139317 (0.6) | 0.28 ± 0.01 |

Rabbits received either vehicle, FR 139317 at $0.2\ mg\ kg^{-1}\ min^{-1}$ preceded by a loading dose of $1.0\ mg\ kg^{-1}$ or FR 139317 at $0.6\ mg\ kg^{-1}\ min^{-1}$ preceded by a loading dose of $3.0\ mg\ kg^{-1}$. Values are given as mean ± s.e.mean of 4–6 observations for each group.

a loading dose of $3\ mg\ kg^{-1}$, i.v.; $n = 4$) had no significant effect on infarct size even in this longer reperfusion model (61 ± 6%, Table 2).

*Electrocardiogram changes:* basal values for the ST-segment in the lead II ECG ranged from 0.004 ± 0.01 mV to 0.03 ± 0.01 mV. In different vehicle-treated control groups, LAL occlusion elicited a peak rise in the ST-segment ranging from 0.24 mV ± 0.07 mV to 0.36 ± 0.02 mV which remained elevated throughout the occlusion period (Table 3). Upon reperfusion, the ST-segment gradually returned to almost basal levels, with end values of 0.01 ± 0.01 mV to 0.05 ± 0.02 mV. FR 139317 ($0.2\ mg\ kg^{-1}\ min^{-1}$ preceded by a loading dose of $1.0\ mg\ kg^{-1}$ or $0.6\ mg\ kg^{-1}\ min^{-1}$ preceded by a loading dose of $3\ mg\ kg^{-1}$, i.v.) had no effect on the ischaemia-induced ST-segment elevation in any of the models studied (Table 3).

## Discussion

Here we demonstrate that the $ET_A$ receptor antagonist, FR 139317, attenuates the coronary vasoconstrictor effects elicited by ET-1 in the isolated perfused heart of the rabbit. However, FR 139317, in doses which attenuate by 83–89% the $ET_A$-mediated pressor effects of exogenous ET-1 ($1\ nmol\ kg^{-1}$) in the anaesthetized rabbit (McMurdo *et al.*, 1993a), has no effect on infarct size and ST-segment elevation in three different models of myocardial ischaemia and reperfusion in the rabbit. This is in contrast to previous studies which suggested that an enhanced formation of endogenous ET-1 contributes to ischaemia/reperfusion injury of the heart. Thus, an antibody to ET-1 (Watanabe *et al.*, 1991) or the endothelin-converting enzyme inhibitor, phosphoramidon (Grover *et al.*, 1992) reduce infarct size in models of coronary artery occlusion and reperfusion in the anaesthetized rat. Furthermore, pretreatment of rabbits with an antibody to ET-1 reduced infarct size caused by 30 min occlusion of the first branch of the left circumflex coronary artery followed by 24 h of reperfusion from 61 ± 5% (control) to 37 ± 5% (treatment, $n = 5$). In this study, the areas at risk in control and treated animals were 43 ± 5% and 44 ± 3%, respectively (Kusumoto *et al.*, 1993).

Why then, does the $ET_A$ receptor antagonist, FR 139317, not reduce infarct size in this study? Clearly, FR 139317 ($0.2\ mg\ kg^{-1}\ min^{-1}$ preceded by a loading dose of $1.0\ mg\ kg^{-1}$) had no effect on infarct size caused by 60 min of LAL occlusion and 2 h of reperfusion. Considering that collateral blood supply to the ischaemic myocardium after complete coronary artery occlusion is minimal in the rabbit heart (Maxwell *et al.*, 1987), one could argue that the degree of the ischaemic insult caused by 60 min of LAL occlusion was too severe to allow therapeutic intervention. However, drugs such as the prostacyclin analogue, iloprost (Chiariello *et al.*, 1988), the fibrinolytic compound, defibrotide (Thiemermann *et al.*, 1989) and the anti-platelet agent, cloricromene (Lidbury *et al.*, 1993) elicit a substantial reduction in infarct size in this model. Moreover, FR 139317 ($0.2\ mg\ kg^{-1}\ min^{-1}$ preceded by a loading dose of $1.0\ mg\ kg^{-1}$) had no effect on infarct size even when the duration of coronary artery occlusion was reduced to 45 min. Investigation of the effects of FR 139317 on the ischaemic injury caused by shorter periods of LAL occlusion was not feasible, for 30 min of LAL occlusion caused only a very small infarction (10 ± 1% of area at risk). One could also argue that the dose of FR 139317 used in this study was too low to antagonize the effects of endogenous ET-1 in the coronary vasculature of the rabbit. However, this is unlikely, for even a three times higher dose of FR 139317 ($0.6\ mg\ kg^{-1}\ min^{-1}$ preceded by a loading dose of $3.0\ mg\ kg^{-1}$) did not reduce the infarct size caused by 45 min of LAL occlusion followed by 2 h of reperfusion. Assuming a blood volume of $60–70\ ml\ kg^{-1}$ the dose of FR 139317 used should result in plasma levels of more than 100 μM at 10 min after the start of the continuous infusion of FR 139317. Clearly this concentration of the antagonist is sufficient to block the vasoconstrictor effects caused by ET-1 in the coronary vasculature of the rabbit (this study).

In anaesthetized rabbits subjected to coronary artery occlusion (30 min) and reperfusion (24 h), plasma levels of ET-1 are maximally elevated (approximately 2.5 fold) at 3 h after reperfusion (Kusumoto *et al.*, 1993). Thus, ET-1 may play a

pathophysiological role after longer periods of reperfusion and hence, 2 h of reperfusion may not have been long enough to see a potential beneficial action of FR 139317. Therefore, we also investigated the effects of FR 139317 ($0.6\ mg\ kg^{-1}\ min^{-1}$ preceded by a loading dose of $3.0\ mg\ kg^{-1}$) in rabbits in which the LAL was occluded for 60 min and subsequently reperfused for 6 h. However, even when the ischaemic insult was followed by this prolonged reperfusion period, FR 139317 did not reduce infarct size. This suggests that if endogenous ET-1 does play a pathophysiological role in the extension of myocardial ischaemia/reperfusion injury, then it does so between 6 and 24 h of reperfusion.

Interestingly, 9 out of 48 rabbits died within 5–20 min of myocardial ischaemia due to ventricular fibrillation. Of these 9 animals, 7 were treated with vehicle and 2 were treated with FR 139317. This result indicates that inhibition of $ET_A$ receptors with FR 139317 reduces the incidence of ventricular fibrillation and, hence, further studies are necessary to elucidate the role of ET-1 in the pathogenesis of ischaemia-induced arrhythmias.

Ischaemia/reperfusion of the rat heart causes an increase in ET-1 receptor binding sites (Liu *et al.*, 1990). We have shown here that under non-ischaemic conditions, $ET_A$ receptors mediate the rise in CPP elicited by ET-1 in the isolated perfused heart of the rabbit. However, it is unknown which endothelin receptor subtype is upregulated following ischaemia-reperfusion injury of the heart. If other (non-$ET_A$) receptors are increased, then this would explain why FR 139317 is ineffective in reducing myocardial infarct size. It would be interesting to examine the effects of an $ET_B$ or non-selective receptor antagonist in this model of coronary artery occlusion and reperfusion.

Thus, we demonstrate here that endogenous ET-1 does not play a major role in the extension of infarct size in these models of LAL occlusion (45 or 60 min) and reperfusion (2 or 6 h) in the anaesthetized rabbit.

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# Binding kinetics and antiplatelet activities of picotamide, a thromboxane $A_2$ receptor antagonist

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**1** Picotamide was shown to inhibit platelet binding of thromboxane $A_2$ ($TxA_2$)-mimetics and to cause a reduction of $TxA_2$ platelet receptors after *in vivo* administration. The present study aimed to investigate directly [$^3$H]-picotamide binding to human platelets and in particular the relationship between binding kinetics and antiaggregating properties.

**2** [$^3$H]-picotamide time-dependently bound to a single class of platelet $TxA_2$ receptors with a $K_D$ of 325 nmol $l^{-1}$ at equilibrium. The binding was displaceable by $TxA_2$ analogues U46619 and ONO11120 ($K_i$ 19 and 28 nmol $l^{-1}$ respectively) but not by prostacyclin ($PGI_2$), prostaglandin $E_2$ ($PGE_2$) and $TxB_2$. Antiaggregating activity and $TxA_2$ formation inhibition paralleled with binding kinetics.

**3** By prolonging the incubation time from 30 to 120 min, picotamide showed a progressively increasing non-displaceable binding, whereas specific displaceable binding decreased in comparison to the values reached at 30 min. Non displaceable binding was specific, temperature-dependent, saturable and followed a Michaelis-Menten kinetic ($V_{maxapp}$ = 130 fmol per $10^8$ platelets $h^{-1}$, $K_{Mapp}$ = 330 nmol $l^{-1}$). Picotamide progressively underwent a specific stable interaction with its platelet receptor.

**4** In conclusion, after an initial reversible binding, a progressive stabilization of picotamide binding takes place resulting in a progressively more stable interaction with platelets.

**Keywords:** Platelets; thromboxane; thromboxane receptor antagonist; thromboxane synthesis inhibitor; binding studies; aggregation; antiplatelet drug

## Introduction

Picotamide (N,N'*bis*(3-picolyl)-4-methoxy-isophthalamide) is a new antiaggregating drug (Violi *et al.*, 1988; Berrettini *et al.*, 1990; Cattaneo *et al.*, 1991) which was found to be able to displace the platelet binding of both a labelled thromboxane $A_2$ ($TxA_2$)-mimetic ([$^3$H]-U46619) and a labelled $TxA_2$ antagonist ([$^{125}$I]-PTAOH) (Modesti *et al.*, 1989). These findings have caused picotamide to be considered as a $TxA_2$/$PGH_2$ competitive receptor inhibitor (Gresele *et al.*, 1989; Modesti *et al.*, 1989). However, no studies have been performed aimed at investigating directly the kinetics of picotamide binding to the $TxA_2$/$PGH_2$ receptors. Moreover $TxA_2$/$PGH_2$ binding sites have been found to be decreased after picotamide administration in man (Modesti *et al.*, 1991) and this observation apparently contrasts with the hypothesis of picotamide as a competitive receptor inhibitor. Indeed this pattern might suggest either that picotamide acts as a non competitive inhibitor with a stable interaction at the $TxA_2$/$PGH_2$ receptor site, or that picotamide follows a particularly low kinetic of dissociation from the $TxA_2$ receptor, with consequent functional inactivation of the $TxA_2$ receptor.

The present study aimed to investigate directly the kinetics of the binding of [$^3$H]-picotamide to human platelets and to assess the relationship between the kinetics of [$^3$H]-picotamide binding and antiplatelet activity.

## Methods

### *Platelet aggregation studies*

Blood was withdrawn by venipuncture from six overnight fasting healthy volunteers, aged 28 to 42 years, between 08 h 00 min–09 h 00 min and anticoagulated with 129 mmol $l^{-1}$ trisodium citrate (9:1 v:v). No subject had taken any drugs for at least 15 days. Platelet aggregation studies were performed in platelet-rich plasma (PRP) with the optical method of Born, using an Elvi 840 dual channel aggregometer (Elvi Logos, Milan, Italy). PRP was prepared by centrifugation at 160 *g* at room temperature for 6 min. Platelet poor plasma (PPP) was obtained by centrifugation of the blood samples at 1,200 *g* at room temperature for 15 min. Platelet count in PRP was adjusted to $3 \times 10^{11}$ platelets $l^{-1}$ with autologous PPP in all experiments.

The stable endoperoxide analogue, U46619 (0.75 μmol $l^{-1}$) and collagen (2.5 μg $ml^{-1}$) were used as inducers. Platelet aggregation was recorded for 15 min and the extent of aggregation was evaluated by measuring the maximal height reached by the aggregation curves.

Percent inhibitions of U46619 and collagen induced platelet aggregation, by increasing concentrations of picotamide (0, 0.1, 1, 10, 100, 500 and 1,000 μmol $l^{-1}$) were calculated from the reduction of the maximal amplitude of the aggregation tracings in relation to the values obtained in the paired solvent experiments. The concentration of picotamide giving 50% inhibition of aggregation ($IC_{50}$) was calculated from the mean of at least four different experiments.

To assess the time-dependency of $IC_{50}$, the inhibitory effects of picotamide were evaluated after 2, 10 and 20 min of platelet incubation with picotamide.

After 15 min stirring in the aggregometer, PRP was centrifuged at 12,000 r.p.m. in an Eppendorf centrifuge and the supernatant stored at − 20°C for $TxB_2$ determination. $TxB_2$ was assayed by an enzymatic immunoassay (Cayman Chemical, Ann Arbor, MI, U.S.A.). Coefficients of variation of intraassay and inter-assay were 6.8% and 9.5%, respectively.

### *Binding studies*

*Blood sampling and platelet isolation* Blood for the receptor binding studies was withdrawn from the same healthy volunteers into a syringe containing indomethacin (10 μmol $l^{-1}$)

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and EDTA (5 mmol $l^{-1}$). PRP, obtained by centrifugation as described above, was centrifuged at 1,800 $g$ for 30 min at 20°C. Platelets were then resuspended in 10 ml of phosphate buffer pH 7.2 (mmol $l^{-1}$: $Na_2HPO_4$ 8, $NaH_2PO_4$ 2, KCl 5, NaCl 135, EDTA 10) and again recentrifuged at 1,800 $g$ for 30 min at 20°C. The supernatant was discarded and the platelets were resuspended in assay Tris-buffer pH 7.4 (NaCl 100 mmol $l^{-1}$, dextrose 5 mmol $l^{-1}$, indomethacin 10 μmol $l^{-1}$ and Tris-HCl 5 mmol $l^{-1}$). If necessary assay Tris-buffer was added to obtain a platelet concentration of $5 \times 10^{11}$ platelets $l^{-1}$.

### *Equilibrium analysis of [methylene$^3$H]-picotamide binding*

Washed platelets ($5 \times 10^7$ platelets) were incubated with 10 nmol $l^{-1}$ (final concentrations are given) [methylene$^3$H]-picotamide and increasing concentrations of unlabelled picotamide (0 to 20 μmol $l^{-1}$) at 22°C in a final volume of 0.2 ml. The binding obtained in the presence of a large excess of picotamide (20 μmol $l^{-1}$) was considered as non-specific binding. Specific binding at each concentration was calculated as the difference between total and non-specific binding. After 30 min incubation four 4 ml aliquots of ice-cold buffer were added to each tube to stop the reaction and the content was rapidly filtered under reduced pressure through Whatman GF/C glass microfibre filters. The entire washing procedure was completed within about 15 s. Filters were dried under air flow and counted in a Beckman gamma counter with an overall efficiency of 50%. Binding data, dissociation constant ($K_D$) and maximum binding capacity ($B_{max}$), were calculated according to Scatchard (1949).

The competition for [methylene$^3$H]-picotamide binding by a thromboxane $A_2$ agonist (U46619), a thromboxane $A_2$ antagonist (ONO11120) (Narumiya *et al.*, 1989), $PGI_2$, $PGE_2$, $TxB_2$ and picotamide were evaluated by incubating 10 nmol $l^{-1}$ [methylene$^3$H]-picotamide with platelets in the absence and in the presence of increasing concentrations (20 nmol $l^{-1}$–2 mmol $l^{-1}$) of competitors in a final volume of 0.2 ml. After 30 min the incubation was stopped by the addition of 5 ml of cold assay buffer and filtered. Inhibition constants of competitors were calculated according to Cheng & Prusoff (1973).

### *Kinetic analysis of [methylene$^3$H]-picotamide specific binding*

The kinetics of association and dissociation of [methylene$^3$H]-picotamide to washed platelets were evaluated as previously described for other $TxA_2$ antagonists (Modesti *et al.*, 1989). To determine the rate of association of [methylene$^3$H]-picotamide, $5 \times 10^7$ washed platelets were suspended in assay buffer containing 10 nmol $l^{-1}$ [methylene$^3$H]-picotamide (at 22°C in a final volume of 0.2 ml. After selected time intervals of incubation (20 s, 1, 2, 3, 5, 10, 15, 20, 30 min) samples were rapidly filtered. Specific binding was determined as the difference between the amount of [methylene$^3$H]-picotamide bound in the absence and in the presence of unlabelled picotamide (20 μmol $l^{-1}$).

To determine the rate of dissociation of [methylene$^3$H]-picotamide from its platelet binding sites, samples were prepared as described above. After 30 min incubation, picotamide (20 μmol $l^{-1}$) was added, and the amount of specific binding was measured after various time periods (20 s, 1, 2, 3, 5, 10, 15, 20, 30 min). Kinetic constants were calculated according to Weiland & Molinoff (1981).

### *Effect of prolonged incubation on [methylene$^3$H]-picotamide displaceable and non displaceable binding*

The time-dependency of [methylene$^3$H]-picotamide displaceable and non displaceable binding was investigated in separate time course experiments.

The total, displaceable specific, non displaceable specific and non-specific binding at the different incubation times were investigated at 37°C by preparing three different sets of tubes.

The first set of tubes was aimed to evaluate the total binding. Tubes containing [methylene$^3$H]-picotamide at increasing concentrations ($10^{-9}$, $10^{-8}$, $5 \times 10^{-8}$, $10^{-7}$, $5 \times 10^{-7}$, $10^{-6}$, $10^{-5}$ mol $l^{-1}$) plus platelets, were prepared as described above and filtered after different incubation times ($t$) (3, 10, 20, 30, 50, 60 and 120 min of incubation).

The second set of tubes was prepared to evaluate the non specific binding. Tubes contained the same incubation mixture (increasing concentrations of [methylene$^3$H]-picotamide and platelets) plus a high concentration (20 μmol $l^{-1}$) of unlabelled picotamide added before starting incubation (time 0). Samples were filtered after different incubation times ($t$) contemporary to the first set of tubes (3, 10, 20, 30, 50, 60 and 120 min of incubation).

In the third set of tubes, containing platelets and increasing concentrations of [methylene$^3$H]-picotamide, a large excess of unlabelled picotamide (20 μmol $l^{-1}$) was added at each time $t$ (3, 10, 20, 30, 50, 60 and 120 min of incubation). Samples were filtered after 30 min and the residual binding was considered as non displaceable binding. The difference between total and non displaceable binding was considered as displaceable specific binding (displaceable receptor binding). The difference between non displaceable and non-specific (evaluated by the addition of 20 μmol $l^{-1}$ picotamide at time 0) binding was considered as specific non displaceable binding (non displaceable receptor binding).

The characteristics ($K_D$, $B_{max}$) of displaceable specific receptor binding at 37°C after 30 min (at equilibrium) and after 60 and 120 min of incubation were calculated according to Scatchard (1949) as previously described.

The affinity constant ($K_{Mapp}$) and the maximal velocity ($V_{maxapp}$) of non-displaceable specific binding were calculated for each subject by the double reciprocal plot of Lineweaver-Burk (Cornish-Bowden & Eisenthal, 1978) and the lines of best fit were calculated by linear regression using the method of the least squares.

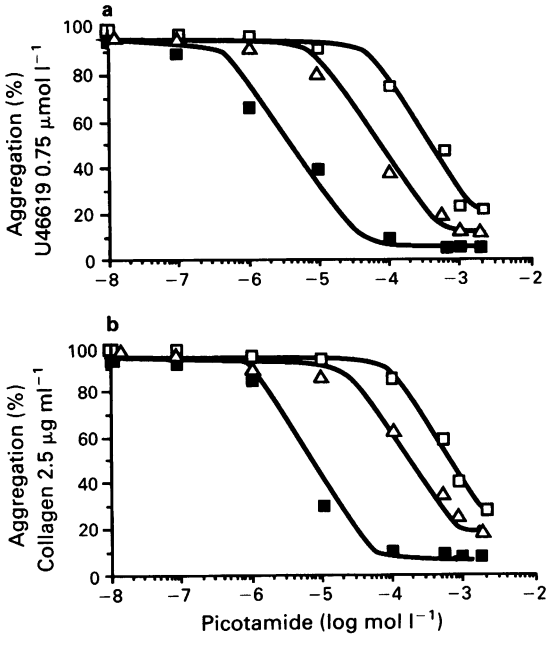


**Figure 1** Platelet aggregation induced by U46619 (0.75 μmol $l^{-1}$) (a) and collagen (2.5 μg $ml^{-1}$) (b) with increasing concentrations of picotamide after different incubation times: (□) 2 min incubation; (△) 10 min incubation; (■) 20 min incubation.

The competition for [methylene$^3$H]-picotamide non-displaceable binding by picotamide, ONO11120, U46619, $PGI_2$, $PGE_2$ and $TxB_2$, was evaluated by incubating 10 nmol $l^{-1}$ [methylene$^3$H]-picotamide with $5 \times 10^7$ platelets in the absence and in the presence of increasing concentrations (20 nmol $l^{-1}$–2 mmol $l^{-1}$) of competitors (added at time 0) at 37°C in a final volume of 0.2 ml. After 60 min the incubation was stopped by the addition of 5 ml of cold assay buffer and platelets were spun down by centrifugation. The pellet was then resuspended in assay buffer and filtered. Incorporated radioactivity was then counted as described.

### *Materials*

ONO11120 (9,11-dimethylmethano-11,12-methane-16-phenyl-13, 14-dihydro-13-aza-15-tetranor-$TxA_2$) (Narumiya *et al.*, 1986) was a kind gift from Prof. Narumiya (Kyoto, Japan). Prostacyclin ($PGI_2$), $PGE_2$ and thromboxane $B_2$ ($TxB_2$) were obtained from Upjohn, Kalamazoo, MI, U.S.A. U46619 (9,11-dideoxy-11 α,9-α-epoxymethano-$PGF_{2\alpha}$) was obtained from SIGMA Chemicals, St. Louis, MO, U.S.A. Picotamide (N, N'*bis* (3-picolyl)-4-methoxy-isophthalamide, batch no. 870311) was kindly provided by the Samil Inc. (Sandoz group, Rome, Italy); [methylene$^3$H]-picotamide (29 Ci mmol $l^{-1}$, Amersham, Buckinghamshire, GB) was a kind gift of LPB (Milan, Italy).

All the other reagents were obtained from Merck (Darmstadt, Germany) and were of analytical grade.

## Results

### *Aggregation studies*

After 2 min of incubation, picotamide dose-dependently inhibited platelet aggregation induced both by collagen and U46619 with $IC_{50}$s of $4.5 \times 10^{-4}$ mol $l^{-1}$ and $6.1 \times 10^{-4}$ mol $l^{-1}$ respectively. Inhibition of platelet aggregation was also time-dependent and peaked after 20 min. Picotamide $IC_{50}$ on collagen-induced platelet aggregation were $6.8 \times 10^{-5}$ mol $l^{-1}$ and $3.7 \times 10^{-6}$ mol $l^{-1}$ after 10 and 20 min of incubation respectively. Picotamide $IC_{50}$ on U46619-induced aggregation was $5.9 \times 10^{-5}$ mol $l^{-1}$ and $4.7 \times 10^{-6}$ mol $l^{-1}$ after 10 and 20 min of incubation respectively (Figure 1).

Picotamide also exerted a time-dependent inhibitory activity on $TxA_2$ production. The $EC_{50}$s for $TxA_2$ production during collagen (7.5 μg $ml^{-1}$) induced aggregation were $5.4 \times 10^{-5}$ mol $l^{-1}$, $2.2 \times 10^{-6}$ mol $l^{-1}$ and $4.3 \times 10^{-7}$ mol $l^{-1}$ after 2, 10 and 20 min of incubation respectively (Figure 2).

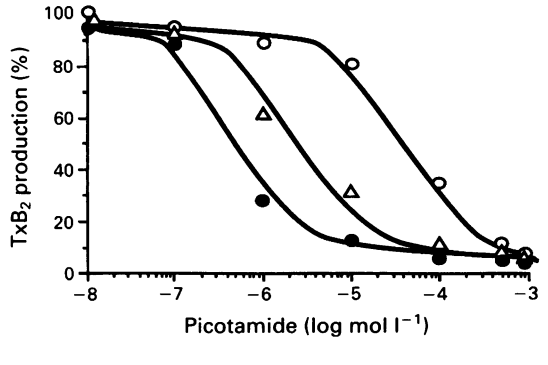


**Figure 2** Effect of increasing concentrations and time of incubation of picotamide on thromboxane $B_2$ ($TxB_2$) synthesis by platelets during collagen induced (7.5 μg $ml^{-1}$) aggregation: (○) 2 min incubation; (△) 10 min incubation; (●) 20 min incubation.

### *[Methylene$^3$H]-picotamide binding*

*Equilibrium studies* The binding of [methylene$^3$H]-picotamide at 22°C was saturable. Scatchard analysis of specific binding at equilibrium (i.e. after 30 min incubation) yielded a straight line, indicating a single class of binding sites for picotamide with $K_D$ of 325 nmol $l^{-1}$ and a $B_{max}$ of 312 fmol per $10^8$ platelets (Figure 3). The Hill coefficient ($n_H$) was 1.12 (Figure 4), suggesting that picotamide binds to a homogeneous individual class of binding sites without cooperativity.

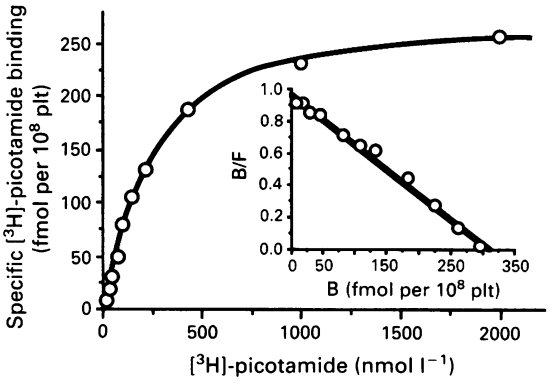


**Figure 3** Saturation curve of the [methylene$^3$H]-picotamide binding to washed human platelets (fmol per $10^8$ platelets) at 22°C. Scatchard analysis of the specific binding.

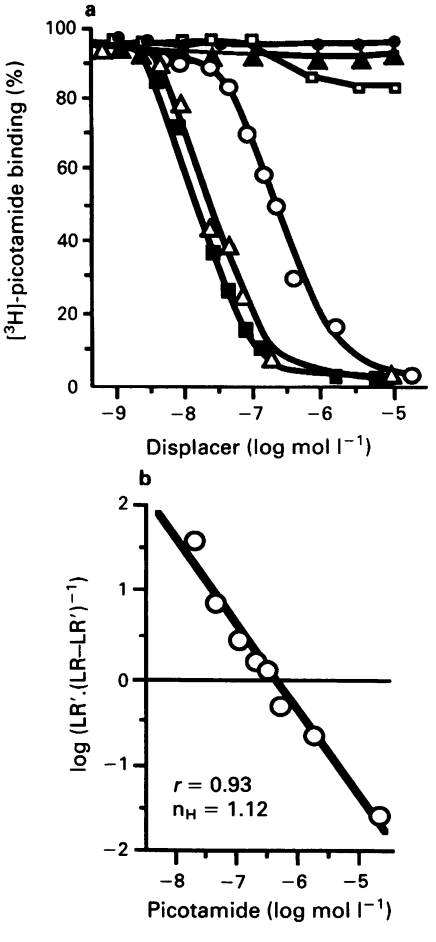


**Figure 4** (a) Displacement of the specific [methylene$^3$H]-picotamide binding by increasing concentrations of different compounds: (■) U46619; (△) ONO11120; (○) picotamide; (□) $TxB_2$; (▲) $PGE_2$; (●) $PGI_2$. Data points represent means of triplicate determinations in at least four independent experiments. (b) Hill plot of the displacement curve by picotamide.

Binding of [methylene$^3$H]-picotamide was displaced by unlabelled picotamide, ONO11120 and by U46619 with a $K_i$ of 361 nmol $l^{-1}$, 28 nmol $l^{-1}$ and 19 nmol $l^{-1}$ respectively. In contrast, $PGI_2$, $PGE_2$ and $TxB_2$ did not inhibit the binding (Figure 4).

*Kinetic analysis* The displaceable specific binding of [methylene$^3$H]-picotamide to platelets reached equilibrium after about 30 min. The observed rate constant of association ($K_{obs}$) was 0.110 $min^{-1}$. The dissociation curve performed after 30 min of incubation showed a displacement of about 65–70% of the total radioactivity bound. The analysis of the first order rate of dissociation showed a linear pattern with a $K_{-1}$ of 0.107 $min^{-1}$ ($n = 6$). The resulting association rate constant ($k_1$) was 0.00028 nmol $l^{-1}$ $min^{-1}$ with a calculated dissociation constant ($K_D$) of 382 nmol $l^{-1}$ (Figure 5).

When the displaceable specific binding of [methylene$^3$H]-picotamide to platelets at 37°C was investigated after 30, 60 and 120 min, a progressive significant reduction of the binding capacity was observed. The $B_{max}$ were 343, 243 and 153 fmol per $10^8$ platelets after 30, 60 and 120 min respectively (2066, 1463, 921 binding sites/platelet respectively) with no significant changes in $K_D$ (275, 251 and 222 nmol $l^{-1}$ respectively) (Figure 6), thus indicating a progressive decrease of platelet receptors.

In contrast the non displaceable binding of [methylene$^3$H]-picotamide to platelets (i.e. the residual radioactivity after addition of 20 μmol $l^{-1}$ picotamide to platelets at each time) at 37°C showed a slow regular increase (Figure 7). This time-dependent increase of non displaceable binding was almost completely inhibited when binding was performed at 4°C. The non displaceable binding of [methylene$^3$H]-picotamide did not increase when unlabelled picotamide was added at time 0 (non specific binding) (Figure 7).

The non displaceable binding at 37°C was concentration-dependent as it increased with increasing concentrations of [methylene$^3$H]-picotamide but was saturable. In fact, a progressive increase in binding velocity was found until a plateau level was reached with a Michaelis-Menten type kinetic of saturation (Figure 8). The Lineweaver-Burk plot was found to be linear (Figure 8) with a $V_{maxapp}$ of 130 fmol per $10^8$ platelets $h^{-1}$ and a $K_{Mapp}$ of 330 nmol $l^{-1}$. Therefore, about 35% of [methylene$^3$H]-picotamide bound to platelet receptors was irreversibly bound after 1 h.

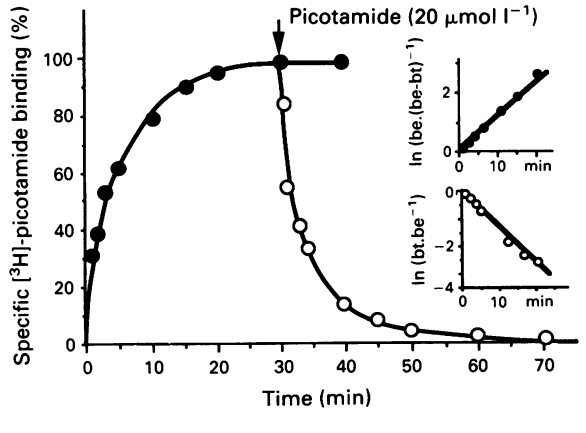


**Figure 5** Time course of the association and dissociation phases of the specific [methylene$^3$H]-picotamide binding to washed human platelets at 22°C. Dissociation was obtained after 30 min incubation upon addition of 20 μmol $l^{-1}$ (final concentration) picotamide to the incubation mixture. Data points represent means of triplicate determinations in at least four independent experiments. Upper inset: Specific binding association is plotted according to the pseudo-first order rate equation ($y = 0.331 + 0.110x$; $k_{obs} = 0.110$ $min^{-1}$). Lower inset: Dissociation is plotted as a first order reaction ($y = -0.461 - 0.107x$; $k_{-1} = 0.107$ $min^{-1}$; $k_1 = 0.00028$ nmol $ml^{-1}$ $min^{-1}$; $K_D + 382$ nmol $l^{-1}$).

The non displaceable binding of [methylene$^3$H]-picotamide was inhibited by unlabelled picotamide, ONO11120 and U46619 when added at time 0 but not by $PGI_2$, $PGE_2$ and $TxB_2$ (data not shown).

## Discussion

The present results indicate that [methylene$^3$H]-picotamide binds time-dependently to specific $TxA_2/PGH_2$ platelet receptors from which it is displaced by the $TxA_2$ agonist, U46619 and antagonist, ONO11120. Moreover, the time-dependent picotamide binding is associated with a progressive reduction

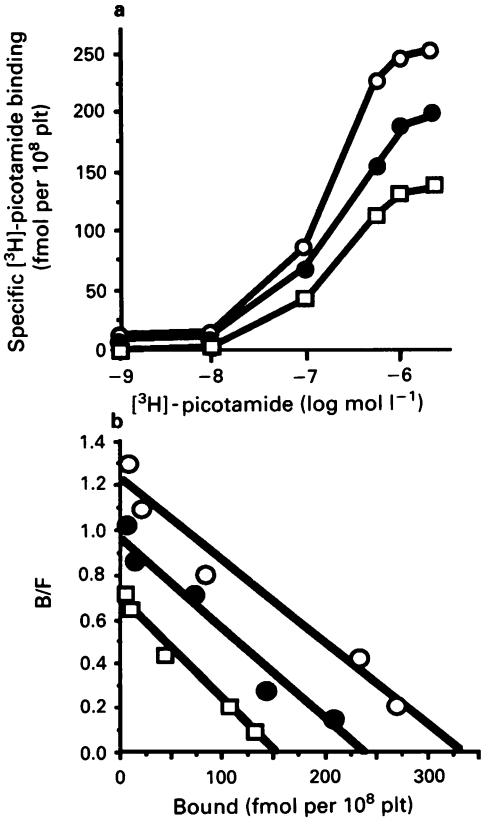


**Figure 6** Saturation curve (a) and Scatchard analysis (b) of the specific [methylene$^3$H]-picotamide binding to washed human platelets (plt) at 22°C after 30 (○), 60 (●) and 120 min (□) of incubation.

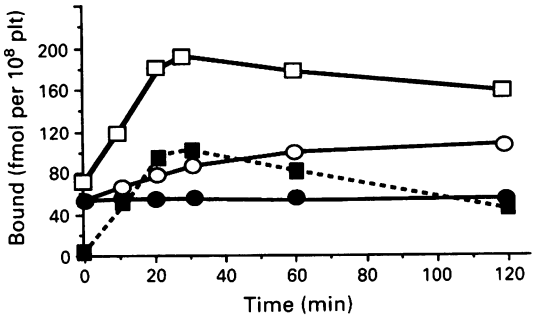


**Figure 7** Time course of the binding of [methylene$^3$H]-picotamide 10 nmol $l^{-1}$ to washed human platelets (plt) at 37°C. Total binding (□); specific displaceable binding at indicated time ($t$) (■) was considered as the radioactivity displaced by adding picotamide 20 μmol $l^{-1}$ (final concentration) at time ($t$). Specific non-displaceable binding was defined as the difference between non displaceable binding (○) and non-specific binding (●).

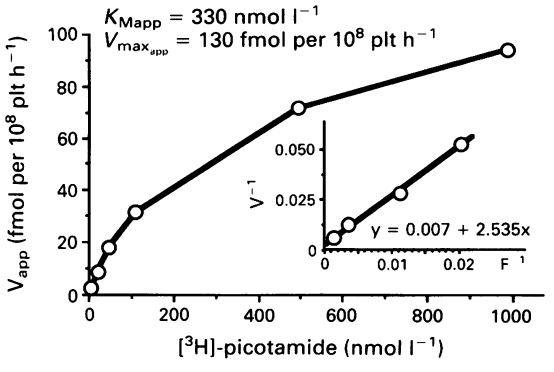


**Figure 8** Analysis of the [methylene$^3$H]-picotamide uptake by human platelets (plt) in experiments performed at 37°C. Results shown are the means of experiments carried out in triplicate. Inset: Lineweaver-Burk's plot of the results.

in displaceable binding and a contemporary increase of specific non-displaceable binding.

These findings confirm previous indirect studies which had shown that picotamide shares the $TxA_2/PGH_2$ receptor (Modesti *et al.*, 1989). In addition they show that both the picotamide binding and the antiaggregating activity of picotamide occur slowly, reaching their maximum level after about 20 min incubation. The slow onset of picotamide binding accounts for the apparently low *in vitro* antiaggregating activity reported in previous studies (Gresele *et al.*, 1989; Berrettini *et al.*, 1990) in which the antiaggregating effect was assessed after only 2–10 min incubation, i.e. before that the binding equilibrium was reached.

The antiaggregating activity of picotamide is associated with reduced $TxA_2$ formation which may be due both to inhibition of $TxA_2$-synthase by picotamide (Gresele *et al.*, 1989) and to the reduced platelet aggregation resulting from $TxA_2$ receptor blockade. Indeed, $TxA_2$ formation during collagen-induced platelet aggregation was found to be reduced by the simple receptor antagonists not provided with $TxA_2$ synthase inhibitory properties (Hornby & Skidmore, 1984).

Previous studies on the antiaggregating effects of picotamide (Gresele *et al.*, 1989; Berrettini *et al.*, 1990) led to the suggestion that picotamide could act as a competitive $TxA_2$ inhibitor. However, the present kinetic analysis of the [methylene$^3$H]-picotamide binding showed a more complex pattern. Indeed [methylene$^3$H]-picotamide was readily and almost completely displaced from the $TxA_2$ receptor during the first 20 min of incubation whereas after the first 20 min the non displaceable amount progressively increased and paralleled the reduction of specific binding. This pattern conformed to that of non-competitive receptor inhibitors (Patscheke, 1990) and suggests a stable interaction of picotamide with the $TxA_2$ platelet receptor. The progressively increasing non displaceable binding of picotamide is unlikely to be due to a simple diffusion of the drug into the platelets because the non displaceable binding of [methylene$^3$H]-picotamide was saturable, reached a plateau and was specifically blocked by the addition of unlabelled picotamide. The non displaceable binding was found to be almost completely inhibited when the binding experiments were performed at 4°C. This fact and the observation that the $K_M$ of the specific non reversible binding was in the same order of magnitude of the receptor binding $K_D$, are indicative of an internalization of the $TxA_2$ receptor-picotamide complex, although a simple non reversible receptor blockade cannot be excluded. Previous studies reported evidence of internalization of the $TxA_2$ receptor after the binding of ONO11120 (an antagonist of $TxA_2$) in human platelets (Modesti *et al.*, 1990) and U46619 (a $TxA_2$-mimetic) in cultured human leukaemic cells (Dorn, 1991).

In conclusion, picotamide binds to the $TxA_2$ receptor on human platelets with peculiar kinetics. After a first stabilizing period when the binding of picotamide is still reversible, a progressive stabilization of the binding takes place, resulting in an irreversible receptor blockade. For these characteristics picotamide is to be considered an inhibitor of platelet activity displaying both competitive and non competitive activity against thromboxane $A_2$-mediated responses.

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# Differential effects of $Na^+,K^+$-ATPase inhibition by ouabain on acid secretory responses to histamine and bethanechol in the mouse isolated stomach

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**1** The effect of $Na^+,K^+$-ATPase inhibition by ouabain on gastric acid secretion was studied in the mouse isolated whole stomach preparation.

**2** Ouabain caused a transient enhancement of histamine-induced gastric acid secretion followed by an inhibitory phase. On the other hand, ouabain caused a rapid reduction of bethanechol-stimulated acid secretion without an enhancement phase.

**3** In dibutyryl cyclic AMP-induced acid secretion, ouabain led to a transient increase in acid secretion followed by a fall, as was seen with the histamine stimulation. Ouabain caused a rapid reduction of A23187-induced acid secretion.

**4** Ouabain by itself increased basal acid secretion, and thereafter slowly suppressed the acid secretion.

**5** Atropine inhibited both the ouabain-induced enhancement of the stimulated gastric acid secretion and the ouabain-induced stimulation of basal acid secretion.

**6** The present study showed that $Na^+,K^+$-ATPase inhibition by ouabain caused a phasic enhancement of the stimulated gastric acid secretion through release of endogenous acetylcholine when the secretagogues act via an intracellular cyclic AMP pathway. It also inhibited the stimulated acid secretion irrespective of secretagogues, probably through its inhibitory effect on $Na^+,K^+$-ATPase in the gastric parietal cell.

**Keywords:** Gastric acid secretion; parietal cell; $Na^+,K^+$-ATPase; ouabain; omeprazole; mouse isolated stomach

## Introduction

Endogenous ouabain and digitalis-like factor have been recently identified in plasma of several mammals, including man (Hamlyn *et al.*, 1991; Mathews *et al.*, 1991). Previous studies have suggested an adrenal origin (Rauch & Buckalew, 1988; Boulanger *et al.*, 1993). The physiological roles of these compounds have not yet been defined, but the amounts of these compounds are elevated in plasma with experimental and clinical hypertension (Hamlyn *et al.*, 1982; 1988). Because digitalis-like activity has been found to be widely distributed among tissues, these humoral substances are likely to influence numerous processes in many tissues. In the present paper, we focus our attention on gastric acid secretion. This study may lead to speculation that endogenous ouabain modifies gastric acid secretion in physiological and pathological conditions.

We have studied the ion transport system in the parietal cell basolateral membrane (Horie *et al.*, 1992; 1993a,b). $Na^+,K^+$-ATPase in the gastric parietal cell regulates intracellular $K^+$ that is required for acid secretion by $H^+,K^+$-ATPase (Sachs *et al.*, 1976). Inhibition of $Na^+,K^+$-ATPase by ouabain is well known to inhibit gastric basal acid secretion in frog gastric mucosa (Cooperstein, 1959; Davenport, 1962). Ouabain also reduced histamine-stimulated acid production in rabbit gastric glands (Berglindh *et al.*, 1980; Koelz *et al.*, 1981). However, the effects of ouabain on the stimulation by acid secretagogues except histamine remain to be clarified because acid secretagogues acting on intracellular $Ca^{2+}$ pathway, such as bethanechol, have little stimulatory effect on acid production in rabbit gastric gland (Soll & Berglindh, 1987). Therefore, we attempted to study further the effect of ouabain on acid secretion stimulated by bethanechol as well as histamine in mouse isolated stomach and compared our results with the effect of $Na^+,K^+$-ATPase inhibition caused by $K^+$-free serosal solution.

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## Methods

### *Measurement of gastric acid secretion in the mouse isolated whole stomach*

Male ddY mice (10–20 g) were used. Gastric acid secretion was measured in the isolated lumen-perfused stomach preparation as described previously (Wan, 1977; Angus & Black, 1979) with a slight modification (Watanabe *et al.*, 1993). Briefly, the oesophagus and the pylorus were ligated under urethane (1.5 g $kg^{-1}$, i.p.) anaesthesia. After a dual cannula was attached to the forestomach, the stomach was quickly removed. The stomach was set in an organ bath containing a buffered serosal solution (20 ml) maintained at 37 ± 1°C and gassed with 95% $O_2$ and 5% $CO_2$. The stomach lumen was continuously perfused with an unbuffered mucosal solution through an inlet tube of the dual cannula connected to a perfusion pump at the rate of 1 ml $min^{-1}$. The perfusate from an outlet of the cannula was recovered as a fraction per 10 min with a fraction collector. The perfusing pressure in the stomach was kept at 20 $cmH_2O$. The acid output in the 10-min perfusate fraction was determined by titrating with 2 mN NaOH to the end point of pH 5.0, by using an automatic titrator (AUT-201, Toa Electronics, Tokyo, Japan). Gastric acid secretion in each 10-min fraction was expressed as a percentage of the maximum response to the secretagogue used in each preparation. The nutrient solutions were prepared as described by Szelenyi (1981). The serosal nutrient solution contained (mM): NaCl 118.1, KCl 4.8, $KH_2PO_4$ 1.0, $Na_2HPO_4$ 16, $MgSO_4$ 1.2, $CaCl_2$ 0.65 and glucose 31.6. The mucosal nutrient solution contained (mM):

NaCl 135.8, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ 1.3 and glucose 31.6, and was adjusted to pH 5.0. $K^+$-free serosal solution was made by substituting KCl and $KH_2PO_4$ for NaCl and $NaH_2PO_4$ on an equimolar basis, respectively. Drugs were applied to the serosal solution.

### *Chemicals*

Chemicals were obtained from the following sources: ouabain, histamine dihydrochloride and atropine sulphate from Nacalai Tesque (Kyoto, Japan); bethanechol chloride and dibutyryladenosine-3':5-cyclic monophosphate sodium salt (db cyclicAMP) from Sigma Chemical (St. Louis, U.S.A.); A23187 from Calbiochem (San Diego, U.S.A.). Omeprazole was kindly gifted from Yoshitomi Pharmaceutical (Osaka, Japan). A23187 was dissolved in absolute ethanol and omeprazole was suspended in a 0.5% methylcellulose-0.2% $NaHCO_3$ solution (pH 9.0). All other drugs were dissolved in water.

### *Data analysis*

Results are expressed as the mean ± s.e.mean of data from $n$ number of mice. Statistical analysis was done with Student's two-tailed $t$ test for unpaired observations or one-way analysis of variance followed by the Bonferroni test. The difference between groups was considered statistically significant at $P<0.05$.

## Results

### *Effects of ouabain on gastric acid secretion stimulated by histamine and bethanechol*

Histamine (500 μM) and bethanechol (10 μM) produced a sustained increase in gastric acid secretion that reached a plateau within 30 min. Ouabain was added to the serosal solution 30 min after the stimulation. Ouabain, at 100 μM only, caused a transient increase in histamine-induced gastric acid secretion immediately after the addition. After that, the gastric acid secretion slowly decreased; total inhibition was observed 30 min later (Figure 1a). On the other hand, all concentrations of ouabain caused a rapid decrease in the bethanechol-stimulated acid secretion (Figure 1b). These inhibitions were concentration-dependent.

### *Effects of $K^+$-free serosal solution and omeprazole on gastric acid secretion stimulated by histamine and bethanechol*

The composition of the serosal solution was changed to $K^+$-free to inhibit $Na^+,K^+$-ATPase. $K^+$-free serosal solution caused a transient enhancement of histamine-induced gastric acid secretion immediately after the solution replacement (Figure 2a). In contrast, $K^+$-free medium caused a rapid reduction of acid secretion by bethanechol (Figure 2b).

Omeprazole produced a rapid reduction of gastric acid secretion stimulated by either histamine or bethanechol (Figure 3a,b).

### *Effects of ouabain on gastric acid secretion stimulated by dibutyryl cyclic AMP (db cyclicAMP) and A23187*

db cyclicAMP (300 μM) or A23187 (30 μM) produced a sustained increase in gastric acid secretion which reached a plateau within 60 min. Ouabain caused a transient increase in db cyclicAMP-induced acid secretion followed by an inhibitory phase (Figure 4a). On the other hand, ouabain produced a rapid decrease in A23187-induced acid secretion (Figure 4b).

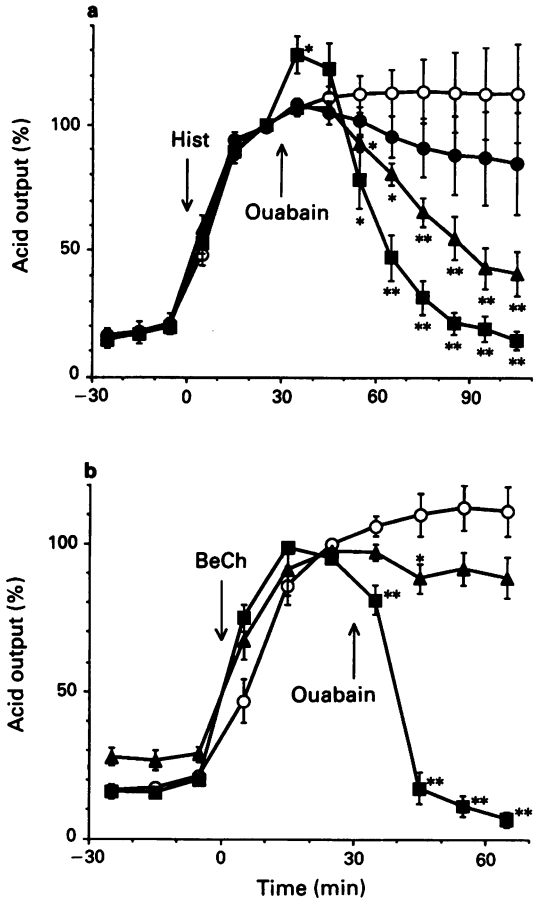


**Figure 1** Effects of ouabain on (a) histamine (Hist)- and (b) bethanechol (BeCh)-stimulated gastric acid secretion in the mouse isolated whole stomach. Ouabain was applied 30 min after Hist (500 μM) or BeCh (10 μM) was added. Gastric acid output per 10 min was expressed as a percentage of the maximum response to the secretagogue used (Hist: 3.36 ± 0.21; BeCh: 2.83 ± 0.26 μEq $10\,min^{-1}$) in each preparation. $*P<0.05$, $**P<0.01$, significantly different from the control group. Each value represents mean ± s.e.mean; $n=6$ for (a); $n=4$–6 for (b). Control (○); ouabain 10 μM (●), 30 μM (▲), 100 μM (■).

### *Effect of ouabain on basal acid secretion*

The isolated whole stomach has a spontaneous output of acid without exogenous stimulation. This preparation constantly secreted basal acid at the rate of 3.22 ± 0.34 μEq $h^{-1}$ over an hour ($n=5$). The basal secretion was inhibited by the proton pump inhibitor, omeprazole (Horie *et al.*, 1993a) but was inhibited neither by atropine (1 μM, 3.41 ± 0.45 μEq $h^{-1}$, $n=5$) nor by famotidine (10 μM, 4.36 ± 0.56 μEq $h^{-1}$, $n=5$). These results are consistent with the previous findings of Black & Shankley (1985). Thus, the basal acid secretion is closely related to the activity of parietal cell function in the resting condition.

Ouabain tended to increase basal acid secretion immediately after its addition. The following secretory response to ouabain was expressed as net increase over the basal acid output in 10-min fraction before the ouabain addition: control, 0.23 ± 0.07 μEq $10\,min^{-1}$; ouabain 30 μM, 0.24 ± 0.09 μEq $10\,min^{-1}$, $P>0.8$ vs. control group; 100 μM, 0.51 ± 0.15 μEq $10\,min^{-1}$, $P=0.24$ vs. control group; 300 μM, 0.60 ± 0.18 μEq $10\,min^{-1}$, $P=0.12$ vs. control group; 1 mM, 1.02 ± 0.23 μEq $10\,min^{-1}$, $P=0.004$ vs. control group; $n=7$ (the Bonferroni test). The ouabain-induced acid secretion was followed by a sustained fall lower than the basal value (Figure 5).

*Effects of atropine on ouabain-induced enhancement of gastric acid secretion*

Atropine (1 μM) inhibited the ouabain- or $K^+$-free serosal solution-induced enhancement of gastric acid secretion stimulated by histamine (Figure 6a,c). Atropine also tended to inhibit the ouabain-induced enhancement of acid secretion stimulated by db cyclicAMP (Figure 6b). Moreover, the ouabain-increased basal acid secretion was abolished by atropine (Figure 5). On the other hand, atropine did not modify the effect of ouabain on A23187-induced acid secretion (data not shown).

## Discussion

We studied the effect of ouabain on gastric acid secretion stimulated by bethanechol as well as histamine in the mouse isolated lumen-perfused stomach preparation. The preparation used has the following advantages: acid output can be measured directly as $H^+$ concentration under more physiological conditions than the gastric gland; secretagogues acting on intracellular $Ca^{2+}$ pathway can produce acid secretion as effectively as those acting on intracellular cyclic AMP pathway.

The present results showed that ouabain caused a transient enhancement of histamine-induced acid secretion followed by an inhibitory phase. The omission of $K^+$ from the serosal solution, in order to inhibit $Na^+,K^+$-ATPase, also provided similar results to the ouabain treatment. Atropine abolished the phasic enhancement of acid secretion. These findings suggest that this response to ouabain or $K^+$-free solution is, in great part, due to endogenous acetylcholine (ACh) release from nerve endings. It has been previously reported that ouabain causes endogenous ACh release due to inhibition of $Na^+,K^+$-ATPase in postsynaptic neurones (Satoh & Nakazato, 1992; Adam-Vizi, 1992; Adam-Vizi *et al.*, 1993). The release of endogenous catecholamines by ouabain has also been reported in other tissues (Karaki *et al.*, 1978; Wallick *et al.*, 1982; Marin *et al.*, 1986). Accordingly, it is suggested that ouabain inhibits $Na^+,K^+$-ATPase in postsynaptic neurones to cause excitation and release of ACh. The ouabain-induced enhancement of acid secretion may be explained by a synergism of action between histamine and released ACh (Berglindh, 1977). It contrast, the effect of ouabain or $K^+$-free solution on the bethanechol-induced acid secretion was only inhibitory. In can, therefore, be speculated that there is an absence of a synergism of action between bethanechol and released ACh. Taken together, we suggested that $Na^+,K^+$-ATPase inhibition leads to the release of endogenous ACh, followed by a decrease in stimulated acid secretion.

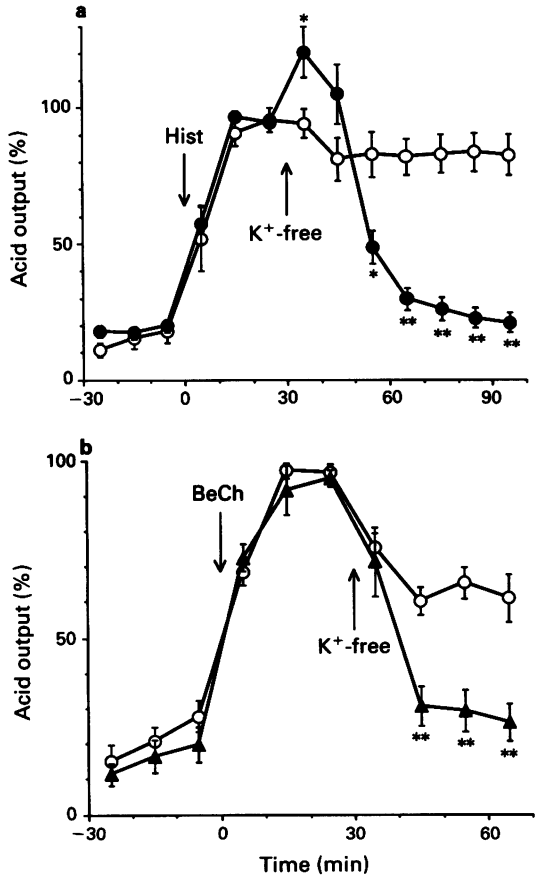


**Figure 2** Effects of $K^+$-free serosal solution on (a) histamine (Hist)- and (b) bethanechol (BeCh)-stimulated gastric acid secretion in the mouse isolated whole stomach. The treatment with $K^+$-free solution was carried out 30 min after Hist (500 μM) or BeCh (10 μM) was added. In a control group, the serosal solution was replaced with fresh solution 30 min after the secretagogue was added for comparison with $K^+$-free serosal solution group. Gastric acid output per 10 min was expressed as a percentage of the maximum response to the secretagogue used (Hist: 2.36 ± 0.24; BeCh: 2.45 ± 0.24 μEq $10\,min^{-1}$) in each preparation. $^*P<0.05$; $^{**}P<0.01$, significantly different from the control group. Each value represents mean ± s.e.mean; $n = 5$. Control (○); $K^+$-free serosal solution (●, ▲).

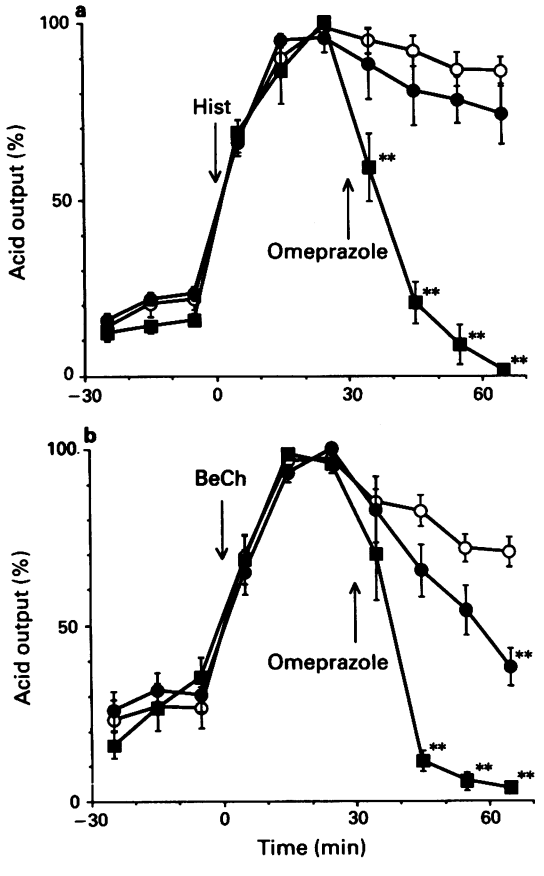


**Figure 3** Effects of omeprazole on (a) histamine (Hist)- and (b) bethanechol (BeCh)-stimulated gastric acid secretion in the mouse isolated whole stomach. Omeprazole was applied 30 min after Hist (500 μM) or BeCh (10 μM) was added. Gastric acid output per 10 min was expressed as a percentage of the maximum response to the secretagogue used (Hist: 3.40 ± 0.19; BeCh: 3.20 ± 0.13 μEq $10\,min^{-1}$) in each preparation. $^{**}P<0.01$, significantly different from the control group. Each value represents mean ± s.e.mean; $n = 5$ for (a); $n = 5$–6 for (b). Control (○); omeprazole 10 μM (●), 100 μM (■).

We studied the effects of ouabain on stimulation by secretagogues acting directly on intracellular signal transduction mechanisms. Ouabain caused a transient rise in the membrane-permeable cyclic AMP analogue db cyclicAMP-induced acid secretion followed by a fall, as was seen with the histamine stimulation. This enhancement may also be related to a synergism of action between db cyclicAMP and the ACh released by ouabain. On the other hand, ouabain produced a rapid decrease in the $Ca^{2+}$ ionophore A23187-induced gastric acid secretion, similar to that seen with bethanechol stimulation. These results suggest that ouabain-induced enhancement of stimulated gastric acid secretion can only be obtained when the secretagogues act via an intracellular cyclic AMP pathway.

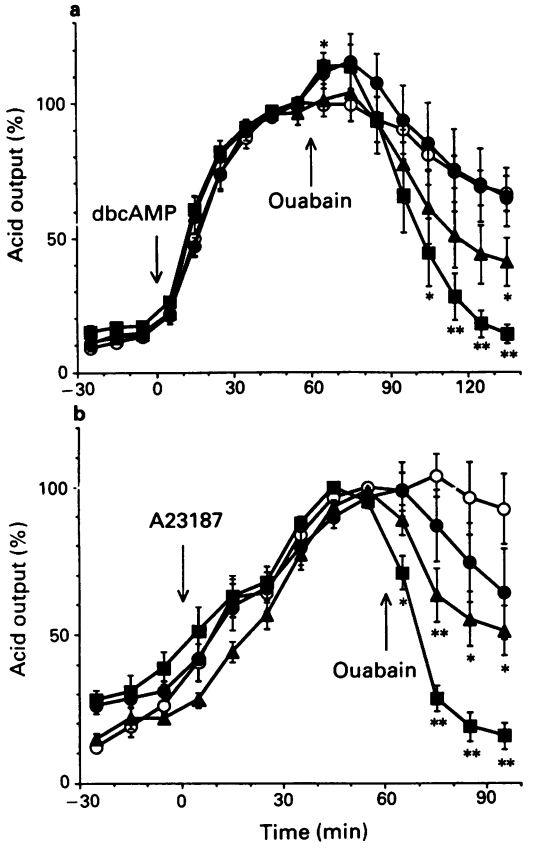


**Figure 4** Effects of ouabain on (a) dibutyryl cyclic AMP (dbcAMP)- and (b) A23187-stimulated gastric acid secretion in the mouse isolated whole stomach. Ouabain was applied 60 min after dbcAMP (300 μM) or A23187 (30 μM) was added. Gastric acid output per 10 min was expressed as a percentage of the maximum response to the secretagogue used (dbcAMP: $4.62 \pm 0.27$; A23187: $2.83 \pm 0.26$ μEq 10 $min^{-1}$) in each preparation. $^{*}P<0.05$; $^{**}P<0.01$, significantly different from the control group. Each value represents mean ± s.e.mean; $n = 6$ for (a); $n = 5$ for (b). Control (○); ouabain 10 μM (●), 30 μM (▲), 100 μM (■).

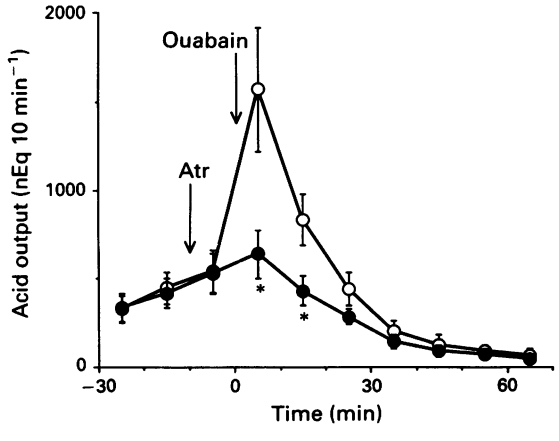


**Figure 5** Effect of ouabain on basal acid secretion and effect of atropine (Atr) on the response to ouabain in the mouse isolated whole stomach. Atr (1 μM) was applied 10 min before ouabain (1 mM) was added. $^{*}P<0.05$, $^{**}P<0.01$, significantly different from the control group. Each value represents mean ± s.e.mean; $n = 6$–7. Control (○); Atr (●).

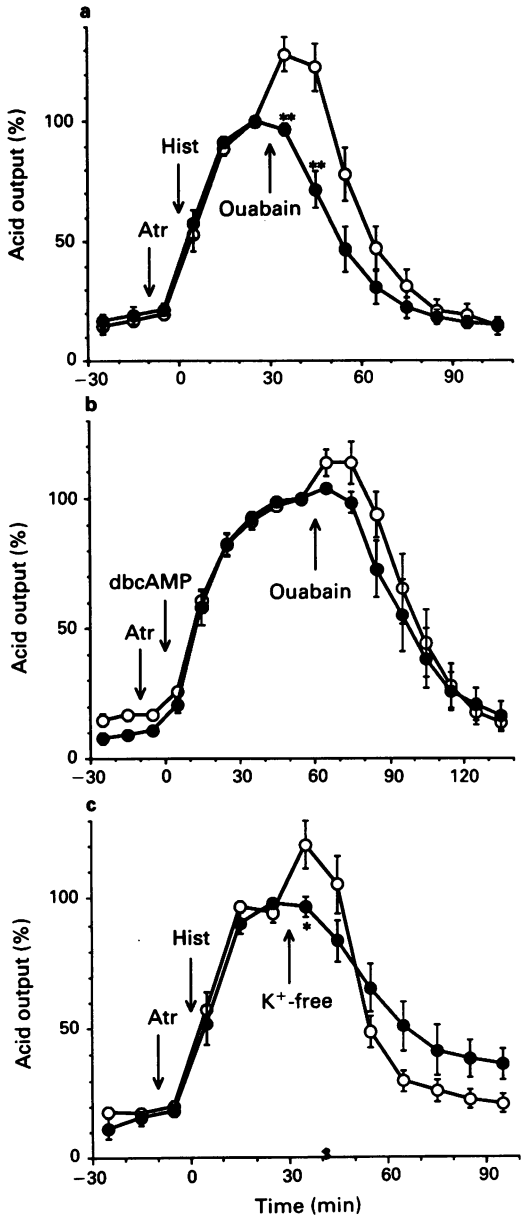


**Figure 6** Effects of atropine (Atr) on (a) the ouabain- and (c) the $K^{+}$-free serosal solution-induced enhancement on gastric acid secretory response to histamine (Hist) in the mouse isolated whole stomach. Atr (1 μM) was applied 10 min before Hist (500 μM) was added, and ouabain (100 μM) or $K^{+}$-free serosal solution was applied 30 min after histamine was added. (b) Effect of Atr on ouabain-induced enhancement of gastric acid secretory response to dibutyryl cyclic AMP (dbcAMP) in mouse isolated whole stomach. Atr (1 μM) was applied 10 min before dbcAMP (300 μM) was added, and ouabain (100 μM) was applied 60 min after dbcAMP was added. Gastric acid output per 10 min was expressed as a percentage of the maximum response to the secretagogue used in each preparation. $^{*}P<0.05$; $^{**}P<0.01$, significantly different from the control group. Each value represents mean ± s.e.mean; $n = 6$ for (a) and (b); $n = 5$ for (c). Control (○); Atr (●).

Ouabain by itself caused an increase in basal acid secretion in an apparently concentration-dependent fashion, but this was statistically significant only at the high concentration. This increased basal acid secretion may also be due to the endogenous ACh. A phasic increase produced by ouabain in acid secretion was followed by a sustained decrease lower than the original basal value. It was previously reported that ouabain or its related compounds inhibited the basal acid secretion in the frog gastric mucosa (Cooperstein, 1959; Davenport, 1962).

As to the direct effect of ouabain on gastric parietal cell function, ouabain is known to inhibit histamine-induced acid formation in the rabbit gastric gland, as judged by the aminopyrine accumulation method (Berglindh *et al.*, 1980; Koelz *et al.*, 1981). The present study not only supports their findings, but also shows that ouabain inhibits stimulated acid secretion irrespective of secretagogues. Treatment with $K^+$-free solution also provided the same results, resulting from the inhibition of $Na^+,K^+$-ATPase in the gastric parietal cell. The mechanisms involved in the antisecretory effect of ouabain are not fully understood, but several mechanisms could be suggested based on previous reports. In particular, because the maintenance of intracellular $K^+$ is mainly due to $Na^+,K^+$-ATPase and $K^+$ is required for acid formation (Sachs *et al.*, 1976), inhibition of $Na^+,K^+$-ATPase would decrease gastric acid secretion. In addition, the inhibition of this enzyme also produces an increase in intracellular $Na^+$ level (Davenport, 1962). Because intracellular $Na^+$ plays an inhibitory role in the acid-transporting system after histamine stimulation (Koelz *et al.*, 1981), the increase in $Na^+$ would reduce gastric acid secretion. Similarly, we reported that monensin, an artificial $Na^+$-$H^+$ exchanger, inhibited stimulated gastric acid secretion, probably through an increase in both intracellular pH and $Na^+$ (Horie *et al.*, 1992).

The characteristic of this antisecretory effect of ouabain is apparently similar to that of omeprazole, an $H^+,K^+$-ATPase inhibitor (Fellenius *et al.*, 1981; Sachs *et al.*, 1988), in that these compounds can inhibit acid secretion stimulated by any secretagogue. However, ouabain inhibited $Na^+,K^+$-ATPase, but not $H^+,K^+$-ATPase (Stekhoven & Bonting, 1981; Sachs, 1987). Furthermore, omeprazole caused a rapid decrease in histamine- and bethanechol-stimulated acid secretion, whereas ouabain inhibited bethanechol- and A23187-induced acid secretion immediately after the addition, but caused a slow inhibition of the histamine- and db cyclicAMP-stimulated acid secretion in the presence of atropine. Negulescu *et al.* (1990) reported that carbachol activated $Na^+,K^+$-ATPase activity in the rabbit isolated parietal cell, but that db cyclicAMP plus isobutylmethylxanthine did not affect the activity. Taken together, it is suggested that the increase in the activity of $Na^+,K^+$-ATPase may be involved in the intracellular $Ca^{2+}$ pathway in gastric acid secretion, and thus ouabain may inhibit the gastric acid secretion stimulated by bethanechol and A23187 more rapidly than that by histamine and db cyclicAMP.

In summary, the present study showed that ouabain caused both the release of endogenous ACh and the inhibition of gastric acid secretion in the mouse isolated stomach. The ouabain-induced phasic enhancement of stimulated gastric acid secretion can be obtained when the secretagogues act via an intracellular cyclic AMP pathway, but not when the secretagogues act via an intracellular $Ca^{2+}$ pathway. Furthermore, ouabain inhibited stimulated acid secretion irrespective of secretagogues, probably through its inhibitory effect on $Na^+,K^+$-ATPase in the gastric parietal cells. These findings suggest the possibility that endogenous ouabain and digitalis-like factor modify gastric acid secretion in physiological and pathological conditions.

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# Comparative analysis of the vagal stimulation of gastric acid secretion in rodent isolated stomach preparations

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1 Electrical field stimulation produced a tetrodotoxin-sensitive, frequency-dependent, release of acid from isolated, lumen-perfused, stomach preparations from mouse, immature rat and guinea-pig.

2 In the guinea-pig and mouse preparations, the frequency-dependent response was abolished by hexamethonium, acetylcholine (ACh) muscarinic (M) and histamine $H_2$-receptor blockade, consistent with the hypothesis that the vagal ACh acts indirectly by stimulating the release of endogenous histamine.

3 In contrast, in the rat preparation the frequency-dependent response was partially refractory to all of these inhibitors. However, a combination of $H_2$- and ACh M-receptor blockade did abolish the effect.

4 We conclude that vagal-stimulated acid secretion in the rat, unlike the other two species, behaves as though there is a direct innervation of the oxyntic cells by either cholinergic or noncholinergic neurones.

**Keywords:** Receptor, muscarinic; gastric acid secretion; vagus nerve; histamine; rodent isolated stomach preparations

## Introduction

The role of the vagus in the regulation of gastric acid secretion is complex. *In vivo*, the response to vagal stimulation may be inhibited by atropine, histamine $H_2$-receptor antagonists (Grossman & Konturek, 1974) or by antrectomy (Olbe, 1964), implicating a role for histamine and gastrin as well as acetylcholine (ACh) acting at ACh muscarinic (M)-receptors.

Previously, the frequency-dependent acid secretion obtained by electrical field stimulation of the isolated, lumen-perfused, stomach preparation from the mouse was concluded to be due to preganglionic stimulation of the vagus nerve resulting in the postganglionic release of ACh as judged by the inhibition produced by tetrodotoxin, hexamethonium and atropine (Angus & Black, 1982). Similar results were found (Baird & Main, 1978) in a gastric mucosal sheet preparation from the rat, although in that assay the stimulation was apparently post-ganglionic because it was refractory to hexamethonium. Subsequently, we presented preliminary data in the mouse stomach preparation showing that the frequency-response was abolished by histamine $H_2$-receptor blockade (Black & Shankley, 1987), although the response to a stable, efficacious, ACh M-receptor agonist, 5-methylfurmethide, was relatively refractory (Black & Shankley, 1985a). These results in the mouse were explained by proposing that neurally-released ACh, perhaps restricted by cholinesterase activity and localized release, acted predominantly to stimulate histamine secretion, whereas 5-methylfurmethide was able to gain access to the M-receptors on the oxyntic cell as well as those on the histamine cell. In contrast, Main & Pearce (1978) reported that the histamine $H_2$-receptor antagonist, metiamide, was ineffective against both methacholine and electrical field stimulation of the vagus in their rat isolated gastric mucosal sheet preparation.

In an attempt to resolve these conflicting results, we have investigated the response to electrical field stimulation in a comparative study in isolated, lumen-perfused, stomach preparations from mouse, immature rat and guinea-pig.

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## Methods

### *Isolated, lumen-perfused, stomach preparations*

*Mouse and rat* Gastric acid secretion was measured in isolated, lumen-perfused, stomach preparations essentially as described previously for the mouse (Black & Shankley, 1985b). Young adult male mice (Charles River 22–26 g), fasted for 18 h prior to experimentation but with free access to water, and pre-weaned rat pups (Wistar 32–38 g corresponding to age range 10–23 days) were used. Animals were killed by cervical dislocation, the abdomen opened and the oesophagus ligated close to the stomach. A polythene cannula (2 mm internal diameter) was inserted into the pylorus via the duodenal bulb, and a small incision made in the fundus through which the stomach contents were gently washed. A second cannula was tied into this incision. The stomachs were then transferred into a 40 ml organ bath containing buffered serosal solution (mM: NaCl 118, KCl 4.8, $MgSO_4$ 1.2, $KH_2PO_4$ 1.14, $NaHPO_4$ 15.9, $CaCl_2$ 0.65 and glucose 31.6) maintained at 37°C and gassed with 95% $O_2$ and 5% $CO_2$. The preparations were continuously perfused from the fundic to the pyloric cannulae with warmed unbuffered mucosal solution (mM: NaCl 135, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ 1.3 and glucose 31.6) gassed with 100% $O_2$, and the perfusate passed over a pH-electrode system adjusted to provide 12 $cmH_2O$ intragastric pressure.

*Guinea-pig* Due to the large size of the guinea-pig stomachs, especially following distension because of the back pressure applied to the lumen-perfusate, 'half' stomach assays were prepared. Male guinea-pigs, weighing 180–220 g, were killed by cervical dislocation, the abdomen opened, the duodenal bulb removed and a polythene cannula inserted into the antral end of the stomach. A cut was made round the stomach from the lesser curvature at the level of the cardiac sphincter, retaining approximately half of the glandular portion, and the stomach contents were gently washed out. The tissue was removed and a further catheter inserted into the aperture created and tied to form a water-tight seal. The guinea-pig preparations were mounted in 40 ml organ baths which were maintained at 34°C because preliminary studies suggested that muscle contraction was reduced at this lower temperature.

### Experimental design

Six preparations were used simultaneously and, after a 60 min stabilization period, any not showing stable basal responses were rejected (less than 5%). Thereafter, drugs were added to the serosal solution according to individual experimental protocols. The total vehicle volume did not exceed 1 ml. A randomized block design was used throughout for allocation of experimental treatments such that, as far as possible, each organ bath received each treatment within the course of an experiment.

Acid secretory responses were expressed as ΔpH, that is the difference between basal pH, measured immediately prior to experimental intervention, and stimulated pH. The stomach preparations were electrically stimulated with a pair of platinum, ring electrodes (ring diameter 2 mm, wire diameter 0.5 mm) placed either side of the stomach in the region of the fundic glands (Black & Shankley, 1986). The intensity of stimulation was standardized at 10 V with square wave pulses of 0.5 ms duration. Single cumulative frequency-effect curves were obtained over a frequency range of 1 to 30 Hz.

### Data analysis

Where possible, the frequency-effect curve data from individual preparations were fitted by means of an iterative least squares minimization programme to a general logistic function to provide estimates of the midpoint location ($\log f_{50}$), midpoint slope parameter (p) and upper asymptote (α), as described previously (Black & Shankley, 1985b). For display purposes the individual computed parameter estimates for each treatment group were expressed as mean ± s.e.mean and single logistic curves simulated shown superimposed upon the experimental data.

Computed logistic curve-fitting parameters were compared using Student's $t$ test. Values of $P<0.05$ were considered significant.

### Drugs

Tiotidine (a gift from Zeneca Ltd.) and famotidine (a gift from Merck, Sharp and Dohme Ltd.) were dissolved in dilute HCl to give 0.2 mM stock solutions. Subsequent dilutions were made in distilled water. All other compounds were dissolved in distilled water and sources were: atropine sulphate, hexamethonium, tetrodotoxin (Sigma); CI-988 ([[3-(1H-indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo[3,3,1,1,$^{3,7}$]dec-2-yl-oxy)carbonyl]amino]propyl]amino]-1-phenyl-ethyl]amino]-4-oxo butanoic acid (a gift from Parke Davis Ltd.), 5-methylfurmethide iodide (a gift from Wellcome Foundation Ltd.); and McN-A 343 (4-(N-[-3-chlorophenyl]carbamoyloxy)-2-butyryl-trimethylammonium chloride) (a gift from McNeil Laboratories USA Ltd.).

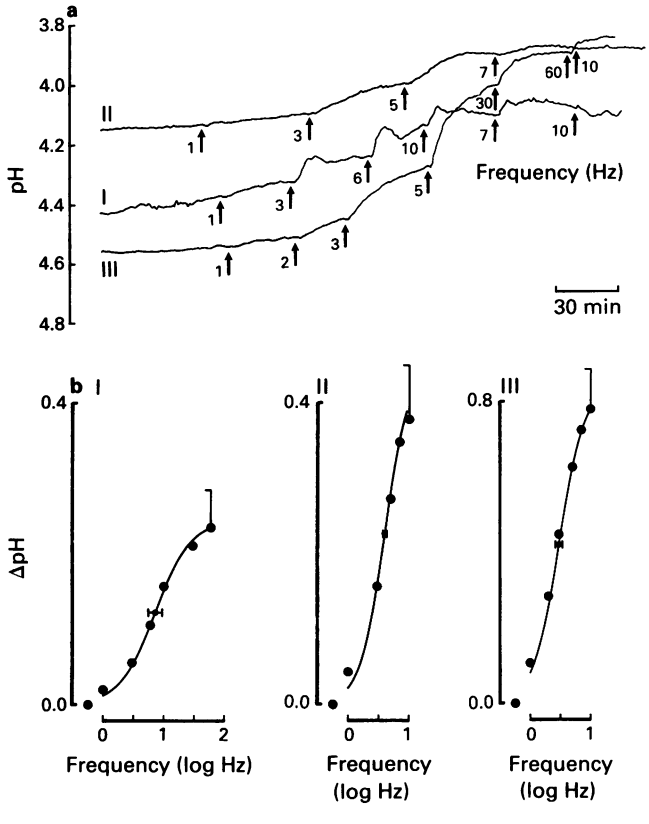


**Figure 1** (a) Examples of experimental traces showing cumulative frequency-dependent changes in the pH of the lumen perfusate during electrical field stimulation of isolated, lumen-perfused, stomach preparations from (I) mouse and immature (II) guinea-pig and (III) rat. (b) Frequency-effect curve data corresponding to (a). The effect is expressed as the change in pH (ΔpH) of the lumen perfusate from the basal unstimulated level and is plotted as a function of the frequency of electrical field stimulation. The curves shown superimposed on the mean data points ($n = 4/5 \pm$ s.e.mean) were obtained using the logistic curve fitting parameters shown in Table 1 according to the methods described in the text.

**Table 1** Logistic curve-fitting parameters (midpoint location, $\log f_{50}$; midpoint slope parameter, p and upper asymptote, α ± s.e.mean) of control frequency-effect curves obtained in isolated, lumen-perfused, stomach preparations from mouse, immature guinea-pig and rat

| | n | $\log f_{50}$ | α(ΔpH) | p |
|---|---|---|---|---|
| Mouse | 5 | 0.86 ± 0.12 | 0.25 ± 0.05 | 1.50 ± 0.16 |
| Guinea-pig | 4 | 0.60 ± 0.03 | 0.45 ± 0.07 | 2.17 ± 0.48 |
| Rat | 5 | 0.48 ± 0.06 | 0.85 ± 0.11 | 2.04 ± 0.26 |

## Results

Electrical field stimulation produced a frequency-dependent release of acid from all three preparations (Figure 1a) which was abolished by 10 μM tetrodotoxin (TTX) indicating neural origin. The inhibition in the presence of TTX of the maximum frequency-dependent response in the guinea-pig, mouse and rat preparations was 100 ± 13, 96 ± 5, 94 ± 3%, respectively. The responses in each assay were stable after 30 min, although an initial peak was frequently observed in the mouse assay (see Figure 1).

The frequency-dependent-response data could be fitted by the logistic function (Figure 1b) and the parameter estimates (Table 1) indicated that the curve obtained in the mouse stomach assay had a lower midpoint slope than that obtained in the immature guinea-pig and rat assays.

In the guinea-pig and mouse preparations, the frequency-dependent responses were also abolished by blocking ganglia (hexamethonium) and ACh M-receptors (atropine). The inhibition in the presence of hexamethonium, at a concentration (100 μM) shown previously to produce selective ganglionic blockade in the mouse stomach preparation by Angus & Black (1982), of the maximum frequency-dependent response in the guinea-pig and mouse preparations was 95 ± 5 and 96 ± 5%, respectively. The inhibition in the presence of atropine at concentrations approximately 1000 fold (20 μM) and 100 fold (2 μM) higher than the $K_B$ values estimated in guinea-pig and mouse preparations (Welsh et al., 1992), of the maximum frequency-dependent response in the guinea-pig and mouse preparations was 95 ± 13 and 93 ± 9%, respectively. Similarly, histamine $H_2$-receptor blockade, achieved with concentrations of famotidine (20 μM) or tiotidine (100 μM) which are approximately 1000 fold their $K_B$ values in these assays (Welsh et al., 1992), also abolished the responses in the guinea-pig and mouse preparations (Figure 2a,b).

In the rat assay, fully-defined frequency-effect curves could still be obtained in the presence of any of these three antagonists (Figures 2c and 3) although the curve maxima were significantly reduced (28 ± 6, 36 ± 6, 47 ± 11% inhibition with hexamethonium, atropine and tiotidine, respectively). However, in the presence of both 100 μM tiotidine and 20 μM atropine the response in the rat was completely inhibited (Figure 4).

The possibility that gastrin was mediating part of the frequency-dependent response in the rat assay was investigated by use of the gastrin/$CCK_B$ receptor antagonist, CI-988 (Horwell *et al.*, 1991). This ligand had no effect on the frequency-effect curve (curve maxima: 0.68 ± 0.08 and 0.81 ± 0.05ΔpH, in the absence and presence of CI-988, respectively) at a concentration (10 μM) greater than 300 fold its reported $K_B$ value (23 nM) at gastrin/$CCK_B$-receptors in an isolated preparation of rat gastric mucosa (Patel & Spraggs, 1992).

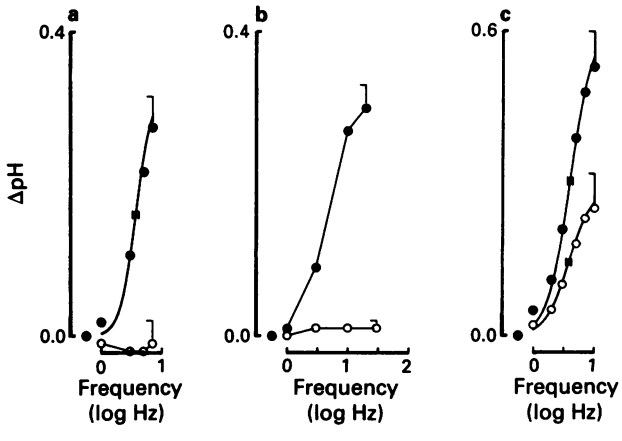


**Figure 2** Frequency-effect curves obtained by electrical field stimulation in isolated stomach assays from (a) immature guinea-pig, (b) mouse and (c) immature rat in the absence (●) and presence (○) of histamine $H_2$-receptor block (100 μM tiotidine or 20 μM famotidine). The curves shown superimposed on the mean data points ($n$ = 3/9 ± s.e.mean) were obtained by logistic curve fitting as described in the text.

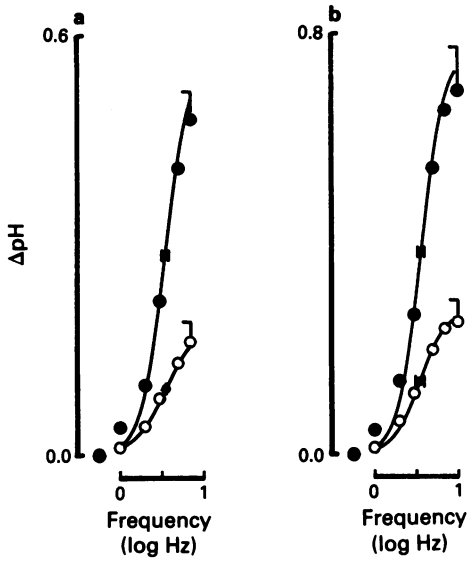


**Figure 3** Frequency-effect curves obtained by electrical field stimulation of isolated stomach preparations from immature rats in the absence (●) and presence (○) of (a) hexamethonium (100 μM) and (b) atropine (20 μM). The curves shown superimposed on the mean data points ($n$ = 7/8 ± s.e.mean) were obtained by logistic curve fitting.

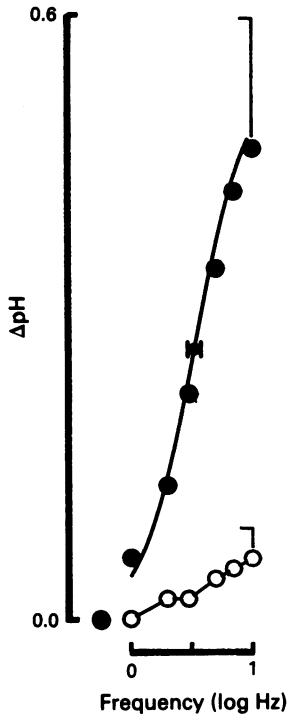


**Figure 4** Frequency-effect curves obtained by electrical field stimulation in rat isolated stomach preparations in the absence (●) and presence (○) of both atropine (20 μM) and tiotidine (100 μM). The curves shown superimposed on the mean data points ($n$ = 5/6 ± s.e.mean) were obtained by logistic curve fitting.

## Discussion

Isolated, lumen-perfused, stomach preparations were chosen for this comparative analysis because they retain the gastric mucosal architecture so that the relationship between the oxyntic, neural and histamine-secreting cells is maintained during neural stimulation (Black & Shankley, 1985b). Electrical field stimulation gave stable and reproducible acid secretion responses in each of the assays using the stimulation parameters previously optimised in the mouse stomach (Angus & Black, 1982; Black & Shankley, 1986). The effects of electrical field stimulation were totally blocked by TTX in all preparations implying that the effects were mediated by neural activity.

In the mouse and guinea-pig preparations we conclude that, (i) as the effects were annulled by hexamethonium then the frequency effects were mediated by preganglionic nerve stimulation, (ii) as atropine also suppressed the frequency effects then ACh was the postganglionic transmitter, (iii) as histamine $H_2$-receptor antagonism also blocked the frequency effects, then neurally-released ACh acted predominantly to release histamine. We have argued previously (Black & Shankley, 1987) that the ACh was restricted to the region of the histamine-secreting cells by neural configuration and by cholinesterase activity. This view was confirmed, in the mouse, by the finding that inhibition of cholinesterase produced tiotidine-refractory frequency effects as though ACh could now diffuse to the location of the oxyntic cells (Black & Shankley, 1987). We were unable to confirm this result in the guinea-pig because cholinesterase inhibition by physostigmine produced contractions of the stomach muscle sufficiently powerful to interfere with lumen-perfusion. Histamine-storing mast-cell-like cells in the subepithelium and enterochromaffin-like-cells (ECL cells) have been described (Hakanson, 1970; Hakanson & Sundler, 1991) although their relationship to cholinergic neurones in the gastric mucosa is apparently not described. However, discrete

innervation of mucosal histamine-containing cells in the small intestine has been reported (Newson *et al.*, 1993).

The present results obtained in the mouse stomach preparation are consistent with those previously reported by Angus & Black (1982). In the rat, however, hexamethonium only partially inhibited the frequency effects. If hexamethonium acts by blocking ganglionic nicotinic receptors this indicates that both pre- and postganglionic fibres were being stimulated or that there was an element of non-nicotinic receptor-mediated ganglionic transmission. Either way, on this evidence, we cannot know whether different nerve pathways are involved. Previously, using a mucosal sheet preparation from rat, Baird & Main (1978) found that responses to electrical field stimulation were completely resistant to hexamethonium. The difference between their results and ours may be due to destruction of the autonomic ganglia during the stripping of the smooth muscle, leaving only the postganglionic fibres intact (Angus & Black, 1982).

The rat also differed from the guinea-pig and mouse in that frequency effects were partially atropine-resistant. Atropine and hexamethonium produced almost identical effects on the frequency-effect curves. In both cases there was a decrease in amplitude of the frequency-effect curves of about 60% without changes in location or slope. This suggests that the effects which are resistant to both hexamethonium and atropine may be mediated by the same nerve fibres which would, therefore, be different from those mediating the drug-sensitive effects. The congruence of hexamethonium and atropine effects in the rat may imply that, as in other species, the preganglionic pathways connect to the cholinergic neurones.

In the rat preparation, histamine $H_2$-receptor blockade only partially inhibited the frequency effects. As with hexamethonium and atropine, tiotidine did not alter the location and slope parameters of the frequency-effect curves but decreased their amplitude by about 60%. However, in the presence of a combination of both atropine and tiotidine, the frequency effects were more or less abolished. Therefore, it seems that the histamine-dependent portion of the response is not the atropine-sensitive portion; that is, it is not, or not entirely, due to ACh M-receptor stimulated histamine release. It is as though atropine removes the component refractory to $H_2$-receptor blockade and tiotidine removes the component refractory to ACh M-receptor blockade, leading to the conclusion that there is both direct cholinergic innervation plus a non-cholinergic innervation which either releases histamine by releasing an unknown transmitter or, more economically, the transmitter is histamine. Histamine and gastrin containing nerves in the stomach submucosa have been described (Uvnas-Wallenstein *et al.*, 1977). Gastrin does not appear to be a candidate because the atropine-resistant frequency effects were not blocked by the gastrin/$CCK_B$-receptor antagonist, CI-988.

In the rat, the partial inhibitory effects of $H_2$-receptor blockade indicate that, unlike other species, direct innervation of oxyntic cells by cholinergic or non-cholinergic neurones is involved in addition to neural release of histamine. These indications raise the question of whether, in the rat, in addition to direct cholinergic and direct histaminergic mechanisms, there is also an indirect cholinergic release of histamine as in the other species.

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# Anticonvulsant effects of the glycine/NMDA receptor ligands D-cycloserine and D-serine but not R-(+)-HA-966 in amygdala-kindled rats

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**1** The effects of the glycine/NMDA receptor partial agonists, D-cycloserine and (+)-HA-966 and the full agonist, D-serine, on focal seizure threshold and behaviour have been determined in amygdala-kindled rats, i.e. a model of focal (partial) epilepsy. The uncompetitive NMDA receptor antagonist, MK-801, was used for comparison.

**2** The high efficacy glycine partial agonist, D-cycloserine, did not alter the threshold for induction of amygdaloid afterdischarges (ADT) at doses of 20–80 mg kg$^{-1}$ i.p., but significant ADT increases were determined after application of higher doses (160 and 320 mg kg$^{-1}$). The ADT increases after these high doses were long-lasting; significant elevations were still observed 2 days after drug injection. Determination of D-cycloserine in plasma and brain tissue showed that it was rapidly eliminated from plasma. Compared to peak levels in plasma, only relatively low concentrations of D-cycloserine were measured in brain tissue.

**3** The low efficacy glycine partial agonist, (+)-HA-966, 10–40 mg kg$^{-1}$ i.p., did not alter the ADT or seizure recordings (seizure severity, seizure duration, afterdischarge duration) at ADT currents. However, the drug dose-dependently increased the duration of postictal behavioural and electroencephalographic depression in kindled rats. At the higher dose tested, postictal immobilization was dramatically increased from 3 min to about 120 min. This might indicate that glutamatergic activity is decreased postictally, which is potentiated or prolonged by (+)-HA-966.

**4** Like D-cycloserine, the glycine receptor full agonist, D-serine, injected bilaterally into the lateral ventricles at a dose of 5 μmol, significantly increased the ADT, while no effect was seen at a lower dose (2.5 μmol).

**5** The anticonvulsant effects observed with D-cycloserine were completely antagonized by combined treatment with (+)-HA-966, indicating that the effects of D-cycloserine were mediated by the glycine/NMDA receptor complex.

**6** MK-801, 0.1 mg kg$^{-1}$, did not alter the focal seizure threshold or seizure recordings at ADT current, but induced marked phencyclidine(PCP)-like behavioural alterations, such as hyperlocomotion, stereotypies and motor impairment. No PCP-like behaviours were observed after D-cycloserine, D-serine or (+)-HA-966. High doses of (+)-HA-966 induced moderate motor impairment in kindled rats.

**7** The long lasting increases in seizure threshold observed after the high efficacy glycine partial agonist, D-cycloserine but not the low efficacy partial agonist, (+)-HA-966, may suggest that the effects of D-cycloserine are mediated by adaptive changes in the NMDA receptor complex in response to glycine receptor stimulation.

**8** Pharmacological intervention at the strychnine-insensitive glycine receptor by high-efficacy partial agonists with systemic bioavailability may be an effective means of increasing seizure threshold without concomitantly inducing PCP-like adverse effects.

**Keywords:** Glycine receptors; NMDA receptors; glutamate; epilepsy; MK-801; dizocilpine; stereotypies; locomotor activity; motor impairment; postictal depression

## Introduction

The strychnine-insensitive glycine site of the N-methyl-D-aspartate (NMDA) receptor complex has generated an enormous amount of interest since it was first described seven years ago by Johnson & Ascher (1987). It is now clear that occupation of the glycine site by an agonist is an absolute requirement for NMDA receptor activation and that glycine is, in effect, a NMDA receptor co-agonist (Kemp & Leeson, 1993). Because of this crucial role of the glycine site for NMDA receptor activation, glycine receptor antagonists are thought to have several therapeutic indications for diseases, including cerebral ischaemia and epilepsy, in which glutamatergic overactivity is thought to be involved (Carter, 1992). One advantage of glycine antagonists in this respect might be that they have a larger therapeutic window than antagonists of the NMDA receptor and associated ion channel (Carter, 1992). Indeed, glycine antagonists, such as 7-chlorokynurenic acid, were shown to antagonize or attenuate convulsions in different seizure models, including seizures induced by NMDA, audiogenic stimulation, electroshock or amygdala-kindling, without producing phencyclidine(PCP)-like behavioural adverse effects (Croucher & Bradford, 1990; Koek & Colpaert, 1990; Tricklebank & Saywell, 1990; Croucher & Bradford, 1991; Baron *et al.*, 1992). Based on the role of NMDA receptors in the initiation and propagation of seizures (Dingledine *et al.*, 1990; Löscher, 1993), the NMDA potentiation by glycine and the anticonvulsant activity of NMDA and glycine antagonists, it has been proposed that glycine or glycine agonists such as D-serine are proconvulsant (Foster & Kemp, 1989). However, in contrast

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to that proposal, several reports have shown that 1-amino-1-carboxycyclopropane, which is almost a full agonist (up to 90% efficacy of glycine), D-cycloserine, which has about 40–70% efficacy of glycine, and glycine itself have anticonvulsant activity when administered to animals in high doses (Wood *et al.*, 1966; Mayer *et al.*, 1971; Lapin, 1981; Toth *et al.*, 1983; Peterson & Boehnke, 1989; Skolnick *et al.*, 1989; Stark *et al.*, 1990; Tricklebank & Saywell, 1990; Peterson, & Schwade, 1993). Furthermore, glycine and D-serine were found to potentiate the anticonvulsant effect of clinically established antiepileptic drugs, while L-serine was ineffective (Peterson, 1991). Since these data conflict with the current theory of the role of glutamate receptors in seizures and anticonvulsant drug actions, it has been proposed that the anticonvulsant effect of glycine agonists and high efficacy partial agonist cannot be explained entirely by their actions at the glycine site of the NMDA receptor (Kemp & Leeson, 1993). However, Peterson (1992) demonstrated that the anticonvulsant effect of D-cycloserine in the maximal electroshock seizure (MES) test in rats could be antagonized by the glycine antagonist, 7-chlorokynurenic acid.

In the present study, we were interested to examine if D-cycloserine also exerts anticonvulsant effects in amygdala-kindled rats, i.e. a widely used model of complex-partial seizures with secondary generalization to clonic convulsions. For comparison with D-cycloserine, we used the uncompetitive NMDA receptor antagonist, MK-801 (dizocilpine), the full glycine receptor agonist, D-serine, and the low efficacy partial glycine agonist (+)-HA-966 (**R**-(+)-3-amino-1-hydroxypyrrolid-2-one), which has less than 8% of the efficacy of the endogenous agonist, glycine (Henderson *et al.*, 1990).

## Methods

### *Animals*

Female Wistar rats (Harlan-Winkelmann, Borchen, Germany), weighing 210–230 g, were used. The animals were purchased from the breeder at a body weight of about 200 g. Following arrival in the animal colony, the rats were kept under controlled environmental conditions (ambient temperature 24–25°C, humidity 50–60%, 12/12 h light/dark cycle, light on at 06 h 00 min) for at least 1 week before being used in the experiments. Standard laboratory chow (Altromin 1324 standard diet) and tap water were allowed *ad lib.* All experiments were done between 08 h 00 min and 12 h 00 min.

### *Preparation of animals*

The rats were anaesthetized with chloral hydrate (360 mg $kg^{-1}$, i.p.) and received stereotaxic implantation (according to the surgery methods described in the atlas of Paxinos & Watson, 1986) of one bipolar electrode in the right basolateral amygdala. Coordinates for electrode implantation were AP −2.2, L −4.8, V −8.5. All coordinates were measured from bregma. Skull screws served as the indifferent reference electrode. The electrode assembly was attached to the skull by dental acrylic cement. In one group of rats, intracerebroventricular (i.c.v.) injection guide cannulae were implanted in addition to the bipolar amygdala electrode. In each of these animals, two 0.6 mm guide cannulae were bilaterally implanted 0.5 mm dorsal to the left and right lateral ventricle. Coordinates for guide cannula implantation were AP −0.8, L ±1.5, V −2.9. A 0.35 mm stylet was placed in the cannulae to prevent clogging when not in use. The guide cannulae were secured together with the electrode assembly with dental acrylic cement. For i.c.v. drug injection, a 0.35 mm injection needle that extended 1 mm beyond the dorsal end of the guide cannula was inserted into each guide cannula. In preliminary experiments, verification of the cannula placements was made by localization of a dye which had been infused into the lateral ventricles.

### *Kindling*

After a postoperative period of 2 weeks, constant current stimulations (500 μA, 1 ms, monophasic square-wave pulses, 50 $s^{-1}$ for 1 s) were delivered to the amygdala at intervals of 1 day until 10 sequential stage 5 seizures were elicited. Seizure severity was classified according to Racine (1972): (1) immobility, eye closure, twitching of vibrissae, sniffing, facial clonus; (2) head nodding associated with more severe facial clonus; (3) clonus of one forelimb; (4) rearing, often accompanied by bilateral forelimb clonus; (5) rearing with loss of balance and falling accompanied by generalized clonic seizures. After kindling acquisition, the electrical susceptibility of the stimulated region for triggering of paroxysmal neuronal activity (threshold for induction of afterdischarges, ADT) was recorded using an ascending step procedure (Freeman & Jarvis, 1981). The initial current intensity was 10 μA, and the current intensity was increased in steps of about 20% of the previous current at intervals of 1 min until an afterdischarge of at least 3 s duration was elicited. Determination of ADTs was repeated at intervals of 2–3 days until all animals exhibited reproducible seizure thresholds. Since almost all fully kindled animals exhibited generalized seizures (stage 4–5) at the ADT current, it was not necessary to determine the threshold for generalized seizures (GST) separately. In fully kindled rats, the ADT determined with interstimulation intervals of 1 day was not different from ADT values determined with interstimulation intervals of 1 min, thus demonstrating that the short interstimulation interval did not bias ADT determinations. In addition to ADT, in fully-kindled rats the following parameters of kindled seizures were measured at stimulation with the ADT current: seizure severity was classified as described above. Seizure duration was the duration of the limbic (stage 1–2) and motor seizures (stage 3–5). Afterdischarge duration was the total time of spikes (with an amplitude of at least twice the amplitude of the prestimulus recording and a frequency greater than 1 $s^{-1}$) in the EEG recorded from the site of stimulation. Furthermore, the period of postictal immobilization ('freezing'; defined as the period of immobility between the end of the afterdischarge until the onset of locomotion) was recorded.

### *Evaluation of drug effects on focal seizure threshold*

The effects of MK-801, D-cycloserine, and (+)-HA-966 were compared in groups of 6–10 fully kindled rats by determination of the ADT, i.e. the most sensitive measure of anticonvulsant effects on focal seizure activity in kindled rats. The drugs were injected i.p. 1 h (D-cycloserine), 2.5 h (MK-801) or 0.5 h ((+)-HA-966) prior to amygdala stimulation. The effect of D-serine on ADT was determined 60 min after bilateral i.c.v. administration of 2.5 or 5 μmol (half of these doses injected per site). These doses of D-serine were infused i.c.v. in a volume of 4 μl (i.e. 2 μl per site) over a period of 1 min per site. The injection needle was left in place for an additional 1-min period at the end of the infusion to allow diffusion of the solution into the ventricles. Dosages and pretreatment times were chosen on the basis of previous dose-effect and time-course experiments with the drugs in rats (Löscher & Hönack, 1991a; Vartanian & Taylor, 1991; Peterson, 1992). The control ADT was determined 2–3 days before and after each drug treatment, and the next drug experiment in the same group of rats was only undertaken if the post-drug ADT was not significantly different from the pre-drug ADT. For control determinations, rats received i.p. or i.c.v. injection of vehicle (saline) with the same pretreatment time used for drug testing. For all drug experiments, at least 4 days were interposed between 2 drug injections in the same group of rats in order to avoid alterations in drug potency due to cumulation or tolerance.

*Evaluation of behavioural effects*

Behavioural alterations after administration of test drugs were determined at different times after injection of each compound up to 2 min before amygdala stimulation. For all observations, rigorous observational protocols described elsewhere were used (Löscher & Hönack, 1991b; 1992). Hyperlocomotion, head weaving (swaying movements of the head and upper torso from side to side for at least one complete cycle; i.e. left-right-left), stereotyped sniffing, biting, licking or grooming, reciprocal forepaw treading ('piano playing'), stereotyped rearing, hyperexcitability (as indicated by increased reactions to noise or handling), tremor, abduction of hind limbs, reduction of righting reflexes, flat body posture, circling, Straub tail and piloerection were scored using a ranked intensity scale where 0 = absent, 1 = equivocal, 2 = present and 3 = intense. Behavioural alterations other than those decsribed above were recorded separately. Ataxia was quantitated by a rating system as described recently (Löscher *et al.*, 1987). In short, animals were taken out of the cage, placed in an open field, observed for about 1 min and ataxia was rated as follows: (1) slight ataxia in hind-legs (tottering of the hind quarters); (2) more pronounced ataxia with dragging of hind legs; (3) further increase of ataxia and more pronounced dragging of hind legs; (4) marked ataxia, animals lose balance during forward locomotion; (5) very marked ataxia with frequent loss of balance during forward locomotion; (6) permanent loss of righting reflexes, but animal still attempts to move forward. In addition to rating of motor impairment in the open field, impaired motor function was quantitated by the rotarod test of Dunham & Miya (1957). The rotarod test was carried out with a foam rubber coated rod of 6 cm diameter which rotated at 8 r.p.m. Neurological deficit was indicated by inability of the animals to maintain their equilibrium for at least 1 min on the rotating rod. The kindled rats were trained prior to drug experiments to remain on the rod. After drug treatment, rats which were not able to maintain their equilibrium on the rod for 1 min were put again on the rod a further two times. Only animals which were not able to remain on the rod during 3 subsequent 1 min trials were considered to exhibit neurological deficit. For time course studies, the rotarod test was performed at different times after drug injection immediately after rating of adverse effects in the open field.

*Determination of D-cycloserine in plasma and brain*

In two groups of 6 rats each (non-kindled but age-matched with the kindled rats), D-cycloserine was injected at a dose of 320 mg $kg^{-1}$. One group of rats was killed after 15 min, the other after 60 min. A third group of rats received i.p. saline and served as control. Blood was sampled for preparation of plasma. The brains were rapidly removed and samples of the frontal cortex were weighed and homogenized by an Ultra-Turrax homogenizer in 2 ml of ice-cold 80% ethanol. Further processing of plasma and brain samples was as described recently (Rundfeldt & Löscher, 1992; Löscher *et al.*, 1993). D-Cycloserine was determined in plasma and brain by high performance liquid chromatography (h.p.l.c.) after precolumn derivatization by means of *o*-phthaldialdehyde/2-mercaptoethanol essentially as described recently for endogenous amino acids (Rundfeldt & Löscher, 1992; Löscher *et al.*, 1993).

*Statistics*

All data are given as means ± s.e.mean. In some experiments, single animals responded differently from the other animals of the group. In order to maintain normal distribution of data, such outliers were not involved in presentation of group means and deviation, but data from such animals are mentioned in the text. Significance of differences between seizure readings in the same group of rats was calculated by the Wilcoxon signed-rank test for paired replicates.

*Drugs*

D-Cycloserine and D-serine were purchased from Sigma (Munich, Germany). (+)-HA-966 (**R**-(+)-3-amino-1-hydroxypyrrolid-2-one) was generously supplied by Merz & Co. (Frankfurt/M, Germany) and MK-801 (dizocilpine maleate) by Merck Sharp & Dohme (Rahway, NJ, U.S.A.). All drugs were freshly dissolved in saline before each experiment and were injected i.p. in a volume of 2–3 ml $kg^{-1}$ or i.c.v. in a volume of 4 µl.

## Results

*Effect of MK-801, D-cycloserine, (+)-HA-966, and D-serine on the focal seizure threshold (ADT) in amygdala-kindled rats*

The uncompetitive NMDA receptor antagonist, MK-801, 0.1 mg $kg^{-1}$, i.p., did not alter the ADT of kindled rats (Figure 1) or seizure severity, duration of seizures and afterdischarges recorded at ADT currents (Table 1). The partial glycine agonist, D-cycloserine, did not significantly alter the ADT at doses of 20, 40 or 80 mg $kg^{-1}$ (Figure 1). At 80 mg $kg^{-1}$, only 1 of 9 kindled rats (not included in the

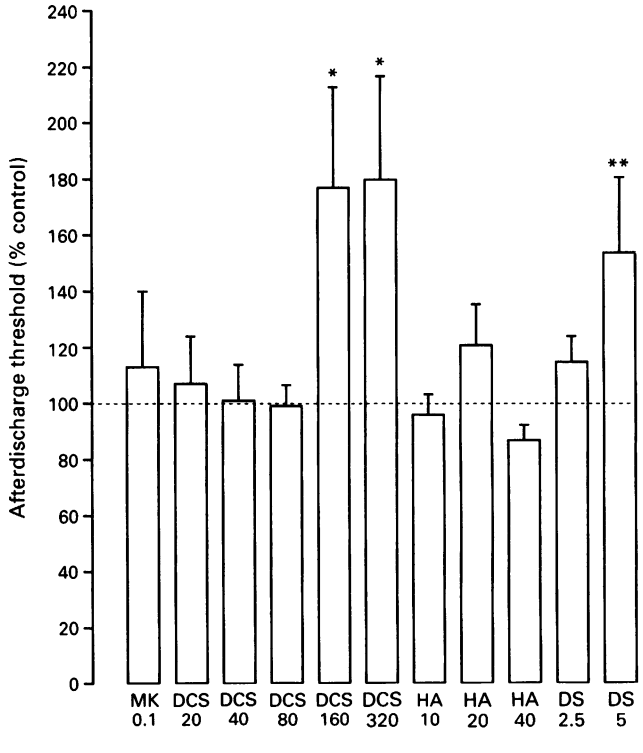


**Figure 1** Effect of MK-801 (MK), D-cycloserine (DCS), D-serine (DS) or (+)-HA-966 (HA) on focal seizure threshold (ADT) determined by electrical stimulation of the amygdala in amygdala-kindled rats. Data are from experiments with groups of 8–11 fully kindled rats (except 40 mg $kg^{-1}$ DCS, which is from a group of 6 animals). The doses of drugs are shown below each column in mg $kg^{-1}$, i.p. (for DS in µmol, i.c.v.). Control ADTs (with i.p. or i.c.v. injection of saline) were determined 2–3 days before each drug experiment; average pre-drug control ADT was 27.8 ± 1.6 µA (mean ± s.e.mean of 11 pre-drug control threshold determinations in groups of 6–11 rats). Drug data are shown as mean percentage (+s.e.mean) of the individual pre-drug control threshold. Significant differences to pre-drug control are indicated by asterisks (*$P<0.05$; **$P<0.01$). Pretreatment times were 2.5 h (MK), 1 h (DCS, DS) or 0.5 h (HA).

**Table 1** Effects of MK-801, D-cycloserine, (+)-HA-966, D-serine, and a combination of D-cycloserine and (+)-HA-966 on ictal and postictal recordings at ADT currents in kindled rats

| | | Seizure recordings at ADT | | | |
|---|---|---|---|---|---|
| *Drug* | *Dose* | *Seizure severity score* | *Seizure duration sec* | *Afterdischarge duration sec* | *Postictal immobilization sec* |
| Control | – | 4.8 ± 0.1 | 43.2 ± 7.2 | 78.0 ± 16.4 | 72.3 ± 13.8 |
| MK-801 | 0.1 mg kg$^{-1}$ i.p. | 4.7 ± 0.2 | 44.7 ± 6.2 | 62.2 ± 16.7 | 64.4 ± 14.3 |
| Control | – | 4.8 ± 0.1 | 65.0 ± 6.8 | 121.7 ± 13.1 | 113.9 ± 23.2 |
| D-Cycloserine | 20 mg kg$^{-1}$ i.p. | 4.7 ± 0.2 | 54.7 ± 5.9 | 95.9 ± 19.7 | 58.9 ± 14.2* |
| Control | – | 4.2 ± 0.7 | 58.5 ± 10.9 | 89.2 ± 20.5 | 56.0 ± 13.2 |
| D-Cycloserine | 40 mg kg$^{-1}$ i.p. | 4.5 ± 0.2 | 51.5 ± 6.4 | 90.2 ± 17.9 | 66.3 ± 18.2 |
| Control | – | 4.9 ± 0.1 | 46.7 ± 6.6 | 77.9 ± 11.3 | 166.7 ± 32.5 |
| D-Cycloserine | 80 mg kg$^{-1}$ i.p. | 4.4 ± 0.4 | 46.7 ± 6.1 | 85.5 ± 12.4 | 186.0 ± 39.9 |
| Control | – | 4.9 ± 0.1 | 52.6 ± 5.4 | 84.2 ± 11.7 | 142.2 ± 26.1 |
| D-Cycloserine | 160 mg kg$^{-1}$ i.p. | 4.2 ± 0.4 | 50.4 ± 5.8 | 92.3 ± 19.5 | 174.9 ± 51.4 |
| Control | – | 4.9 ± 0.1 | 55.8 ± 4.2 | 86.2 ± 10.0 | 172.6 ± 27.9 |
| D-Cycloserine | 320 mg kg$^{-1}$ i.p. | 4.3 ± 0.3 | 48.1 ± 5.7 | 106.3 ± 28.1 | 196.8 ± 34.8 |
| Control | – | 4.6 ± 0.3 | 50.9 ± 4.7 | 81.2 ± 10.3 | 218.2 ± 39.2 |
| (+)-HA-966 | 10 mg kg$^{-1}$ i.p. | 5 | 46.9 ± 4.9 | 67.9 ± 9.8 | 511.3 ± 235.4 |
| Control | – | 4.9 ± 0.1 | 45.3 ± 4.6 | 79.0 ± 12.8 | 143.2 ± 26.9 |
| (+)-HA-966 | 20 mg kg$^{-1}$ i.p. | 4.0 ± 0.6 | 41.1 ± 5.6 | 66.9 ± 11.8 | 1839.3 ± 878.2* |
| Control | – | 5 | 49.9 ± 3.2 | 81.6 ± 6.2 | 206.6 ± 37.6 |
| (+)-HA-966 | 40 mg kg$^{-1}$ i.p. | 4.3 ± 0.4 | 38.4 ± 4.4 | 59.9 ± 7.9* | 7411.9 ± 1661.4** |
| Control | – | 5 | 56.8 ± 4.3 | 80.8 ± 10.0 | 181.9 ± 12.8 |
| D-Serine | 2.5 μmol i.c.v. | 5 | 55.8 ± 3.7 | 82.9 ± 5.5 | 219.1 ± 11.6 |
| Control | – | 5 | 53.7 ± 3.2 | 82.5 ± 4.5 | 190.7 ± 11.5 |
| D-Serine | 5.0 μmol i.c.v. | 4.9 ± 0.09 | 55.4 ± 4.9 | 81.5 ± 4.5 | 201.4 ± 18.1 |
| Control | – | 4.5 ± 0.3 | 55.4 ± 3.5 | 114.7 ± 25.5 | 150.3 ± 26.5 |
| D-Cycloserine plus (+)-HA-966 | 160 mg kg$^{-1}$ i.p. 20 mg kg$^{-1}$ i.p. | 4.5 ± 0.3 | 57.5 ± 2.3 | 82.4 ± 7.6 | 285.3 ± 48.9** |

Data are from experiments with groups of 9–11 fully kindled rats (except 40 mg kg$^{-1}$ D-cycloserine, which is from a group of 6 animals). Control ADTs (with i.p. or i.c.v. injection of saline) were determined 2–3 days before each drug experiment; average pre-drug control ADT was 27.5 ± 1.1 μA (mean ± s.e.mean of 12 pre-drug control threshold determinations in groups of 6–11 rats). Pretreatment times were 2.5 h (MK-801), 1 h (D-cycloserine, D-serine) or 0.5 h ((+)-HA-966). In case of combined treatment with D-serine and (+)-HA-966, both drugs were given 60 min prior to ADT determination. At the individual pre-drug and drug ADT current, severity and duration of seizures as well as duration of amygdaloid afterdischarges were recorded. Furthermore, the duration of postictal immobilization ('freezing') was determined. All data are shown as means ± s.e.mean. Significant differences to pre-drug control are indicated by asterisks (*$P<0.05$; **$P<0.01$). ADT = afterdischarge threshold.

average data shown in Figure 1) exhibited a marked increase in ADT in response to D-cycloserine. When the dose of D-cycloserine was increased to 160 or 320 mg kg$^{-1}$, significant ADT increases were found 1 h after administration (Figure 1). Seizure severity, seizure duration and afterdischarge duration recorded at ADT currents were not changed by treatment with D-cycloserine (Table 1).

The low efficacy partial glycine agonist, (+)-HA-966, did not significantly alter the focal seizure threshold at doses of 10, 20 or 40 mg kg$^{-1}$ (Figure 1). At the two higher doses, only 1 of 9–10 animals per group (not included in the average data shown in Figure 1) displayed marked threshold increases in response to (+)-HA-966. The severity or duration of seizures recorded at ADT currents was not altered by (+)-HA-966 (Table 1).

With the full glycine agonist, D-serine, significant increases in ADT were recorded 1 h after i.c.v. injection of a dose of 5 μmol, but not 2.5 μmol (Figure 1). Seizure parameters recorded at ADT currents were not altered by treatment with D-serine (Table 1).

The significant increase in ADT seen after high doses of D-cycloserine was very long-lasting. Thus, it was still present 2 days after drug administration (Figure 2). In order to prove the reproducibility of this long lasting alteration in ADT, the experiment with 160 mg kg$^{-1}$ D-cycloserine was repeated in two other groups of kindled rats: in both groups, the ADT was significantly increased 2 days after drug administration (not illustrated). In contrast, no ADT increases were seen with MK-801, (+)-HA-966, D-serine or vehicle at post-drug (or post-vehicle) control recordings (not illustrated).

### *Effect of combined treatment with D-cycloserine and (+)-HA-966 on the focal seizure threshold (ADT) in amygdala-kindled rats*

In this experiment, (+)-HA-966, 20 mg kg$^{-1}$, and D-cycloserine, 160 mg kg$^{-1}$, were administered together, and the ADT was recorded 1 h after injection as well as 2 and 5 days later (Figure 3). In contrast to the findings with D-cycloserine alone (Figures 1 and 2), the ADT was significantly decreased after 1 h and not increased after 2 days (Figure 3). Thus, (+)-HA-966 completely antagonized the anticonvulsant effect in response to treatment with D-cycloserine.

### *Effect of (+)-HA-966 on postictal immobilization*

Following termination of a fully kindled (stage 4–5) seizure, amygdala-kindled rats show a typical period of behavioural depression ('freezing'), during which the animals do not move but infrequently show facial clonus or head nodding. This postictal immobilization is associated with postictal depression (i.e. flattened activity) in the electroencephalographic recording from the amygdala; the depressed electroencephalographic activity being interrupted by short periods

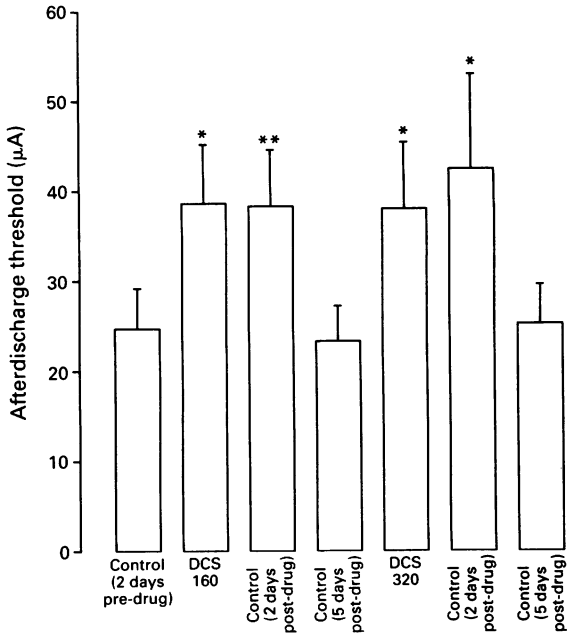


**Figure 2** Duration of the increase in focal seizure threshold (ADT) after administration of high doses of D-cycloserine (DCS) in amygdala-kindled rats. Data are mean ± s.e.mean of a group of 9 fully kindled rats with reproducible ADTs. DCS was administered i.p. at a dose of 160 mg kg$^{-1}$ 2 days after pre-drug ADT control recording. The ADT shown for 'DCS 160' was determined 1 h after drug administration. Two days after drug injection (2 days post-drug), the ADT was still significantly elevated. Five days after drug injection (5 days post-drug), ADTs were not different from pre-drug control. Two days later, DCS was administered at a dose of 320 mg kg$^{-1}$, i.p. and ADT was determined after 1 h ('DCS 320'). Similar to the experiment with 160 mg kg$^{-1}$, the increase in ADT was still present 2 days after drug injection (2 days post-drug), but reached control level after 5 days. Significant differences from pre-drug control are indicated by asterisks (*$P<0.05$; **$P<0.01$). The time of day of ADT determinations was the same for all experiments. At all control days, saline was injected 1 h prior to ADT determination. Saline injection alone did not induce any alterations in ADTs (not illustrated).

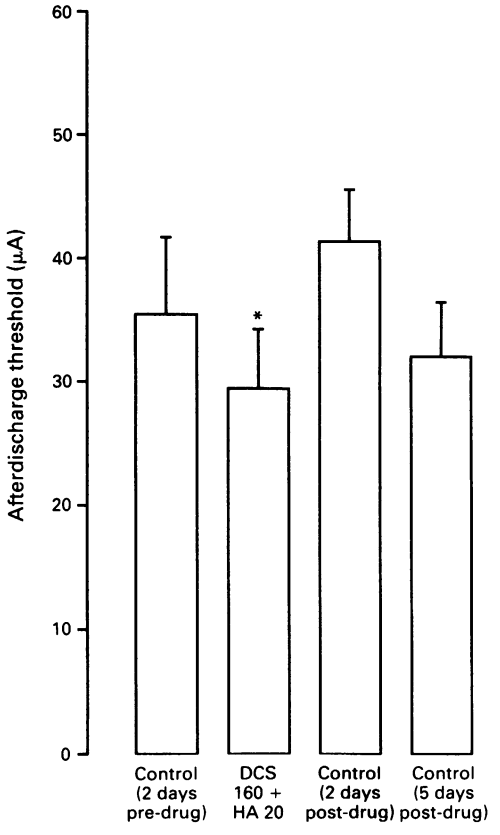


**Figure 3** Effects of combined treatment with D-cycloserine (DS; 160 mg kg$^{-1}$) and (+)-HA-966 (HA; 20 mg kg$^{-1}$) on focal seizure threshold (ADT) in kindled rats. Data are means+s.e.mean of a group of 10 fully kindled rats with reproducible ADTs. Both drugs were administered consecutively 2 days after pre-drug ADT control recording. The ADT shown for 'DCS 160 + HA 20' was determined 1 h after i.p. drug administration. Significant difference from pre-drug control is indicated by asterisk (*$P<0.05$). Two and 5 days after drug administration, the ADT was not significantly different from pre-drug control. The time of day of ADT determinations was the same for all experiments. At all control days, saline was injected 1 h prior to ADT determination.

of spiking only during the infrequent head nodding or facial movements. As shown in Table 1, the postictal behavioural depression normally lasts for about 1–4 min after termination of a kindled seizure and afterdischarge. Thereafter, animals resume normal behaviour and electroencephalographic activity. (+)-HA-966 significantly increased the duration of postictal immobilization in a dose-dependent manner (Table 1). At the highest dose, viz. 40 mg kg$^{-1}$, (+)-HA-966 increased the duration of freezing almost 40 fold, resulting in a total freezing time of 124 min! Concomitantly with the prolongation of behavioural depression, the flattened activity in the EEG (postictal depression) was prolonged (not illustrated). In contrast to (+)-HA-966, MK-801, D-cycloserine or D-serine did not increase the duration of postictal immobilization (Table 1) or postictal EEG depression. D-Cycloserine significantly decreased the period of postictal behavioural depression at 20 mg kg$^{-1}$ but not at any of the other doses tested (Table 1). After combined treatment with (+)-HA-966 and D-cycloserine, the effect of (+)-HA-966 on postictal immobilization appeared to be reduced (Table 1), but it has to be considered that pretreatment time of (+)-HA-966 was 60 min in this experiment instead of 30 min in the experiments with (+)-HA-966 alone.

### *Behavioural effects of MK-801, D-cycloserine, (+)-HA-966 and D-serine in kindled rats*

MK-801, 0.1 mg kg$^{-1}$, induced the characteristic PCP-like behavioural syndrome, consisting of hyperlocomotion, head weaving, ataxia, piloerection, and infrequent circling, abducted hind limbs, reciprocal forepaw treading, flat body posture and Straub tail. Most of these behavioural alterations reached their maximal intensity after 1.5–2.5 h. The most intense behaviours, i.e. motor impairment, hyperlocomotion and stereotypies (head weaving) are shown in Figure 4. Motor impairment was characterized by ataxia in the open field and inability of the rats to remain on the rotarod. None of these MK-801-induced behavioural alterations were seen after saline injection (not illustrated). D-Cycloserine was almost devoid of any behavioural effects (Figure 4). Some animals showed moderate piloerection and Straub tail after high doses but nothing more. (+)-HA-966 induced moderate motor impairment at the highest dose tested (Figure 4). Furthermore, piloerection and Straub tail were observed in some rats. Combined treatment with D-cycloserine and (+)-HA-966 did not induce more marked adverse effects than single drug treatment (Figure 4). Neither D-cycloserine nor (+)-HA-966, nor combined treatment with both drugs induced PCP-like behaviours, such as hyperlocomotion or stereotypies, at any dose tested. Furthermore, no signs of proconvulsant activity were seen with D-cycloserine at any time after treatment, including the 1-h interval prior to ADT determination. In this period, electroencephalographic recordings from the amygdala showed normal activity without any indication of paroxysmal activity (not illustrated). Similarly, no proconvulsant activity was seen after i.c.v. injection of D-serine. Indeed, all rats behaved normally after D-serine administration (Figure 4).

*Plasma and brain concentrations of D-cycloserine after i.p. injection in rats*

Following i.p. administration of D-cycloserine, 320 mg kg$^{-1}$, high plasma levels of 3427 nmol ml$^{-1}$ (350 μg ml$^{-1}$) were determined after 15 min (Table 2). Plasma levels rapidly declined to 2199 nmol ml$^{-1}$ after 60 min. From this decline, a plasma half-life of about 70 min can be estimated. In contrast to plasma, levels of D-cycloserine in brain tissue increased from 15 to 60 min after administration (Table 2). Consequently, the brain/plasma ratio was only 0.13 after 15 min but 0.36 after 60 min. Peak brain levels obtained after 60 min were 800 nmol g$^{-1}$ (80 μg g$^{-1}$), corresponding to about 23% of peak plasma levels.

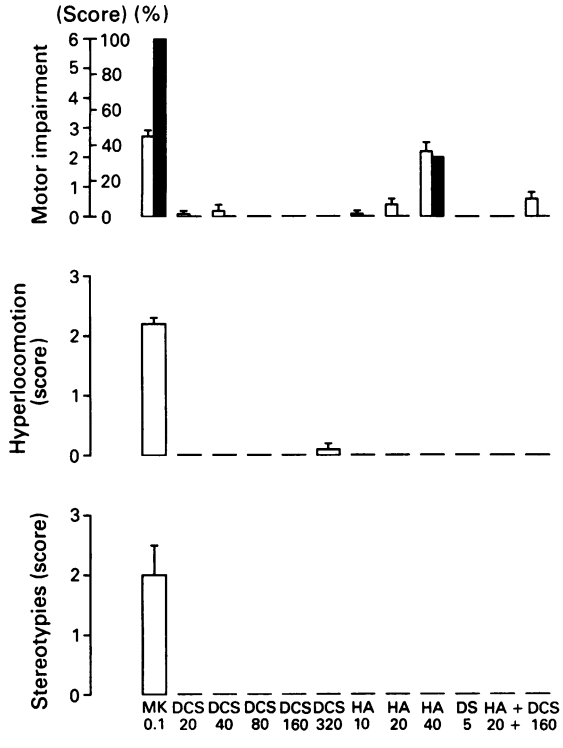


**Figure 4** Behavioural adverse effects of MK-801 (MK), D-cycloserine (DCS), D-serine (DS), (+)-HA-966 (HA), or combined treatment with HA and DCS in amygdala-kindled rats. The doses of drugs are shown below each column in mg kg$^{-1}$, i.p. (for DS in μmol, i.c.v.). Data are mean + s.e.mean of 9–11 fully kindled rats per experiment (except data for DCS 40, which are from 6 animals) and were determined immediately prior to ADT determinations. The severity of motor impairment was scored by behavioural ratings (left side of ordinate scale; open columns) and also determined in the rotarod test (right side of ordinate scale; solid columns). For the rotarod test, the percentage of animals which did not pass the test is given. Hyperlocomotion and stereotypies (head weaving) were scored by severity ratings. Other behavioural alterations not included in the figure, such as circling and flat body posture, were only infrequently observed with MK-801 and not at all with any of the other drugs. For pretreatment times of drugs and further details, see legend to Figure 1.

**Table 2** Levels of D-cycloserine in plasma and brain tissue after i.p. administration of D-cycloserine in rats

| *Time after administration* | *Concentration of D-cycloserine* *Plasma* (nmol ml$^{-1}$) | *Frontal cortex* (nmol g$^{-1}$) | *Brain/plasma ratio* |
|---|---|---|---|
| 15 min | 3427 ± 81.9 | 450 ± 118 | 0.13 |
| 60 min | 2199 ± 75.1 | 800 ± 122 | 0.36 |

Data (means ± s.e.mean) are from experiments with two groups of 6 rats each. D-Cycloserine was administered at a dose of 320 mg kg$^{-1}$, i.p.

## Discussion

D-Cycloserine was discovered in 1954 in a culture of *Streptomyces orchidaceus*. Found to be a broad-spectrum antibiotic with a moderate degree of effectiveness *in vitro* against the tubercle bacillus, its most important clinical application has been in the treatment of tuberculosis (Mandell & Sande, 1990). Although the drug was found to be relatively nontoxic in experimental animals (Tettenborn, 1988), in clinical use the administration of high doses of D-cycloserine (1 g per day) to tuberculosis patients has been attended by symptoms of neurotoxicity, such as drowsiness, somnolence, hyperflexia, mental confusion, psychotic states and tonic-clonic or absence seizures (Storey & McLean, 1957; Mandell & Sande, 1990). Anticonvulsant effects of D-cycloserine were first reported by Fust *et al.* (1958) and Mayer *et al.* (1971) who found that the drug had a moderate anticonvulsant effect in fatal pentylenetetrazole (PTZ) shock in mice. More recently, Peterson & Schwade (1993) showed that D-cycloserine (50–300 mg kg$^{-1}$, i.p.) dose-dependently blocked tonic but not clonic PTZ seizures in rats with an $ED_{50}$ of 109 mg kg$^{-1}$, i.p. The anticonvulsant effect was stereospecific in that L-cycloserine was ineffective. Similar results were reported for the MES test in rats, in which D-cycloserine, administered either i.p. or i.c.v., induced significant anticonvulsant activity with a time of peak effect of 1–2 h and an $ED_{50}$ of 153 mg kg$^{-1}$, i.p. or 5 μmol, i.c.v. (Peterson, 1992).

Shortly after the discovery of the stimulatory action of glycine at the NMDA receptor, it was found that D-cycloserine binds to the glycine/NMDA modulatory site ($K_i$ 2.3 μmol l$^{-1}$ compared to 0.2 μmol l$^{-1}$ for glycine) and acts as a partial agonist of relatively high activity (cf., Carter, 1992; Kemp & Leeson, 1993). In *in vivo* experiments in mice and rats, D-cycloserine was found to possess cognition-enhancing properties, which were related to the partial agonistic effects of this drug at the glycine/NMDA site (Flood *et al.*, 1992; Schuster & Schmidt, 1992; Sirvio *et al.*, 1992). Maximum cognition-enhancing effects were seen at i.p. doses of 10–20 mg kg$^{-1}$. In the present experiments in kindled rats, no behavioural or anticonvulsant effects were observed after administration of D-cycloserine at doses of 20, 40 or 80 mg kg$^{-1}$. Similarly, Peterson (1992) did not obtain anticonvulsant effects in the MES test at doses below 100 mg kg$^{-1}$ D-cycloserine. Significant increases in focal seizure threshold in kindled rats were found when the dose of D-cycloserine was increased to 160 or 320 mg kg$^{-1}$, which is consistent with the dose-range reported by Peterson (1992) and Peterson & Schwade (1993) to exert anticonvulsant effects in the MES and PTZ tests in rats. In contrast to the present data, Peterson & Schwade (1993) found that D-cycloserine had no significant effect on kindled amygdala seizures in doses up to 400 mg kg$^{-1}$. However, Peterson & Schwade (1993) used amygdala stimulation with a fixed suprathreshold current of 400 μA, whereas we determined the individual focal seizure threshold before and after application of D-cycloserine, which may explain the differences between experimental results.

After systemic application, D-cycloserine is distributed throughout body fluids and tissues. There seems to be no appreciable blood-brain barrier to the drug, since cerebrospinal fluid concentrations in tuberculosis patients are about 50% of those in plasma (Iwainsky, 1988). However, the present data on D-cycloserine levels in plasma and brain demonstrate that in rats, only about 20% of peak plasma concentrations appear in the brain, which may explain the relatively high doses of D-cycloserine necessary to induce anticonvulsant effects. A similar low brain penetration rate has previously been determined for D-cycloserine in rats by Crema & Berté (1960). Consistent with the present data, peak brain levels were determined after 60 min, and brain levels rapidly declined thereafter with a half-life of about 2.2 h (Crema & Berté, 1960). In view of the limited penetration of D-cycloserine from blood to brain in intact animals, the

higher neurotoxicity of D-cycloserine in tuberculosis patients compared to laboratory animals (Tettenborn, 1988) may thus be a consequence of disturbed blood-brain-barrier function.

As shown by the present data, the increase in focal seizure threshold in kindled rats following D-cycloserine lasted for several days. In view of the rapid elimination of D-cycloserine in rats, this long-lasting increase in seizure threshold is difficult to explain by a direct anticonvulsant effect of the drug mediated by the glycine site. However, the fact that both the acute and the long-lasting increase of focal seizure in response to D-cycloserine were antagonized by treatment with the selective glycine receptor ligand, (+)-HA-966, strongly indicate that these effects of D-cycloserine were due to interactions with the glycine receptor.

One explanation for the long-lasting increase in seizure threshold following administration of D-cycloserine would be adaptive changes such as desensitization of glycine/NMDA receptors in response to the high efficacy partial agonist. Recent experiments with the glycine partial agonist ACPC (1-aminocyclopropane-1-carboxylic acid), which has similar intrinsic activity (60–80%) as D-cycloserine (Rao *et al.*, 1990) have suggested that the NMDA receptor complex can be desensitized by treatment with a high efficacy partial glycine agonist, resulting in significant protection against cerebral ischaemia and NMDA-induced convulsions and death (von Lubitz *et al.*, 1992; Skolnick *et al.*, 1992). Such adaptive changes in the NMDA receptor complex would also explain the anticonvulsant effect of D-cycloserine found by Peterson (1990) and the anticonvulsant effects of other glycine agonists or high efficacy partial agonists reported by other groups (see Introduction). However, the present finding that no prolonged increase in seizure threshold is induced by the glycine/NMDA receptor full agonist D-serine (see below) would argue against receptor desensitization as the responsible mechanism for the long duration of D-cycloserine's anticonvulsant effect.

Alternative explanations for the 'paradoxical' anticonvulsant effects of the glycine high efficacy partial agonist, D-cycloserine, include the possibility that it might act by potentiating the activity of an endogenous glutamate antagonist, such as kynurenic acid (Kemp & Leeson, 1993), since recent experiments by Norris *et al.* (1992) have shown that D-cycloserine, 320 mg $kg^{-1}$, significantly potentiated the ability of MK-801 to raise seizure threshold. Furthermore, it has been suggested that NMDA receptor-mediated release of neurotransmitters such as noradrenaline and GABA may be involved in the anticonvulsant effects of agonists or high efficacy partial agonists at the strychnine-insensitive glycine site (Peterson, 1991).

Interestingly, as with D-cycloserine, significant increases in focal seizure threshold were also produced by the glycine receptor full agonist D-serine, but the duration of this effect was much shorter than with D-cycloserine. D-Serine is a selective agonist of the strychnine-insensitive glycine receptor with a similar affinity for the receptor to glycine (Kemp & Leeson, 1993). The effects of D-cycloserine and D-serine in kindled rats substantiate the theory that the strychnine-insensitive glycine receptors are not saturated at physiological concentrations of glycine and that exogenously administered glycine agonists can influence the glycine receptor activity *in vivo* (Singh *et al.*, 1990b; Peterson, 1991; Carter, 1992; Kemp & Leeson, 1993). To our knowledge, the present data are the first demonstration of a direct anticonvulsant effect of D-serine. In contrast to D-cycloserine, the blood-brain barrier is almost impermeable to D-serine so that the compound has to be administered centrally. However, oral administration of very large doses of D-serine (2100 mg $kg^{-1}$) in rats was shown to enhance the anticonvulsant effect of phenobarbitone, carbamazepine and phenytoin (Peterson, 1991).

If stimulation of glycine/NMDA receptors is essential for the anticonvulsant effect observed after administration of the high efficacy partial glycine agonist D-cycloserine and the full agonist D-serine, then a low efficacy partial agonist should be devoid of such anticonvulsant activity. This was proven with (+)-HA-966, a highly selective ligand for the strychnine-insensitive glycine site (Singh *et al.*, 1990a). Tested in doses of 10–40 mg $kg^{-1}$, (+)-HA-966 did not exert any significant effects on focal seizure threshold or severity and duration of seizures recorded at seizure threshold. Observation of behaviour showed that (+)-HA-966 induced motor impairment in this dose-range, indicating that pharmacologically active doses had been tested. Indeed, (+)-HA-966 was recently reported to exert effects via NMDA receptors at doses of 3–30 mg $kg^{-1}$ in rats (Hutson *et al.*, 1991; Dunn *et al.*, 1992; Bristow *et al.*, 1993). In the only previous study in which (+)-HA-966 was examined in amygdala-kindled rats, the compound was injected into the amygdala (Croucher & Bradford, 1991). Moderate (20–30%) increases in GST were found 20 min after intra-amygdaloid injections of 50–100 nmol (+)-HA-966, while seizure duration or afterdischarge duration were not altered (Croucher & Bradford, 1991). Although we did not determine the GST in our experiments, most animals showed generalized motor seizures at ADT currents, which were not affected by systemic treatment with (+)-HA-966. Thus, the present experiments demonstrated that, in contrast to D-cycloserine and D-serine, (+)-HA-966 was not capable of exerting anticonvulsant effects in the kindling model. Similar findings were reported for i.c.v. administration of the glycine antagonist, 7-chlorokynurenic acid in amygdala-kindled rats (Morimoto & Sato, 1992), thus suggesting that glycine antagonists or low efficacy partial agonists are ineffective in this model of partial epilepsy. Instead, as shown by the present data, (+)-HA-966 antagonized the anticonvulsant effect induced by D-cycloserine.

Previous studies with systemic administration of (+)-HA-966 in other seizure models have shown that very high doses are needed to block NMDA-induced seizures ($ED_{50}$ 900 mg $kg^{-1}$) or tonic seizures in the MES test ($ED_{50}$ 106 mg $kg^{-1}$) in mice (Singh *et al.*, 1990a; Vartanian & Taylor, 1991). We did not try such high doses in kindled rats, since in the dose range of 10–40 mg $kg^{-1}$, (+)-HA-966 already caused marked increases in the duration of postictal immobilization. At the highest dose tested in the present study (40 mg $kg^{-1}$), rats were immobilized for more than 2 h following the kindled seizure. The behavioural and EEG depression following kindled and other types of seizures is thought to represent decreased or inhibited neuronal firing following seizure activity (Goddard *et al.*, 1986). Several neurotransmitter systems, including GABA, adenosine and opioids, have been involved in the mechanisms underlying these postictal alterations (Dragunow, 1986; Goddard *et al.*, 1986; Löscher, 1989). For instance, similar prolongation of postictal depression as obtained in the present study with (+)-HA-966 was previously found with systemic administrations of morphine and adenosine analogues in kindled rats (Frenk *et al.*, 1979; Whitcomb *et al.*, 1990). It has been proposed that the mechanisms involved in postictal behavioural and EEG depression are responsible for spontaneous seizure arrest and may forestall the onset of the next seizure or reduce the severity of subsequent seizures (Dragunow, 1986; Goddard *et al.*, 1986; Löscher, 1989). In addition to behavioural and electroencephalographic depression, the postictal phase of kindled rats is characterized by intermittent postictal spiking, which has been related to a paroxysmal activation of inhibitory systems (Frenk *et al.*, 1979; Engel *et al.*, 1981). However, as reported previously (e.g. Engel *et al.*, 1981; Löscher & Hönack, 1990a), this postictal spiking is often associated with signs of limbic seizure activity (facial clonus, head nodding) which could indicate an overlap of both ictal and postictal phenomena during the phase of behavioural depression. The finding that the low efficacy partial glycine agonist, (+)-HA-966, markedly enhanced the duration of postictal behavioural and EEG depression in kindled rats may indicate that the glycine modulatory site is involved in these postictal events and that (+)-HA-966 possibly acts as a

glycine antagonist by potentiating and/or prolonging a postictal decrease of glutamatergic activity.

The lack of anticonvulsant activity of the uncompetitive NMDA receptor antagonist, MK-801, in fully kindled rats confirmed previous reports on this drug (McNamara *et al.*, 1988; Löscher & Hönack, 1991a). MK-801 was primarily included in the present experiments for direct comparison of PCP-like behavioural effects with adverse effects in response to the glycine site ligands. Neither D-cycloserine nor (+)-HA-966 produced PCP-like behavioural alterations in kindled rats, confirming previous observations with these drugs in non-kindled rodents (Hutson *et al.*, 1991; Peterson, 1992; Bristow *et al.*, 1993). Only in one study with i.c.v. injection in mice, 5,7-dichlorokynurenic acid, (+)-HA-966 and D-cycloserine were reported to induce head weaving, but the peak intensity of the response was never more than 12% of that seen with MK-801 (Tricklebank & Saywell, 1990). We have recently shown that amygdala-kindled rats are more susceptible to PCP-like adverse effects, including head weaving, of NMDA antagonists than non-kindled rats, most probably because of the kindling-induced changes in glutamate receptor function (Löscher & Hönack, 1991b,c). The present data seem to indicate that this higher susceptibility of kindled rats to behavioural adverse effects of NMDA receptor antagonists does not extend to glycine site ligands, although motor impairing effects of (+)-HA-966 were observed at lower doses than reported previously for non-kindled rats (Bristow *et al.*, 1993).

In conclusion, the present findings indicate that the strychnine-insensitive glycine receptor can be manipulated by high efficacy partial agonists such as D-cycloserine to effect changes in the NMDA receptor complex leading to long-lasting decreases in seizure susceptibility. As recently suggested from experiments with the partial glycine agonist ACPC (von Lubitz *et al.*, 1992; Skolnick *et al.*, 1992), this strategy might be interesting for treatment of disorders associated with hyperactivation of NMDA receptor function, such as epilepsy and ischaemia. However, in view of the fact that high doses of D-cycloserine induce seizures in man (Storey & McLean, 1957), this strategy should be explored with caution, although glycine site antagonists, such as 7-chlorokynurenic acid and NMDA receptor antagonists are known to induce (pro)convulsant effects at high doses as well (Myslobodsky *et al.*, 1981; Klockgether *et al.*, 1988; Koek & Colpaert, 1990; Löscher & Hönack, 1990b). The anticonvulsant activity of D-cycloserine and D-serine and the unexpected findings with the low efficacy partial glycine agonist (+)-HA-966 in kindled rats substantiate that we are far from understanding the complex interactions between NMDA receptor functions and ictal and postictal events. In view of the fact that amygdala-kindling is thought to represent a predictive model of complex partial seizures, i.e. the most common type of epileptic seizures in man (Löscher, 1993), the lack of anticonvulsant activity of (+)-HA-966 combined with the pronounced increase in postictal depression found in the present study might suggest that glycine antagonists or low efficacy partial agonists such as (+)-HA-966 are of limited value for clinical development as antiepileptic drugs. However, in view of the fact that the tools currently in use are far from perfect, with affinities for the glycine/NMDA receptor often well in excess of 1 μM, final judgment about the usefulness of glycine site antagonists should await development of compounds with high affinity and selectivity.

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# Presence of $P_2$-purinoceptors in the rat pineal gland

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**1** The effects of noradrenaline, ATP, adenylyl-imidodiphosphate (AMP-PNP), adenosine, α,β-methylene-ATP and the $P_2$-purinoceptor antagonist, suramin on N'-acetyl-5-hydroxytryptamine production were studied in cultured denervated rat pineal glands.

**2** Noradrenaline (3 nM–1 μM) increased N'-acetyl-5-hydroxytryptamine production as measured both in the gland and the culture medium.

**3** In noradrenaline (10 nM)-stimulated pineal glands, ATP (0.03 nM–1 mM) or AMP-PNP (0.1 μM–1 mM) increased N'-acetyl-5-hydroxytryptamine production in a concentration-dependent manner.

**4** α,β-Methylene-ATP at the concentration of 0.1 mM, but not 3 μM, attenuated the enhancement by ATP (0.1 mM) of noradrenaline (10 nM)-induced N'-acetyl-5-hydroxytryptamine production.

**5** Suramin (0.1 mM) blocked the potentiating effect of ATP (0.1 mM), but not the potentiating effect of adenosine (0.1 mM) in glands incubated with noradrenaline (10 nM).

**6** These findings suggest that the rat pineal gland possesses $P_2$-purinoceptors which when stimulated potentiate the effect of noradrenaline but do not, by themselves, induce an increase in N'-acetyl-5-hydroxytryptamine production.

**Keywords:** $P_2$-purinoceptor; pineal gland; N'-acetyl-5-hydroxytryptamine production

## Introduction

Noradrenaline released from sympathetic nerve terminals in the pineal gland triggers the nocturnal peak of melatonin due to activation of pineal arylalkylamine-N-acetyltransferase (NAT, EC 2:3.1.87), the rate-limiting enzyme that converts 5-hydroxytryptamine (5-HT) to the immediate precursor of melatonin, N'-acetyl-5-hydroxytryptamine (N'-acetyl-5-HT) (e.g. Sugden, 1989). This activation is due to noradrenaline acting through $\beta_1$- and $\alpha_1$-adrenoceptors. β-Adrenoceptor activation is an absolute requirement, while $\alpha_1$-adrenoceptor activation potentiates the β-adrenoceptor stimulation (Klein *et al.*, 1983).

ATP is thought to be released along with noradrenaline during neurotransmission and has been suggested as a co-transmitter in postganglionic sympathetic neurones (Burnstock, 1976). Released ATP may be metabolized by ectoenzymes to adenosine (Nikodijevic & Klein, 1989) or may act directly on postsynaptic receptors (Burnstock, 1976). Receptors that mediate effects of adenosine and adenine nucleotides are classified into $P_1$- and $P_2$-purinoceptors based mainly upon the order of potency of various agonists (Burnstock, 1978). Biochemical, pharmacological and receptor-binding studies have led to a subdivision of the adenosine receptors ($P_1$-purinoceptors) into $A_1$ and $A_2$ receptors (van Calker *et al.*, 1979), and of $P_2$-purinoceptors into $P_{2x}$ and $P_{2y}$ purinoceptors (Burnstock & Kennedy, 1985).

Stimulation of adenosine $A_2$ receptors by adenosine or its analogues (Sarda *et al.*, 1989; Gharib *et al.*, 1992) elevates the pineal content of adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Nikodijevic & Klein, 1989; Nonaka *et al.*, 1991). Noradrenaline, acting through β-adrenoceptors, activates NAT by means of an increase in cyclic AMP (Klein *et al.*, 1981). The effect of adenosine on the NAT pathway, however, is controversial. Some authors describe an increase in N'-acetyl-5-HT or in melatonin production in pineal glands in culture (Vacas *et al.*, 1989; Gharib *et al.*, 1992) or after *in vivo* administration (Gharib *et al.*, 1989), while others did not observe an increase in NAT activity or melatonin production by adenosine analogues in cultured glands (Nonaka *et al.*, 1991).

No attempt has been made so far to search for a direct action of ATP on $P_2$-purinoceptors in the pineal gland. The aim of the present paper was to investigate the effect of $P_2$-purinoceptor stimulation on N'-acetyl-5-HT production in cultured rat pineal glands.

## Methods

Male and female Wistar rats (180–200 g) were kept under light-dark cycle of 12:12 h with water and food *ad libitum*. The animals were killed by decapitation between 09 h 00 min–11 h 00 min and their pineal glands rapidly dissected and immediately placed in ice-cold chemically defined BGJb medium to which was added 2 mM glutamine, 0.1 mg $ml^{-1}$ ascorbic acid and 10 ng $ml^{-1}$ ampicilin.

The glands were incubated (37°C; 95% $O_2$:5% $CO_2$) in the same medium (1 gland per well, 200 μl per well) in a 24 multiwell plate for 48 h prior to treatment. Presynaptic elements degenerate during this period, resulting in a completely denervated preparation (Parfitt *et al.*, 1976). The medium was changed after 24 h. After 48 h, the glands were placed in fresh medium for 1 h and then incubated with agonists during 5 h. The agonists ATP, AMP-PNP or adenosine were added simultaneously with noradrenaline. α,β-Methylene-ATP and suramin, when present, were added 10 min and 15 min, respectively, prior to other treatments. At the end of the incubation period, glands and medium were placed in different microtubes and stored at −70°C.

The N'-acetyl-5-HT content in the gland and incubation medium was determined by high performance liquid chromatography (h.p.l.c.) with electrochemical detection based on the method described by Mefford & Barchas (1980). The indoleamine was separated on a Resolve $C_{18}$ reversed-phase column (5 μm, 150 × 3,9 mm i.d. from Waters, Milford, Mass., U.S.A.). The chromatographic system (Shimadzu, Kyoto, Japan) was isocratically operated with the following mobile phase: 0.1 M sodium acetate, 0.1 M citric

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acid, 0.15 mM EDTA, 8% methanol, pH 3.7 at a flow rate of 0.5 ml $min^{-1}$. The detector potential was adjusted to + 0.90 V (vs. Ag/AgCl reference electrode). Each gland was homogenized (5 s) in ice cold 0.1 M perchloric acid (120 µl), containing 0.02% EDTA and 0.02% sodium bisulphite. Protein and cell debris were removed by centrifugation (13,000 *g*, 5 min, 4°C) and 20 µl of the clear supernatant or the incubation medium was injected into the chromatographic system.

Adenylyl-imidodiphosphate (AMP-PNP) was purchased from Boehringer, Mannheim, Germany; ascorbic acid from Hoechst, Brazil; citric acid, EDTA, sodium acetate, sodium bisulphite, methanol and perchloric acid from Merck, Brazil; adenosine, ATP, N-acetylserotonin, noradrenaline from Sigma, St Louis, U.S.A.; α,β-methylene-ATP from RBI, Natick, U.S.A.; BGJb medium from Gibco-BRL, Gaithersburg, U.S.A.: suramin from Bayer, Leverkusen, Germany.

Results are expressed as ng per pineal or ng per well. All data are presented as mean ± s.e.mean. Statistical comparisons were made by Student's *t* test.

## Results

### *Effect of noradrenaline and noradrenaline in the presence of ATP*

Noradrenaline (3 nM–1 µM) increased N'-acetyl-5-HT production in a concentration-dependent manner as measured in the tissue as well as in the incubation medium (Figure 1). Addition of ATP (1 mM) significantly increased the N'-acetyl-5-HT content in glands incubated with concentrations of noradrenaline lower than 1 µM (Figure 1a). In the medium, after this same treatment, the amount of N'-acetyl-5-HT accumulated after 5 h of incubation was augmented at all concentrations of noradrenaline used (Figure 1b). ATP by itself did not change pineal and medium N'-acetyl-5-HT levels (controls: $<0.3$ ng per pineal and $1.89 \pm 0.53$ ng per well, $n = 12$; ATP: $<0.3$ ng per pineal and $1.94 \pm 0.48$ ng per well, $n = 8$).

### *Studies with $P_2$ agonists*

In pineals stimulated with noradrenaline (10 nM), ATP (0.03 mM–1 mM) increased N'-acetyl-5-HT production in a concentration-dependent manner (Figure 2).

AMP-PNP bears a close structural resemblance to ATP, yet is resistant to hydrolysis by most ATPases (Yount *et al.*, 1971). It mimics ATP effects in snail neurones (Yatani *et al.*, 1982), rat dorsal horn neurones (Jahr & Jessell, 1983) and rabbit basilar artery (von Kügelgen & Starke, 1990). In pineals stimulated with noradrenaline (10 nM), AMP-PNP (0.1 µM–1 mM) increased tissue and incubation medium N'-acetyl-5-HT content in a concentration-dependent manner, similar to ATP. Like ATP, AMP-PNP by itself had no effect on N'-acetyl-5-HT levels ($<0.3$ ng per pineal and $1.24 \pm 0.37$ ng per well, $n = 8$).

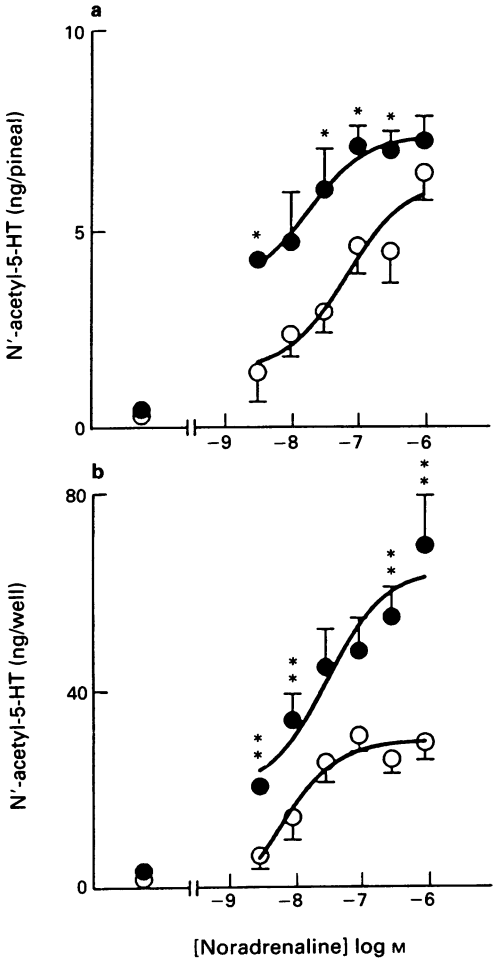


**Figure 1** Effect of noradrenaline (3 nM–1 µM, ○, $n = 4$–12) and noradrenaline (3 nM–1 µM) plus ATP (1 mM, ●, $n = 4$–10) on N'-acetyl-5-hydroxytryptamine (N'-acetyl-5-HT) production as measured in tissue (a) and incubation medium (b). Glands were incubated with drugs for 5 h. The points on the left represent control (absence of drugs) and ATP alone. Values are mean with s.e.mean. $*P<0.05$; $**P<0.01$ (● versus ○).

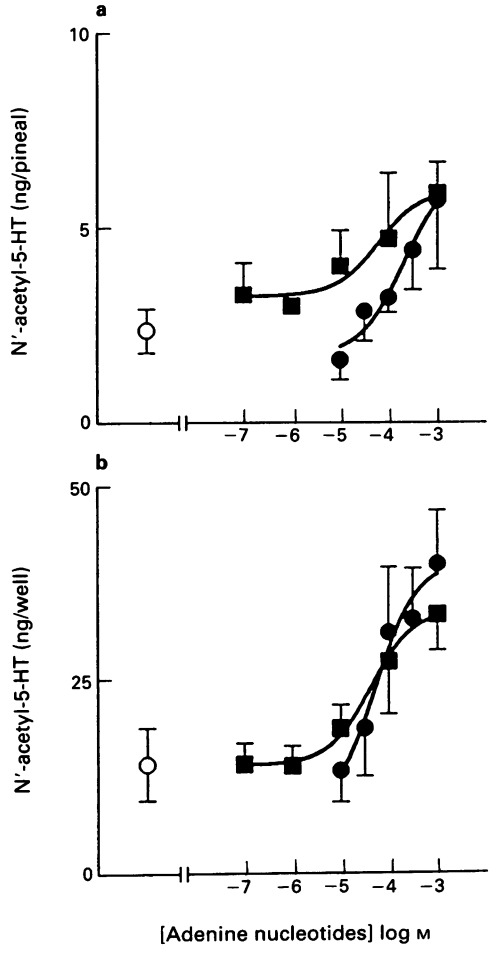


**Figure 2** Effect of ATP (0.03 mM–1 mM, ●, $n = 5$–7) and AMP-PNP (0.1 µM–1 mM, ■, $n = 4$–7) on N'-acetyl-5-hydroxytryptamine (N'-acetyl-5-HT) production induced by noradrenaline (10 nM) as measured in tissue (a) and incubation medium (b). Glands were incubated with drugs for 5 h. The point on the left represents noradrenaline (10 nM, ○, $n = 12$) alone. Values are mean with s.e.mean.

α,β-Methylene-ATP at a concentration of 3 μM was ineffective either in potentiating the increase by noradrenaline of N'-acetyl-5-HT production or in inhibiting the potentiating effect of ATP. However, at a concentration of 0.1 mM, α,β-methylene-ATP attenuated the potentiating effect of ATP (Figure 3).

### *Studies with suramin*

Suramin possesses antagonistic properties at $P_2$-purinoceptors (Dunn & Blakely, 1988). Suramin decreased the potentiating effect of ATP on N'-acetyl-5-HT production induced by noradrenaline (Figure 4). In contrast, the potentiating effect of adenosine (0.1 mM) was not blocked by suramin (Figure 4).

## Discussion

Incubation of cultured denervated pineal glands for 5 h with noradrenaline led to an increase in N'-acetyl-5-HT production dependent on the concentration of the agonist. The concentration-range of noradrenaline was similar to that shown to increase the activity of the enzyme NAT, responsible for N'-acetyl-5-HT synthesis, in pineal glands cultured for 24 h (Klein & Weller, 1973).

ATP potentiated, in a concentration-dependent manner, the production of N'-acetyl-5-HT induced by noradrenaline. The experiments with purinoceptor agonists and suramin indicate that this potentiation is mediated by $P_2$ purinoceptors. The effect of ATP was mimicked by the less-hydrolysable analogue AMP-PNP and blocked by the competitive antagonist, suramin. α,β-Methylene-ATP, which selectively desensitizes $P_2$-purinoceptors in several tissues, also blocked the potentiating effect of ATP. On the other hand, the effect of adenosine, which is mediated by $A_2$-purinoceptors (Sarda *et al.*, 1989; Nikodijevic & Klein, 1989), was not blocked by suramin. Thus, the denervated pineal gland possesses $P_2$-purinoceptors which when stimulated potentiate the effect of noradrenaline but do not, by themselves, induce an increase in N'-acetyl-5-HT production.

Extracellular ATP serves as messenger in many tissues (Burnstock, 1990). Specific receptors for ATP mediate a variety of effects, some involving second messenger pathways (endothelial cells, Pirotton *et al.*, 1987; hepatocytes, Charest *et al.*, 1985; adrenal chromaffin cells, Sasakawa *et al.*, 1989) and others involving the entry of extracellular calcium via direct activation of ion channels (arterial smooth muscle, Benham & Tsien, 1987; chick skeletal muscle, Thomas & Hume, 1990; urinary bladder smooth muscle, Schneider *et al.*, 1991), or opening of calcium voltage-dependent channels (rat vas deferens, French & Scott, 1981). In all the cases mentioned there is an increase of intracellular calcium.

In the pineal gland, agents that elevate cytosolic calcium, including potassium, ouabain, ionomycin, ionophore A23187 or $\alpha_1$-adrenoceptor agonists (Sugden *et al.*, 1986; 1987), are

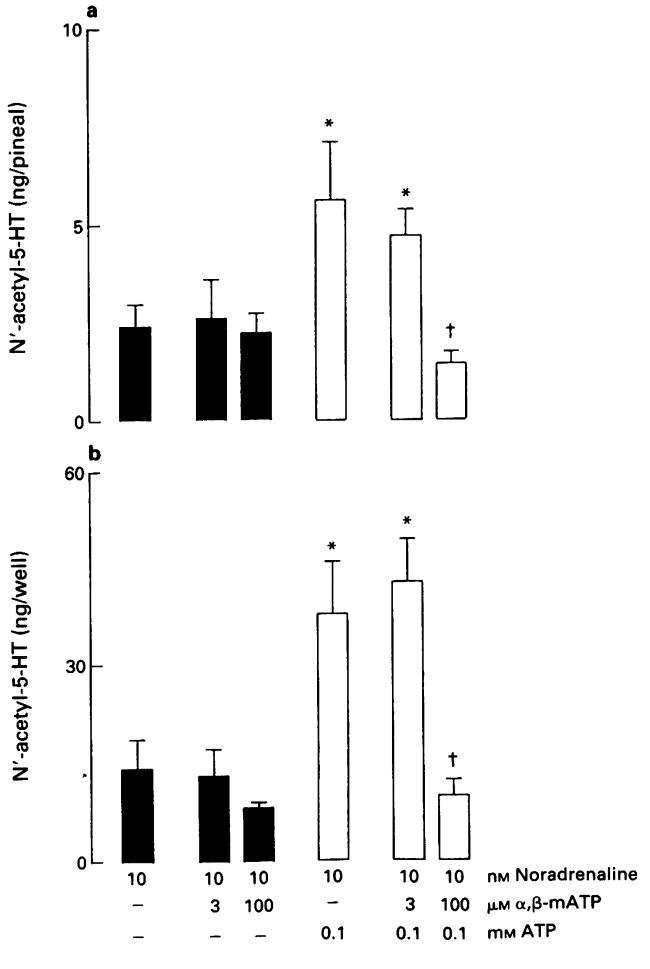


**Figure 3** Effect of α,β-methylene-ATP (α,β m-ATP, 3 μM and 100 μM) on N'-acetyl-5-hydroxytryptamine (N'-acetyl-5-HT) production induced by noradrenaline (10 nM) and noradrenaline (10 nM) plus ATP (0.1 mM) as measured in tissue (a) and incubation medium (b). α,β-mATP was added 10 min prior to other treatments. Values are the mean with s.e.mean; $n = 5-12$. $^*P<0.05$ compared to noradrenaline; $\dagger P<0.05$ compared to noradrenaline plus ATP.

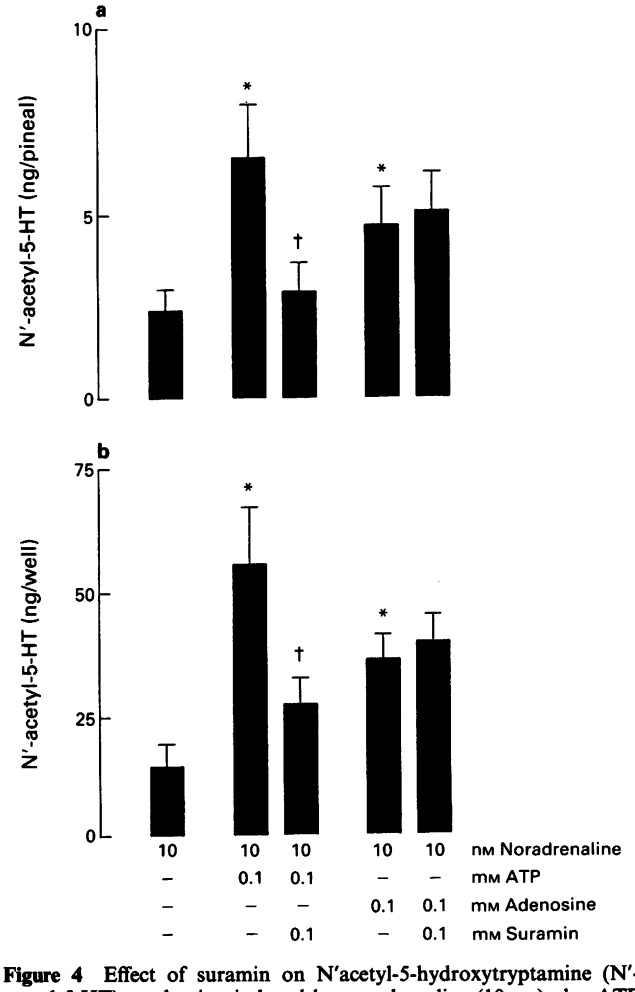


**Figure 4** Effect of suramin on N'acetyl-5-hydroxytryptamine (N'-acetyl-5-HT) production induced by noradrenaline (10 nM) plus ATP (0.1 mM) or plus adenosine (0.1 mM) as measured in tissue (a) and incubation medium (b). Suramin (0.1 mM) was added 15 min prior to other treatments. Values are the mean with s.e.mean; $n = 6-12$. $^*P<0.05$ compared to noradrenaline; $\dagger P<0.05$ compared to noradrenaline plus ATP.

ineffective by themselves, but potentiate the β-adrenoceptor-mediated cyclic AMP increase and stimulation of NAT (Klein *et al.*, 1981). The potentiating effect of $\alpha_1$-adrenoceptor agonists depends on the activation of protein kinase C and an increase in intracellular calcium (Sugden *et al.*, 1986; 1987). If stimulation of $P_2$-purinoceptors increases the cytosolic calcium concentration in the pineal gland, as it does in other tissues, then ATP or analogues should not increase N'-acetyl-5-HT production by themselves, but should, potentiate noradrenaline-induced N'-acetyl-5-HT production as observed. It must be pointed out that noradrenaline, by stimulating $\alpha_1$-adrenoceptors, increases intracellular calcium concentration; thus, if ATP is acting via calcium it should further increase the intracellular calcium concentration.

In summary, ATP and the less-hydrolysable analogue AMP-PNP potentiate noradrenaline-induced N'-acetyl-5-HT production in the pineal gland but have no effect alone. ATP and AMP-PNP act by stimulating $P_2$-purinoceptors.

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# Drug modulation of antigen-induced paw oedema in guinea-pigs: effects of lipopolysaccharide, tumour necrosis factor and leucocyte depletion

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**1** In guinea-pigs previously sensitized with ovalbumin, the intra-plantar administration of the antigen induced dose-dependent and sustained oedema. An intense infiltrate of neutrophils and eosinophils was observed at the peak of the oedema (4 h).

**2** Oedema induced by ovalbumin at the doses of 50 or 200 μg/paw was not inhibited by antihistamines (meclizine and cetirizine), a PAF antagonist (BN 50730), a cyclo-oxygenase inhibitor (indomethacin), a lipoxygenase inhibitor (MK-886), a dual type lipo- and cyclo-oxygenase inhibitor (NDGA), a bradykinin antagonist (Hoe 140) or the combination of cetirizine, MK-886, indomethacin and BN 50730. These drugs did inhibit paw oedema induced by their specific agonists or by carrageenin. These results suggest that histamine, PAF, prostaglandins, leukotrienes or bradykinin are not important in the development of immune paw oedema in guinea-pigs.

**4** Dexamethasone (10 mg $kg^{-1}$) inhibited oedema induced by ovalbumin (50 or 200 μg/paw, $P<0.05$). This effect apparently does not result from inhibition of arachidonate metabolism, since indomethacin, MK-886 and NDGA were without effect.

**5** Oedema induced by ovalbumin (50 or 200 μg/paw) was also inhibited by azelastine. This effect was not due to the anti-histaminic property of azelastine since two other potent-antihistamines, meclizine and cetirizine, were ineffective.

**6** Intravenous injection of lipopolysaccharide (LPS) dose-dependently inhibited the oedema induced by ovalbumin (200 μg/paw). This effect could not be attributed to hypotension or leucopenia since the maximal dose applied (81 μg $kg^{-1}$) did not induce significant changes in the blood pressure or in the white blood cell levels of the animals. It is suggested that the effect of LPS is mediated by the endogenous release of cytokines, including tumour necrosis factor (TNFα). Murine TNFα dose-dependently (9–81 μg $kg^{-1}$) inhibited the paw oedema induced by ovalbumin.

**7** The anti-oedematogenic effects of LPS and/or TNFα are possibly associated with their capacity to inhibit leucocyte emigration. Accordingly, guinea-pigs rendered leucopenic with vinblastine exhibited less intense oedema after ovalbumin. Vinblastine did not affect oedema induced by PAF or bradykinin, indicating that vascular responsiveness was not involved.

**Keywords:** Immunological oedema; guinea-pig paw oedema; leucocyte-dependent oedema; dexamethasone; azelastine; LPS; TNF

## Introduction

Guinea-pigs are commonly used in the study of immediate hypersensitivity, particularly that involving the bronchopulmonary system (Cortijo *et al.*, 1989; Pretolani *et al.*, 1992). However, there have been no studies of paw oedema in the guinea-pig, specifically induced by antigens, as a model for allergy in connective tissue. Histamine, leukotrienes and PAF are the main mediators of the early response of the bronchopulmonary tissue of the guinea-pig to the administration of antigen *in vivo* or *in vitro* (Daffonchio *et al.*, 1987; Desquand *et al.*, 1990).

In the present study employing specific antagonists, we initially investigated the importance of various putative mediators of inflammation in the development of the oedema induced by ovalbumin, in the paw of sensitized guinea-pigs. This oedema was not modified by antagonists or inhibitors of histamine, bradykinin, PAF or arachidonate metabolites, but was inhibited by dexamethasone and by azelastine, an anti-allergic drug used clinically (Magnussen, 1987).

Considering that in the ovalbumin injected paw, there is a massive migration of neutrophils and eosinophils to the extravascular tissues, and given that dexamethasone blocks the migration of leucocytes to the inflammatory site (Cunha *et al.*, 1985), we evaluated the contribution of leucocyte migration to the development of such allergic oedema. Lipopolysaccharide (LPS) has been shown to suppress leucocyte recruitment to inflamed tissues, and cell-dependent oedema in the rat and rabbit (Rosenbaum *et al.*, 1983; Rocha & Ferreira, 1986). Several of the effects of LPS are mediated by tumour necrosis factor (TNF) which, when injected intravenously, also inhibits neutrophil emigration to the inflammatory site (Cunha & Tamashiro, 1992). To evaluate the importance of the blockade of leucocyte migration in the development of allergic oedema, sensitized guinea-pigs were pretreated with intravenous injections of either LPS or murine TNF-α (muTNF-α) and were challenged with ovalbumin. Both muTNF and LPS significantly inhibited ovalbumin-induced oedema. We also show that in leucopenic guinea-pigs, the development of oedema induced by ovalbumin is reduced.

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## Methods

### Animals

Male, short-haired guinea-pigs (250–350 g) were housed in temperature-controlled rooms and received food and water *ad libitum* until use.

### Procedures for active sensitization

On day 0 the animals received a single dorsal, subcutaneous, injection of 1.0 ml of phosphate buffered saline (PBS), containing 20 μg of ovalbumin, dispersed in 1 mg $Al(OH)_3$. The animals were boosted with a similar injection of antigen on days 14, 21 and 28. In all experiments, the animals were used 35 days after the initial injection. Control groups were injected with PBS containing $Al(OH)_3$ alone.

### Measurement of ovalbumin-specific IgG by ELISA

The titres of ovalbumin-specific IgG in the plasma of sensitized and control guinea-pigs were measured by enzyme-linked immunoabsorbent assay (ELISA).

### Quantification of paw oedema

Thirty five days after the first injection of antigen or $Al(OH)_3$ (control), the animals received an intraplantar injection of 0.5, 5, 50 or 200 μg of ovalbumin, diluted in 100 μl of PBS. Oedema was measured plethysmographically (7150 plestimomether, Ugo Basile) 2, 4, 6, 8, 24 and 48 h after the challenge. In non-immunized animals, paw oedema was measured 4 h after the intraplantar injection of carrageenin (300 μg/paw), or 2 h after histamine (10 μg/paw) or PAF (2 μg/paw). The increase in paw volume (Δ volume) was obtained by subtracting the paw volume measured prior to the application of stimuli from the volumes for the different time-points.

### Histopathological study

The animals were killed by cervical dislocation 4 h after the paw challenge. The paws were removed, washed with tap water and immediately fixed in Millonig's solution. Paraffin blocks were prepared by conventional techniques and sections stained with Giemsa.

### Effects of drugs on ovalbumin-induced paw oedema

Drugs were administered at the times indicated below, prior to the intraplantar injection of ovalbumin (50 or 200 μg/paw). Their effects on the subsequent oedema were evaluated 4 h later: meclizine (25 mg $kg^{-1}$, s.c. 30 min), cetirizine (20 mg $kg^{-1}$, s.c., 30 min), MK-886 (10 mg $kg^{-1}$, p.o., 2 h), nordihydroguaiaretic acid (NDGA; 100 mg $kg^{-1}$, i.p., 1 h), indomethacin (10 mg $kg^{-1}$, s.c., 30 min), BN 50730 (10 mg $kg^{-1}$, s.c., 1 h), dexamethasone (10 mg $kg^{-1}$, s.c., 1 h), azelastine (1, 10 and 30 mg $kg^{-1}$, i.p., 1 h), Hoe 140 (1 mg $kg^{-1}$, i.p., 30 min) and the combination of cetirizine (20 mg $kg^{-1}$, 1 h) + MK-886 (10 mg $kg^{-1}$, 1 h) + indomethacin (10 mg $kg^{-1}$, 1 h) + BN 50730 (10 mg $kg^{-1}$, 1 h). The effects of identical pretreatment with MK-886, NDGA, indomethacin, Hoe 140, dexamethasone on paw oedema induced by carrageenin were also evaluated. The effects of meclizine, cetirizine and azelastine were evaluated against histamine (10 μg/paw) and that of BN 50730 against PAF (2 μg/paw).

### Effects of LPS and muTNFα on ovalbumin-induced paw oedema

Ovalbumin-sensitized and control animals received an i.v. injection through the penian venous sinus of LPS (9, 27 and 81 μg $kg^{-1}$) or muTNFα (9, 27 and 81 μg $kg^{-1}$) 1 h before the intraplantar injection of 200 μg/paw of ovalbumin. Paw oedema was measured 2, 4 and 6 h after challenge.

### Evaluation of blood pressure

The effect of 81 μg $kg^{-1}$ of LPS on the blood pressure of conscious guinea-pigs was also investigated. Normal guinea-pigs (350–450 g) were anaesthetized with sodium pentobarbitone (20 mg $kg^{-1}$, i.p.) and the carotid artery and the jugular vein were cannulated with a polyethylene tube (PE 10). Twenty-four hours later, the conscious animals were prepared for the recording of carotid blood pressure on a Beckman polygraph (Model R611, transducer 9872), before and after the jugular vein injection of acetylcholine (1.5 μg $kg^{-1}$). When the blood pressure returned to normal, LPS (81 μg $kg^{-1}$) was injected i.v. (jugular vein) and arterial pressure was measured 1, 2, 3 and 4 h later, followed by acetylcholine injection.

### Effects of LPS and azelastine on circulating leucocytes

The animals were injected i.v. with LPS (81 μg $kg^{-1}$, penial venous sinus) or i.p. with azelastine (30 mg $kg^{-1}$) and the leucogram performed 4 h after. For this, blood samples (1 ml) were collected by cardiac puncture from ether-anaesthetized animals and total and differential cell counts were performed by standard methods (Souza & Ferreira, 1985). Results are expressed by means ± s.e.mean × $10^6$ cells $ml^{-1}$ of blood. The number of animals per group was five.

### Procedures for the induction of leucopenia

Groups of normal or sensitized guinea-pigs were injected s.c. with vinblastine (0.15 mg $kg^{-1}$) and a leucogram performed 5 days later. Blood samples (1 ml) were collected by cardiac puncture from ether-anaesthetized animals. Blood was diluted in Türk solution and the number of leucocytes counted in Neubauer chambers. Leucopenic or normal (control) animals were injected with bradykinin (10 μg/paw), PAF (2 μg/paw), or with ovalbumin and paw oedema was measured 0.5, 2 or 4 h later, respectively.

### Drugs and solutions

The composition of the Millonig solution was (in mM): $NaH_2PO_4.H_2O$ (130), NaOH (470) and formaline 10% v/v. The reagents used were (sources in parentheses); carrageenin (Marine Colloids, U.S.A.); lipopolysaccharide (LPS) B from *E. coli* 026 B6, control number 792386 (Difco Laboratories, U.S.A.); chicken ovalbumin (Merck, Brazil); dexamethasone, Decadron (Merck Sharp & Dohme, Brazil); sodium pentobarbitone, histamine, indomethacin and nordihydroguaiaretic acid, NDGA (Sigma, U.S.A.); cetirizine (UCB, Belgium); azelastine (Asta, Germany); meclizine (Pfizer, Brazil); PAF (Bachem, Switzerland); MK-886 (L-663,536(3-[1-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid) (Merck, Canada); BN 50730 ([3-1,1-dimethylethyl]hexahydro-1,4,4,7b-trihydroxy-8-methyl-9H-1,7α(epoxymethano)-1H, 6αH-cyclopenta-(c) furo [2,3b] furo[3,2,3,4]cyclpenta[1,2-d]furan-5,9,12 (4H)trione) (Institut Henri Beaufour, France); acetylcholine (Merck, Germany); muTNFα (Genentech, U.S.A.); vinblastine sulphate, Velban (Eli Lilly, Brazil); Hoe 140 (D-Arg-[$Hyp^3$,$Thi^5$,D-$Tic^7$,$Oic^8$]bradykinin) (Hoescht, Germany).

### Statistical analyses

Student's unpaired t test or an analysis of variance (ANOVA) followed by Bonferroni's test were used. Statistical differences were considered significant at $P \leqslant 0.05$.

## Results

### *Time course of paw oedema induced by ovalbumin and measurement of specific anti-ovalbumin immunoglobulin G in serum*

The intraplantar injection of ovalbumin at doses up to 200 μg induced dose-dependent oedema in sensitized animals (Figure 1a). At the highest dose, oedema peaked after 4 h and disappeared 48 h later (Figure 1b). Non-sensitized control animals were not affected by ovalbumin. The plasma titre of IgG specific against ovalbumin in sensitized animals was 1/4390; non-sensitized control animals had a titre of approximately 1/2, i.e., no anti-ovalbumin IgG was found in the serum. Isolated preparations of ileum strips from sensitized guinea-pigs contracted on the addition of ovalbumin to the organ bath (results not shown). The challenge with ovalbumin of the paw of naive guinea-pig which has been injected 9 days before with heated serum obtained from immunized animals gave an oedematogenic response similar to that observed in animals treated with non heated serum (results not shown).

### *Histological study of the paw after injection with ovalbumin*

Histological analysis of the paws injected with ovalbumin revealed a massive infiltration of neutrophils and eosinophils in the hypodermis (data not shown).

### *Response of ovalbumin-induced oedema to standard antagonists*

Table 1 shows that meclizine (25 mg $kg^{-1}$), cetirizine (20 mg $kg^{-1}$), MK-886 (10 mg $kg^{-1}$), NDGA (100 mg $kg^{-1}$), indomethacin (10 mg $kg^{-1}$), BN 50730 (10 mg $kg^{-1}$), and Hoe 140 (1 mg $kg^{-1}$) failed to prevent oedema induced by ovalbumin (50 or 200 μg/paw). The combination of citirizine + MK 886 + indomethacin + BN 50730 (doses indicated above) also failed to reduce the oedema induced by ovalbumin (50 μg/paw). Table 1 shows that these drugs inhibited the oedema induced by the specific agonists or inflammatory agents (histamine for meclizine and cetirizine; carrageenin for MK-886, NDGA, indomethacin, Hoe 140; and PAF for BN 50730), thus validating the negative results against ovalbumin.

### *Protection by dexamethasone against ovalbumin-induced oedema*

Dexamethasone (10 mg $kg^{-1}$, s.c.) inhibited oedema induced by ovalbumin at 50 or 200 μg/paw (Figure 2b). This concentration of dexamethasone also inhibited oedema induced by carrageenin (300 μg/paw) in non-sensitized guinea-pigs (Figure 2a), but failed to inhibit the oedema induced by histamine (Control: histamine 10 μg/paw = 300 ± 30 μl; Dexamethasone treated = 290 ± 40 μl ($n$ = 5)).

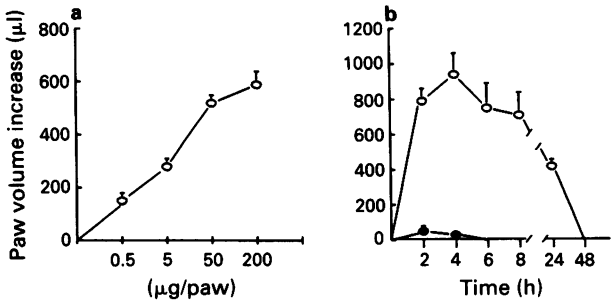


**Figure 1** Dose-dependence (a) and time-course (b) of paw oedema induced by ovalbumin in the immunized guinea-pig. (a) Ovalbumin was injected intraplantar at the doses indicated and oedema was measured after 4 h; (b) time-course of oedema induced by ovalbumin (200 μg/paw) injected in sensitized (○) or non-sensitized guinea-pigs (●). The volume injected per paw was 100 μl. Data are the mean ± s.e.mean of 5–8 animals per group.

**Table 1A** Failure of standard agents to inhibit paw oedema in ovalbumin-sensitized guinea-pigs

| *Antagonist* | (mg $kg^{-1}$) | *Intensity of oedema* (μl) Ovalbumin (50 μg/paw) | Ovalbumin (200 μg/paw) |
|---|---|---|---|
| Solvent | | 520 ± 30 (6) | 680 ± 30 (8) |
| Meclizine | 25 | 500 ± 20 (6) | 630 ± 40 (6) |
| Cetirizine | 20 | 430 ± 40 (6) | 550 ± 60 (6) |
| MK-886 | 10 | 550 ± 30 (6) | 600 ± 20 (6) |
| NDGA | 100 | 530 ± 20 (6) | 560 ± 20 (6) |
| Indomethacin | 10 | 530 ± 20 (6) | 630 ± 20 (6) |
| BN 50730 | 10 | 504 ± 15 (6) | 660 ± 70 (5) |
| Hoe 140 | 1 | 430 ± 60 (5) | $NP^+$ |
| Combination† | – | 450 ± 40 (5) | $NP^+$ |

**B** Inhibitory effect of standard agents on oedema induced by different stimuli

| *Agonist* | *Antagonist* | (mg $kg^{-1}$) | *Intensity of oedema* (μl) |
|---|---|---|---|
| Histamine (10 μg/paw) | | | 360 ± 40 (5) |
| | Meclizine | 25 | 90 ± 10* (5) |
| | Cetirizine | 10 | 30 ± 20* (5) |
| Carrageenin (300 μg/paw) | | | 450 ± 30 (5) |
| | MK-886 | 10 | 160 ± 20* (5) |
| | NDGA | 100 | 280 ± 40* (4) |
| | Indomethacin | 10 | 170 ± 30* (5) |
| | Hoe 140 | 0.1 | 240 ± 60* (4) |
| | Combination† | – | 150 ± 30* (4) |
| PAF (2 μg/paw) | | | 280 ± 17 (6) |
| | BN 50730 | 10 | 110 ± 22* (5) |

Oedema induced by carrageenin, PAF and ovalbumin was evaluated 4 h after challenge, and histamine after 2 h. $NP^+$ = not performed. Combination† = cetirizine + MK-886 + indomethacin + BN 50730 in the indicated doses. Values are the mean ± s.e.mean, $n$ shown in parentheses, $^*P \leq 0.05$ compared to value for agonist alone.

### *Dose-dependent protection by azelastine against ovalbumin-induced oedema*

Figure 3b shows that oedema induced by 50 or 200 μg of ovalbumin per paw was inhibited dose-dependently by azelastine (Azel, 10 and 30 mg kg$^{-1}$, i.p.). The concentration of 1 mg kg$^{-1}$, which was ineffective against ovalbumin, antagonized oedema by histamine (10 μg/paw) in non-sensitized guinea-pigs (Figure 3a).

### *Time course and dose-dependent protection by LPS and muTNFα against ovalbumin-induced oedema*

As seen in Figure 4a, oedema induced by ovalbumin was dose-dependently blocked by LPS (9, 27 and 81 μg kg$^{-1}$, i.v.) and muTNFα (9, 27 and 81 μg kg$^{-1}$, i.v.). A significant inhibition induced by LPS was noted at all time points (Figure 4b). Administration of LPS (81 μg kg$^{-1}$, i.v.) did not reduce the blood pressure of conscious guinea-pigs under conditions where ACh (1.5 μg kg$^{-1}$, i.v.) was effective (Table 2).

### *Effect of LPS and azelastine on circulating white blood cells*

Administration of LPS (81 μg kg$^{-1}$, i.v.) or of azelastine (30 mg kg$^{-1}$, i.p.) did not reduce the circulating leucocytes in control (PBS, i.p.) animals, neutrophils 1.0 ± 0.3, mononuclear cells 6.5 ± 0.9; in LPS-pretreated animals, neutrophils 1.3 ± 0.6, mononuclear cells 6.1 ± 1.0 or in azelastine-pretreated animals, neutrophils 1.8 ± 0.4; mononuclear cells 10 ± 1.0 × 10$^6$ cells ml$^{-1}$ blood.

### *Effect of vinblastine on ovalbumin -induced paw oedema*

Vinblastine (0.15 mg kg$^{-1}$, s.c.) induced drastic leucopenia after 5 days (normal 7550 ± 1445, $n = 8$; vinblastine-treated 2381 ± 854 leucocytes mm$^{-3}$ of blood, $n = 7$). However the paw oedema induced by bradykinin and PAF were as intense in leucopenic as in normal animals, although oedema induced by ovalbumin (200 μg/paw) was reduced (Figure 5).

## Discussion

In the first part of this study, we demonstrated that dose-dependent and prolonged, anaphylactic paw oedema followed the intra-plantar administration of ovalbumin in sensitized

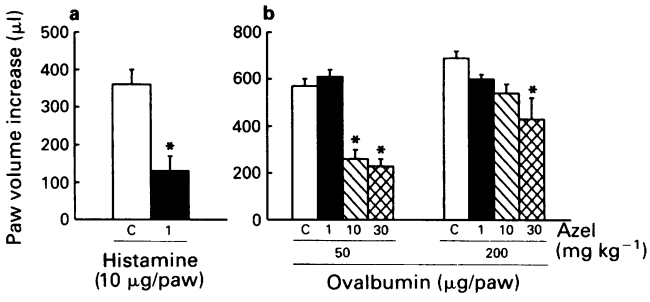


**Figure 3** Inhibition by azelastine (Azel) of paw oedema induced by ovalbumin in the immunized guinea-pig. Oedema was induced in non-sensitized guinea-pigs with histamine (10 μg/paw; a) and in immunized guinea-pigs with ovalbumin (50 or 200 μg/paw, b). Oedema was measured 2 h after the intraplantar injection of histamine or 4 h after ovalbumin challenge. Open and hatched bars represent oedema in guinea-pigs pretreated with PBS (c) or with azelastine at doses of 1, 10 and 30 mg kg$^{-1}$, i.p. Columns are the mean ± s.e.mean of 6–11 animals per group. Asterisks indicate significant differences compared to respective controls ($P \leq 0.05$, compared to untreated animals; ANOVA followed by Bonferroni's test).

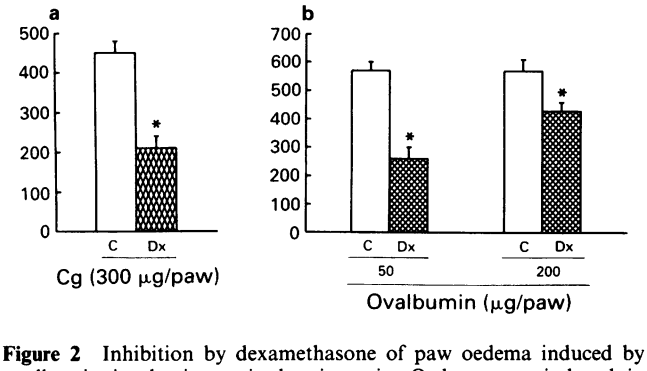


**Figure 2** Inhibition by dexamethasone of paw oedema induced by ovalbumin in the immunized guinea-pig. Oedema was induced in non-sensitized guinea-pigs with carrageenin (Cg, 300 μg/paw; a) and in immunized guinea-pigs with ovalbumin (50 or 200 μg/paw, b). Open and cross-hatched bars represent oedema in guinea-pigs pretreated with PBS or dexamethasone (Dx, 10 mg kg$^{-1}$, s.c.), respectively. Oedema was measured 4 h after the intraplantar challenge. Columns are the mean ± s.e.mean of values from six animals per group. Asterisks indicate significant differences compared to respective controls (c, untreated animals) ($P \leq 0.05$, Student's unpaired $t$ test).

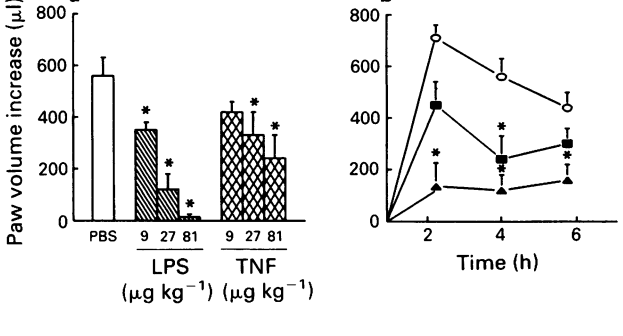


**Figure 4** Inhibition by lipopolysaccharide (LPS) and murine tumour necrosis factor (muTNFα) of paw oedema induced by ovalbumin in the sensitized guinea-pig. (a) The sensitized animals were pretreated i.v. with PBS, LPS or muTNFα at the doses indicated before the injection of ovalbumin (200 μg/paw). Paw oedema was measured 4 h after challenge. (b) Time-course of paw oedema induced by ovalbumin (200 μg/paw) in PBS-pretreated animals (○), TNF-pretreated animals (81 μg kg$^{-1}$, ■) or LPS-pretreated animals (81 μg kg$^{-1}$, ▲). In (a) and (b), data are the mean ± s.e.mean of 4–6 animals per group. Asterisks indicate significant differences compared to respective controls ($P \leq 0.05$ compared to untreated animals; ANOVA followed by Bonferroni's test).

**Table 2** Blood pressure (mmHg) of normal guinea-pigs injected with lipopolysaccharide (LPS, 81 μg kg$^{-1}$); the animals were injected with acetylcholine (1.5 μg kg$^{-1}$) at the beginning and end of each experiment

| | | *Time after LPS injection* (h) | | | | | |
|---|---|---|---|---|---|---|---|
| *Normal* | *ACh* | 0 | 1 | 2 | 3 | 4 | *ACh* |
| 71.25 ± 7.37 | 36.25* ± 3.61 | 71.25 ± 7.37 | 63.33 ± 5.77 | 65.00 ± 8.66 | 66.67 ± 2.89 | 68.75 ± 13.75 | 32.50* ± 5.00 |

Acetylcholine (ACh) and LPS were injected i.v. Data are the mean ± s.e.mean, $n = 4$; *$P \leq 0.05$ compared to normal group.

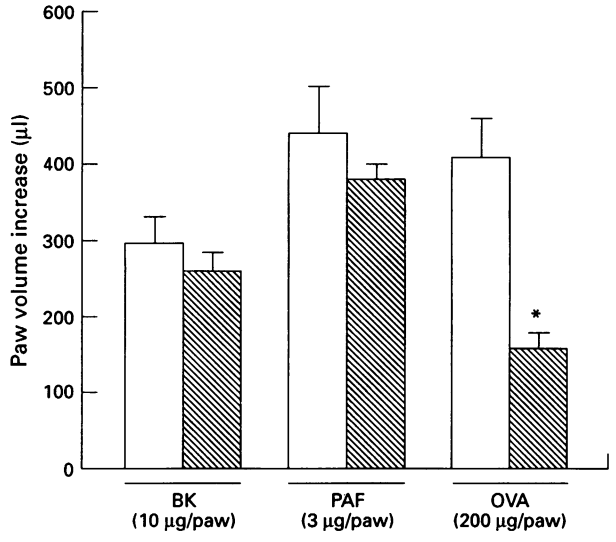


**Figure 5** Effect of vinblastine on paw oedema induced by bradykinin (BK), PAF or ovalbumin. BK, PAF and ovalbumin were injected intraplantar at the doses indicated in normal or immunized guinea-pigs and the oedema was measured 0.5, 2 or 4 h later, respectively. The open and hatched columns represent oedema in PBS- or vinblastine- (0.15 mg $kg^{-1}$; 5 days prior to challenge) treated animals, respectively. Data are the mean ± s.e.mean of 5–6 animals per group. Asterisk indicates significant difference compared to respective control ($P \leq 0.05$; Student's unpaired $t$ test).

guinea-pigs. Sensitized animals had a high titre of specific IgG in the plasma and isolated ileum preparations from these animals contracted in response to ovalbumin, indicating that homocytotropic antibodies were present in the tissues. Moreover the heating of the serum obtained from immunized animals did not abolish its capacity to transfer to naive animals the ability to react to an intraplantar injection of ovalbumin, suggesting that the antibodies involved in the process are not IgE. However, in the present investigation we have not discarded the possibility that in addition to the participation of the homocytotropic antibodies, there is also an additional Arthus type response.

The accumulation of neutrophils in the tissues during the early phase of the inflammatory process is a common phenomenon in acute, non-immunological states and in various immunological inflammatory reactions (Sedgwick & Willoughby, 1985). Four hours after ovalbumin injection, a massive infiltration of neutrophils and eosinophils was noted concomitant with the oedema. Eosinophils are frequently found in tissues provoked by immunological stimuli (Lellouch-Tubiana *et al.*, 1988; Sanjar *et al.*, 1990). To our knowledge, this is the first demonstration that this event occurs in immunologically sensitized guinea-pig paws challenged with antigen. The significance of these cells, however, is unclear, since their presence may either aggravate the expression of anaphylaxis or down-regulate the process. Eosinophils release chemotactic stimuli which may amplify the inflammatory reaction or, since they contain enzymes which degrade inflammatory mediators, they may help to reduce the process (Wasserman *et al.*, 1975; Kay *et al.*, 1976). Agents effective in the guinea-pig against histamine, PAF or carrageenin were ineffective against ovalbumin-induced oedema. The combination of these agents also were ineffective, suggesting that histamine, arachidonate metabolites, PAF and bradykinin are not important in this specific oedema, in contrast to findings in guinea-pig skin, where combination of antagonists inhibited oedema (Weg *et al.*, 1991).

Since guinea-pigs are claimed to be resistant to glucocorticoseroids (Claman, 1972), a high dose of dexamethasone (10 mg $kg^{-1}$) was used which significantly blocked oedema induced by either carrageenin or ovalbumin (50 µg and 200 µg/paw), but not induced by histamine. Several effects of glucocorticoids may be explained by their capacity to inhibit the formation of arachidonate metabolites via lipocortin (Flower, 1988) and/or the release of cytokines with inflammatory properties (Besedovsky *et al.*, 1986; Dinarello, 1991). The inhibitory effect of dexamethasone on oedema of the guinea-pig paw induced by ovalbumin is probably not due to interference with eicosanoid formation, since drugs that block cyclo-oxygenase and lipoxygenase were ineffective. The absence of effect of dexamethasone on histamine paw oedema supports the suggestion that in this model the glucocorticoids do not interfere with the vascular responsiveness. Considering that the release of cytokines is inhibited by glucocorticoids (Besedovsky *et al.*, 1986) and given that we have already demonstrated the association of oedema and leucocyte recruitment (Rocha & Ferreira, 1986), the inhibitory effect of dexamethasone on guinea-pig paw oedema may result, at least in part, from inhibition of the release of the inflammatory cytokines. In fact, the inhibitory effect of dexamethasone has been shown already on the release of cytokines like TNF, interleukin-1 (IL-1), and IL-8 which are chemotactic for neutrophils (Faccioli *et al.*, 1990; Ribeiro *et al.*, 1991), or of IL-8, IL-5 and IL-2, which are chemotactic for eosinophils (Rand *et al.*, 1991; Smith *et al.*, 1991; Iwama *et al.*, 1993).

In the second part of the study, we demonstrated that LPS and TNFα dose-dependently inhibited the oedema induced by ovalbumin. This protective effect was not due to hypotension, since the maximal dose of LPS used (81 µg $kg^{-1}$) failed to induce hypotension. The LPS effect was also not due to a leucopenia in the animals, since the dose of 81 µg $kg^{-1}$ did not change the white blood cell counts of the animals. LPS inhibits the oedema and leucocyte migration induced by carrageenin in rats (Rocha & Ferreira, 1986) as well as anaphylaxis in guinea-pigs (Vannier *et al.*, 1991). Several effects of LPS are mediated by the release of cytokines; the intravenous injection of TNF alone or associated with other cytokines, such as IL-1, mimics LPS effects (Beutler *et al.*, 1985; Tracey *et al.*, 1987). TNFα inhibits neutrophil migration in mice (Otsuka *et al.*, 1990) and in rats (Cunha & Tamashiro, 1992) as well as the chemotaxis of human neutrophils *in vitro* (Salyer *et al.*, 1990). Thus it is possible that the inhibition of ovalbumin-induced oedema by intravenous injections of LPS was due, at least partially, to impairment of leucocyte migration and/or the adhesiveness of the leucocytes in the endothelium, via the release of TNF. To test further the contribution of leucocytes to the ovalbumin-induced oedema, the animals were pretreated with vinblastine which depleted the level of circulating leucocytes by 70% and significantly reduced ovalbumin-induced oedema. This treatment did not affect PAF- or bradykinin-induced oedema which are thought to be cell independent. However, we have not discarded the possibility that vinblastine may be affecting the release of mediators by resident cells such as macrophages or mast cells.

In addition to dexamethasone, azelastine also inhibited the paw oedema induced by ovalbumin. Although azelastine has a well established anti-histamine activity (Magnussen, 1987), its anti-edematogenic effect in the sensitized guinea-pig may not necessarily be associated with this property. In fact, similarly to cetirizine and meclizine, azelastine effectively inhibited histamine-induced oedema, although only azelastine blocked ovalbumin-induced oedema. Furthermore, an effective dose against histamine (1 mg $kg^{-1}$) was ineffective against ovalbumin-induced oedema. Azelastine is known to have a significant effect in various other models of inflammation such as the bronchoconstriction induced by PAF or metabolites of arachidonic acid in the sensitized guinea-pig, which appears to be dependent on the inhibition of leukotriene synthesis (Pretolani *et al.*, 1992). In our model, leukotrienes apparently play no relevant role in oedema formation as demonstrated by the absence of effect of MK-886 and NDGA. Azelastine inhibits immunologically-induced, pleural exudation by a mechanism not yet known (Lima *et al.*, 1991). In our model, the mode of action of azelastine is also not

clear but is not due to a decrease in the number of circulating leucocytes. The possible inhibition of neutrophil and/or eosinophil migration by azelastine in our test is currently under investigation.

In conclusion, our results show that antigen-induced paw oedema is associated with infiltration of neutrophils and eosinophils. This oedema is not dependent on histamine release, eicosanoid, bradykinin or PAF formation, since standard antagonists and inhibitors were ineffective in preventing it. Dexamethasone and azelastine were effective by mechanisms unrelated to arachidonate metabolites and antagonism of histamine respectively. Ovalbumin-induced oedema was inhibited dose-dependently by LPS and TNF, thus suggesting an effect on leucocyte migration, confirmed by the reduction of ovalbumin-induced oedema in leucopenic guinea-pigs. The mediator(s) responsible for oedema formation in this model is as yet unknown.

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# Subtypes of purinoceptors in rat and dog urinary bladder smooth muscles

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**1** Both adenosine and adenosine 5'-triphosphate (ATP) (10 μM and 100 μM) relaxed 10 μM acetylcholine (ACh)-induced contraction of rat bladder strips, which was completely antagonized by 100 μM 8-(*p*-sulphophenyl) theophylline. In dog bladder neither adenosine nor ATP inhibited ACh-induced contraction.

**2** $P_{2x}$-purinoceptor agonists contracted both rat and dog bladder strips with the potency order of α,β-MeATP > ATP > ADP.

**3** α,β-MeADP (100 μM) induced a contraction of the rat bladder strip even after desensitization of $P_{2x}$-purinoceptors but failed to contract the dog bladder strip.

**4** 2-MeSATP (1 μM to 300 μM) concentration-dependently induced contraction of rat bladder strips; this contraction was significantly inhibited after desensitization of $P_{2x}$-purinoceptors. Cibacron blue 3GA (100 μM) antagonized the drug at concentrations lower than 30 μM, whereas it augmented the response to the drug at concentrations above 30 μM.

**5** ADPβS (1 μM to 1 mM) concentration-dependently induced contraction of rat bladder strips after desensitization of $P_{2x}$-purinoceptors; a contraction which was significantly antagonized by cibacron blue 3GA (100 μM).

**6** It is concluded that three subtypes of purinoceptors, $P_1$ (mediating relaxation), and $P_{2x}$ and another type of $P_2$ (mediating contraction), exist in rat urinary bladder smooth muscle, whereas a single subtype of the receptor, $P_{2x}$-purinoceptor (mediating contraction) occurs in dog urinary bladder smooth muscle.

**Keywords:** $P_1$-purinoceptors; $P_2$-purinoceptors; urinary bladder smooth muscle; adenosine 5'-*o*-2-tiodiphosphate; 2-methylthioadenosine 5'-triphosphate; cibacron blue 3GA

## Introduction

Physiological and pharmacological effects of purines have been investigated in a number of smooth muscles (Burnstock *et al.*, 1978; Muramatsu *et al.*, 1980; Fedan *et al.*, 1982), and have become a matter of importance. These effects are mediated via two main classes of receptors, designated as $P_1$ and $P_2$, which respond primarily to adenosine and ATP, respectively (Burnstock *et al.*, 1978). $P_1$-purinoceptors in smooth muscles usually mediate relaxation (Burnstock, 1978), although in a number of smooth muscle preparations adenosine causes contraction via these receptors (Kenakin & Pike, 1987; Bailey *et al.*, 1992). $P_2$-purinoceptors in these tissues are subdivided into $P_{2x}$ which mediates contraction and $P_{2y}$ which mediates relaxation (Burnstock & Kennedy, 1985).

However, in the rat colon, contraction mediated by $P_{2y}$-purinoceptors has recently been reported (Bailey & Hourani, 1990). A new type of $P_2$-purinoceptor mediating contraction was found in rat ileum (Wiklund & Gustafsson, 1988); this type of receptor showed $P_{2y}$-type order of potency of agonists, although it was not sensitive to $P_{2y}$-purinoceptor antagonists but sensitive to $P_{2x}$-purinoceptor antagonists. The authors designated the receptor as $P_{2s}$. These findings do not fit the classification of purinoceptors by Burnstock & Kennedy (1985). In addition, it was reported that two different types of purinoceptors, both of $P_{2x}$ and $P_{2y}$, existed in rabbit mesenteric artery smooth muscle (Burnstock & Warland, 1987). This means that ATP could induce either contraction or relaxation, depending on the type of receptor occupied by ATP. Thus, in smooth muscles the classification of purinoceptors has become complicated and the physiological role of purines is still obscure.

It is well known that there is a variety of receptors in urinary bladder smooth muscles (Hisayama *et al.*, 1988; Iacovou *et al.*, 1990). The existence of $P_{2y}$-purinoceptors was confirmed in guinea-pig urinary bladder (Iacovou *et al.*, 1988) and in rat urinary bladder, $P_1$- and $P_{2x}$-purinoceptors (Bhat *et al.*, 1989; Nicholls *et al.*, 1992) were shown to exist. In mouse urinary bladder it has recently been reported that $P_{2x}$- and $P_{2y}$-purinoceptors coexist (Boland *et al.*, 1993). In the present study, the effects of various purinoceptor agonists were investigated in rat and dog urinary bladder smooth muscles in order to clarify the subtypes of purinoceptors.

## Methods

Male Wistar strain rats (weighing 200 to 250 g) and mongrel dogs of either sex (weighing 15 to 20 kg) were anaesthetized with pentobarbitone sodium (40 mg $kg^{-1}$), and the urinary bladder was rapidly removed. The bladder was transferred into $Ca^{2+}$, $Mg^{2+}$-free Tyrode solution (4°C) and then cut into small tissue strips of 7.5 × 2 mm. The strip was suspended in an organ bath which contained 10 ml of control Tyrode solution. The solution was aerated with atmospheric air and maintained at 37°C. Responses to drugs were monitored by measuring isotonic tension under the resting load of 0.5 g, after the strip had been equilibrated for 60 min. After each application of the drug, it was washed out with more than 100 ml of control Tyrode solution, and the next application of the drug was made 30 min later. The composition of control Tyrode solution was (mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 2.0, $MgCl_2$ 2.0, $MgCl_2$ 0.5, $NaH_2PO_4$ 0.33, HEPES 5.0 and glucose 5.0 (pH = 7.4). $Ca^{2+}$, $Mg^{2+}$-free Tyrode solution was made by omitting both $Ca^{2+}$ and $Mg^{2+}$ ions from the control Tyrode solution.

### *Drugs*

Adenosine (Adn), adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), α,β-methylene adenosine 5'-diphos-

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phate (α,β-MeADP), α,β-methylene adenosine 5'-triphosphate (α,β-MeATP), adenosine 5'-*o*-2-tiodiphosphate (ADPβS) and cibacron blue 3GA were purchased from Sigma Chemical Company. 2-methylthio-adenosine 5'-triphosphate (2-MeSATP) and 8-(*p*-sulphophenyl) theophylline (8-SPT) were purchased from Research Biochemicals. Acetylcholine chloride (ACh) was purchased from Daiichiseiyaku. All drugs were prepared freshly before each experiment by dissolving them in control Tyrode solution.

### *Statistical analysis*

Results are expressed as mean ± s.e.mean. One-way analysis of variance (one-way ANOVA) was used to test for statistical significance. Probability of 0.05 or less was considered significant.

## Results

### *Relaxant effects of Adn and ATP on rat and dog urinary bladder smooth muscles*

We examined whether Adn and ATP induced relaxation in rat and dog urinary bladder smooth muscles (Figure 1). Relaxant responses were measured in strips contracted with ACh (10 μM). As shown in Figure 1a, 10 μM ACh induced tonic-type contraction of the rat bladder strip. Adn concentration-dependently induced relaxation of the strip contracted with 10 μM ACh; Adn 10 μM produced relaxation and 100 μM induced further relaxation of the strip ($n = 8$). ATP also concentration-dependently induced relaxation of the rat bladder strip contracted with 10 μM ACh ($n = 8$). ATP 10 μM induced relaxation of the strip and at 100 μM induced transient contraction of the strip followed by relaxation. Although we did not compare quantitatively the relaxant effects of Adn and ATP, Adn seemed to show more potent relaxant effects than ATP. In three experiments α,β-MeATP (100 μM) did not induce relaxation of the strip contracted by 10 μM ACh (data not shown). In dog urinary bladder smooth muscle (Figure 1b), although 10 μM ACh induced tonic-type contraction, neither Adn (100 μM) nor ATP (100 μM) showed relaxant effects ($n = 8$).

### *Inhibitory effects of 8-SPT on relaxation of rat urinary bladder smooth muscles by Adn and ATP*

We examined whether 8-SPT, a specific blocker of $P_1$-purinoceptors (Collis *et al.*, 1987), inhibited the relaxant effects of Adn and ATP in the rat urinary bladder smooth muscle. Figure 2a shows the inhibitory effects of 8-SPT of various concentrations on the Adn-induced relaxation: 1 μM 8-SPT did not significantly affect the Adn-induced relaxation of the strip; in the presence of 10 μM 8-SPT, the relaxation induced by 100 μM Adn was slightly inhibited though the relaxation by 10 μM Adn was obviously inhibited. With 100 μM antagonist, relaxant effects of adenosine were almost completely inhibited ($n = 5$).

Figure 2b shows the inhibitory effects of 8-SPT on the ATP-induced relaxation in rat urinary bladder smooth muscle. As already shown in Figure 1, ATP concentration-dependently induced relaxation of the strip contracted by ACh, and 100 μM ATP induced a transient contraction of the strip followed by the relaxation. 8-SPT (100 μM) completely inhibited the relaxant effects of ATP, although it did not inhibit the ATP-induced transient contraction observed at 100 μM. We also examined whether a $P_{2y}$-receptor agonist, 2-MeSATP (Burnstock & Kennedy, 1985), induced relaxation of the strip contracted by ACh ($n = 3$); 2-MeSATP produced no significant relaxation of the strip at 100 μM (data not shown). These results suggested that Adn and ATP induced relaxation of rat bladder strip via $P_1$-receptors.

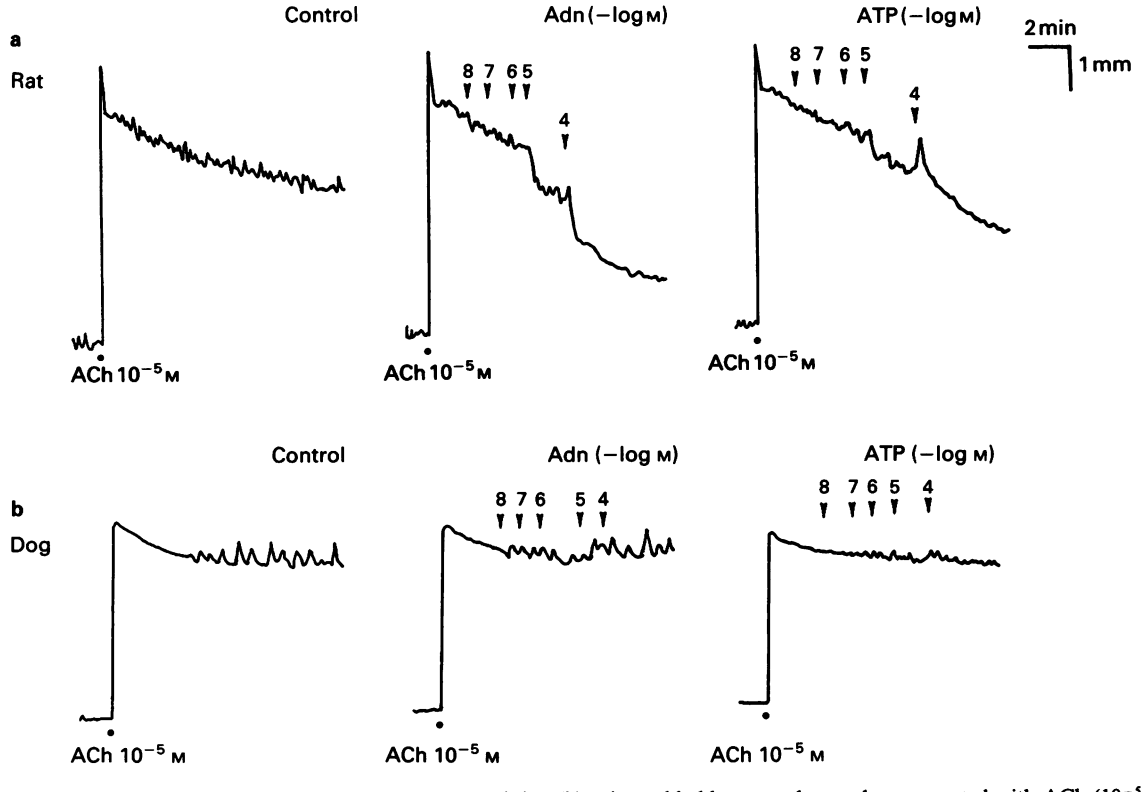


**Figure 1** Relaxant effects of Adn and ATP on rat (a) and dog (b) urinary bladder smooth muscles contracted with ACh ($10^{-5}$ M). Control ACh-induced contractions (left), effects of Adn (middle) and those of ATP (right) are shown in both panels. ACh was applied at (●) and Adn and ATP were cumulatively applied at (▼). Numbers in each panel indicate the concentrations of Adn and ATP in − log M. Traces in each panel were obtained from the same strip, respectively. Horizontal bar indicates 2 min, and vertical 1 mm.

### *Contractile effects of $P_2$-receptor agonists on rat and dog urinary bladder smooth muscles*

We compared contractile responses to various $P_2$-receptor agonists in rat and dog urinary bladder smooth muscles. As shown in Figure 3, in rat bladder strips, ATP (1 mM), ADP (1 mM) and α,β-MeATP (10 μM) induced phasic contractions. The rank order of potency was α,β-MeATP > ATP > ADP. An ADP-analogue, α,β-MeADP (100 μM) induced rather tonic-type contraction of the rat bladder strip which showed a small maximum shortening velocity and lasted for a relatively long time (see also Figure 4).

In dog urinary bladder smooth muscle, ATP (1 mM), ADP (1 mM) and α,β-MeATP (10 μM) also induced phasic contractions (Figure 3). The rank order of potency was also α,β-MeATP > ATP > ADP in dog bladder strips. However, in dog bladder strips, α,β-MeADP (100 μM) did not induce contraction ($n = 4$). It should be noted that strips unresponsive to α,β-MeADP were contracted by subsequent application of ADP (1 mM) ($n = 3$). These results indicated that the α,β-MeADP-induced contraction was only observed in the rat urinary bladder smooth muscle.

### *Effects of α,β-MeADP on rat urinary bladder smooth muscle*

We investigated the type of receptor that mediated the contractile response of the rat bladder strip by α,β-MeADP (Figure 4). Since it is known that α,β-MeATP is a specific agonist of $P_{2x}$-purinoceptors and desensitizes the receptor (Kasakov & Burnstock, 1983), we first examined the desensitization of the muscle to α,β-MeATP. As shown in Figure 4a, the response to cumulative application of α,β-MeATP (final concentration = 20 μM) was 17.36 ± 2.33% ($n = 5$) of that obtained by the first application of the drug (10 μM), suggesting that $P_{2x}$-receptors were almost, though not completely, desensitized by α,β-MeATP, 10 μM.

We next examined whether α,β-MeADP induced contraction after desensitization of $P_{2x}$-receptors. The left side of Figure 4b shows the contraction of the rat bladder strip induced by α,β-MeADP (100 μM). The drug induced tonic-type contraction similar to that observed in Figure 3. The right side of Figure 4b shows the contraction induced by α,β-MeADP (100 μM) of the same strip after pretreatment with α,β-MeATP (10 μM). Although $P_{2x}$-purinoceptors were almost desensitized in this condition, the application of α,β-MeADP (100 μM) 7.5 min after exposure to α,β-MeATP induced a large contraction of the rat bladder strip. The response to α,β-MeADP after desensitization of $P_{2x}$-receptors was 86.25 ± 4.59% ($n = 5$) of that in the control. These results suggested that α,β-MeADP contracted the rat bladder strip mainly via the receptors other than $P_{2x}$-receptors.

### *The contraction induced by 2-MeSATP in rat urinary bladder smooth muscle*

Since it has been suggested in the previous section that the contraction of rat urinary bladder smooth muscles by α,β-MeADP is mediated by receptors other than $P_{2x}$-receptors, we examined the effect of 2-MeSATP on the rat bladder strip.

In Figure 5a the contraction of the rat bladder strip by cumulative application of 2-MeSATP is shown. 2-MeSATP induced phasic contraction of the strip concentration-dependently. In this particular experiment, the drug at concentrations higher than 3 μM induced obvious contraction. In Figure 5b we examined whether the contractile response of the rat bladder strip to 2-MeSATP was affected after desensitization of $P_{2x}$-receptors. As shown in the figure, the cumulative application of 2-MeSATP 12 min after exposure to α,β-MeATP (10 μM) induced smaller contraction of the rat bladder strip than that observed in the absence of α,β-MeATP. After desensitization of $P_{2x}$-receptors, 2-MeSATP at

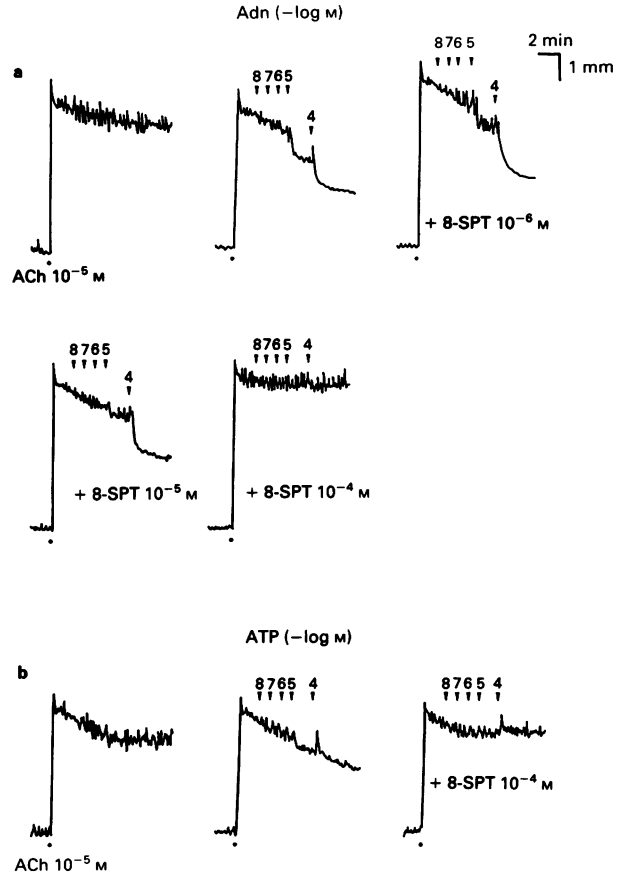


**Figure 2** Inhibitory effects of 8-SPT on Adn- and ATP-induced relaxation of rat urinary bladder smooth muscles. (a) Effects of Adn ($10^{-8}$ to $10^{-4}$ M) in the absence and presence of 8-SPT ($10^{-6}$ to $10^{-4}$ M). (b) Effects of ATP ($10^{-8}$ to $10^{-4}$ M) in the absence and presence of 8-SPT ($10^{-4}$ M). Traces in (a) and (b) were obtained from the same strip, respectively. Effects of 8-SPT were observed after strips had been incubated with the drug for 10 min. Symbols, numbers and calibrations are the same as those in Figure 1. Similar results were obtained in five experiments.

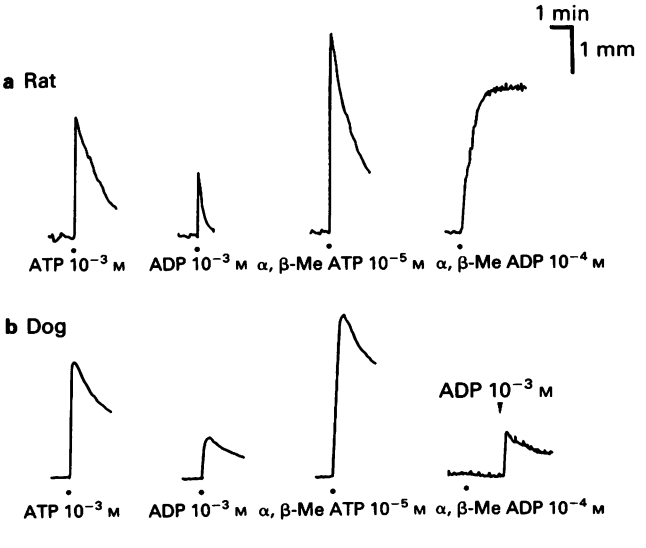


**Figure 3** Contractile effects of various purines on rat (a) and dog (b) urinary bladder smooth muscles. Contractions were induced by $10^{-3}$ M ATP, $10^{-3}$ M ADP, $10^{-5}$ M α,β-MeATP and $10^{-4}$ M α,β-MeADP. In each panel contractions induced by ATP, ADP and α,β-MeATP were obtained from the same strip, whereas those induced by α,β-MeADP was obtained in a different strip. Drugs were applied at (●) or (▼). Horizontal bar and vertical bar indicate 1 min and 1 mm, respectively.

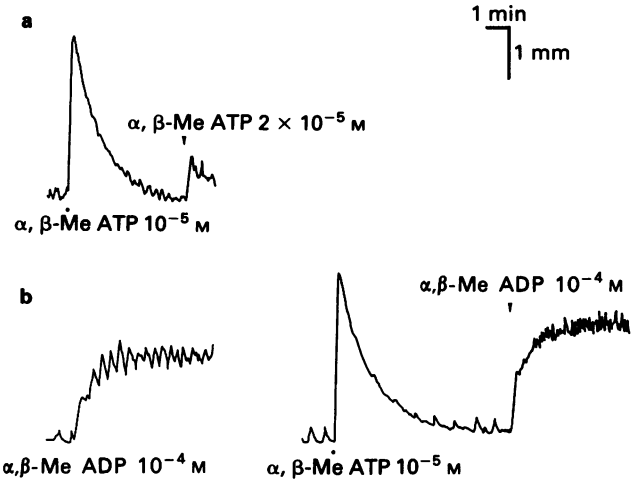


**Figure 4** Contractions of rat urinary bladder smooth muscles induced by α,β-MeATP (a) and α,β-MeADP (b) after desensitization of $P_{2x}$-purinoceptors. In (a) the contraction by cumulative application of α,β-MeATP (final concentration = $2 \times 10^{-5}$ M) is shown. At the left side of (b), the control contraction induced by α,β-MeADP ($10^{-4}$ M) is shown. At the right side the contraction induced by α,β-MeADP ($10^{-4}$ M) after pretreatment with α,β-MeATP ($10^{-5}$ M) is shown. Records were obtained from the same strip. Symbols and calibrations are the same as those in Figure 3. Similar results were obtained in five experiments.

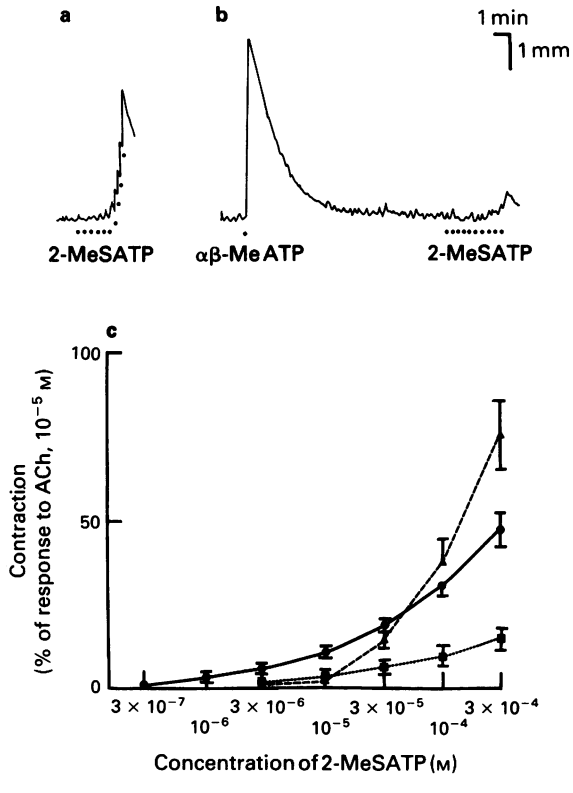


**Figure 5** Contraction induced by cumulative application of 2-MeSATP and effects of desensitization of $P_{2x}$-purinoceptors and effects of cibacron blue 3GA on the 2-MeSATP-induced contraction in rat urinary bladder smooth muscles. (a) Trace shows contraction induced by cumulative application of 2-MeSATP ($10^{-8}$, $3 \times 10^{-7}$, $10^{-7}$, $3 \times 10^{-7}$, $10^{-6}$, $3 \times 10^{-6}$, $10^{-5}$, $3 \times 10^{-5}$, $10^{-4}$ and $3 \times 10^{-4}$ M). (b) The effect of pretreatment with α,β-MeATP (10 μM) on contraction induced by cumulative application of 2-MeSATP is shown. Concentrations of 2-MeSATP used in this experiment were the same as those in (a). The trace was obtained from a different preparation from (a). In (a) and (b) symbols and calibrations are the same as those in Figure 3. (c) Cumulative concentration-response curves to 2-MeSATP in the control, after desensitization of $P_{2x}$-receptors and in the presence of cibacron blue 3GA ($10^{-4}$ M). Responses to 2-MeSATP in control (●), in the presence of cibacron blue 3GA (▲) and after desensitization of $P_{2x}$-purinoceptors (■) are plotted against the concentration of 2-MeSATP (M). Each point represents mean ± s.e.mean ($n = 5$), expressed as percentage of response to $10^{-5}$ M ACh.

concentrations higher than 30 μM induced contraction in this experiment.

Figure 5c shows the concentration-response curves of 2-MeSATP in the absence and the presence of α,β-MeATP, and in the presence of cibacron blue 3GA (100 μM). The contractile effect of 2-MeSATP was observed at 1 μM in the absence of α,β-MeATP, although in its presence 30 μM 2-MeSATP was required to contract the rat bladder strips. At 300 μM 2-MeSATP the response was 47.79 ± 11.81% of that to ACh (10 μM) in the absence of α,β-MeATP, which was decreased to 15.58 ± 6.55% in its presence. In the presence of 100 μM cibacron blue 3GA, 2-MeSATP also induced contraction of rat bladder strips concentration-dependently. The contractile responses of strips to 2-MeSATP at concentrations lower than 30 μM were significantly inhibited by cibacron blue 3GA ($P<0.01$), though those observed at concentrations higher than 30 μM were enhanced by cibacron blue 3GA. The responses at 100 μM and 300 μM 2-MeSATP were increased by 21.1% and 57.9%, respectively, in the presence of cibacron blue 3GA. The augmentation of the contraction by cibacron blue 3GA was significant at 300 μM 2-MeSATP ($P<0.05$).

### *The effect of cibacron blue 3GA on contractions induced by α,β-MeATP and ADPβS in rat urinary bladder smooth muscles*

Figure 6a shows effects of cibacron blue 3GA (10 μM and 100 μM) on the concentration-response curve to α,β-MeATP. α,β-MeATP induced contraction of rat bladder strips at concentrations higher than 30 nM and the contractile effect of the drug was almost maximal at 300 μM; 10 μM cibacron blue 3GA significantly inhibited the contractile response to α,β-MeATP 30 nM ($P<0.01$) and 100 nM ($P<0.01$), but did not significantly inhibit the contraction induced by α,β-MeATP at concentrations greater than 100 nM. At 10 μM, cibacron blue 3GA seemed to increase slightly the contractile response of strips to α,β-MeATP above 3 μM; 100 μM cibacron blue 3GA inhibited the response to α,β-MeATP more significantly than 10 μM. In the presence of 100 μM cibacron blue 3GA the contractile response to the agonist was significantly inhibited up to 3 μM ($P<0.01$ between 30 nM and 1 μM and $P<0.05$ at 3 μM). Similarly to 10 μM, 100 μM cibacron blue 3GA significantly enhanced the contractile responses to the agonist at 100 μM and 300 μM ($P<0.05$). In the presence of 100 μM cibacron blue 3GA the responses to the agonist at 100 μM and 300 μM increased by 17.9% and 17.6%, respectively.

Figure 6b shows effects of cibacron blue 3GA (10 μM and 100 μM) on the concentration-response curve to ADPβS. ADPβS induced contraction of rat bladder strips at concentrations higher than 1 μM. The contractile effect of the drug was not maximal even at 1 mM. In the presence of 10 μM cibacron blue 3GA, 10 μM ADPβS was required to induce an obvious contraction. Cibacron blue 3GA significantly inhibited the contractile response induced by 10 μM ($P<0.05$) and 100 μM ($P<0.05$) ADPβS, whereas it did not significantly inhibit the effect of ADPβS at concentrations higher than 100 μM. Cibacron blue 3GA, 100 μM, completely antagonized the effects of ADPβS lower than 1 mM ($P<0.05$ at 3 μM and $P<0.01$ between 10 μM and 300 μM of ADPβS). At 300 μM ADPβS, cibacron blue 3GA, inhibited the contraction by 68%; at 1 mM ADPβS the drug did not inhibit the contraction at all. These results suggested that low concentration of ADPβS (1 to 100 μM) induced contraction of the rat urinary bladder smooth muscle mainly via receptors other than $P_{2x}$-receptors.

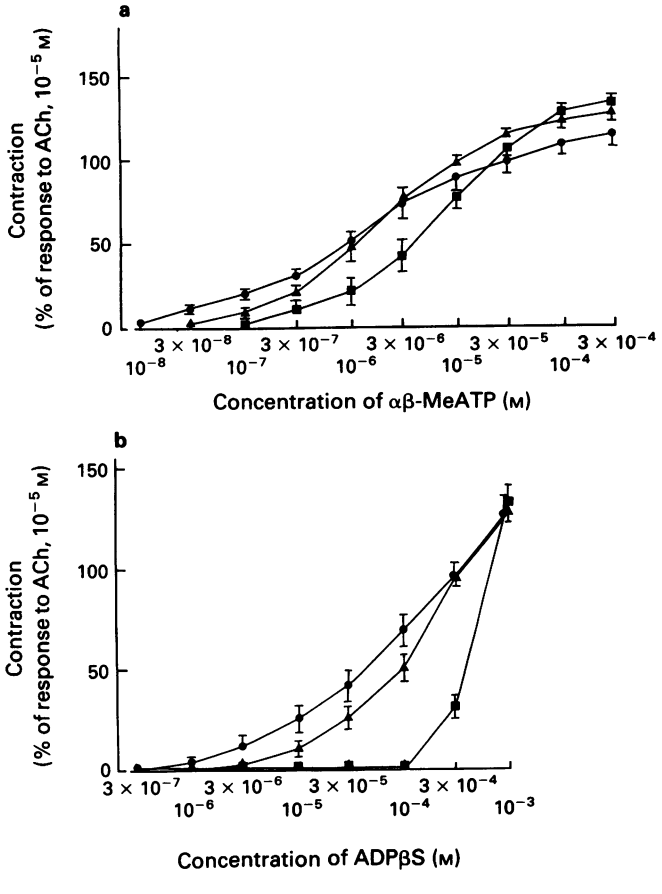


**Figure 6** Effects of cibacron blue 3GA on cumulative concentration-response curves to α,β-MeATP and ADPβS in rat urinary bladder smooth muscles. (a) Responses to α,β-MeATP in the absence of cibacron blue 3GA (●) and in the presence of $10^{-5}$ M (▲) and $10^{-4}$ M (■) cibacron blue 3GA are plotted against the concentration of α,β-MeATP (M). (b) Responses to ADPβS in the absence of cibacron blue 3GA (●) and in the presence of $10^{-5}$ M (▲) and $10^{-4}$ M (■) cibacron blue 3GA are plotted against the concentration of ADPβS (M). In (a) and (b) each point represents mean ± s.e.mean ($n = 6$), expressed as percentage of response to $10^{-5}$ M ACh.

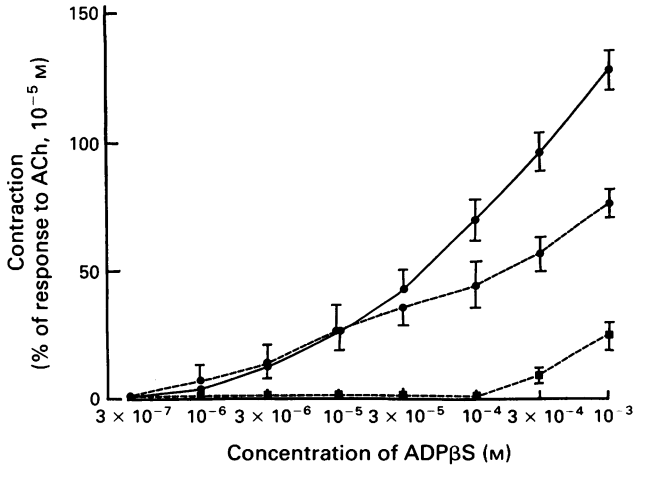


**Figure 7** Effects of cibacron blue 3GA on the cumulative concentration-response curve to ADPβS after desensitization of $P_{2x}$-purinoceptors by α,β-MeATP in rat urinary bladder smooth muscles. Responses to ADPβS in the control (●———●), those after desensitization of $P_{2x}$-purinoceptors (●----●) and those in the presence of cibacron blue 3GA ($10^{-4}$ M) after desensitization of $P_{2x}$-purinoceptors (■----■) are plotted against the concentration of ADPβS (M). Each point represents mean ± s.e.mean ($n = 6$), expressed as percentage of response to $10^{-5}$ M ACh.

### *The contraction by ADPβS and its inhibition by cibacron blue 3GA after desensitization of $P_{2x}$-purinoceptors*

Figure 7 shows the concentration-response curve to ADPβS in the control, after desensitization of $P_{2x}$-receptors by pretreatment with α,β-MeATP (10 μM) and in the presence of cibacron blue 3GA (100 μM) after desensitization of $P_{2x}$-receptors. At relatively low concentrations of ADPβS (1 to 100 μM) there was no significant change in response to the drug between α,β-MeATP-pretreated and non-treated strips. At concentrations higher than 300 μM, the contraction was significantly inhibited in α,β-MeATP-pretreated strips ($P < 0.01$). When we examined the inhibitory effect of cibacron blue 3GA (100 μM) on the contraction induced by ADPβS in α,β-MeATP-pretreated strips, as expected from previous experiments, the contractile effects of ADPβS between 3 μM and 100 μM were completely antagonized by the drug ($P < 0.05$ at 3 μM and $P < 0.01$ between 10 μM and 100 μM). Responses induced by 300 μM and 1 mM ADPβS were inhibited by 84.2% and 67.4% respectively by the drug ($P < 0.01$).

## Discussion

It is known that in smooth muscles Adn and ADP induce relaxation via $P_1$-purinoceptors and $P_{2y}$-purinoceptors, respectively (Burnstock, 1978; Burnstock & Kennedy, 1985). In the present study, both Adn and ATP induced relaxation of rat urinary bladder strips, and the relaxant effects of both drugs were antagonized by the $P_1$-purinoceptor antagonist, 8-SPT. These findings suggest that in the rat urinary bladder smooth muscle Adn and ATP induce relaxation via $P_1$-purinoceptors, probably $A_{2b}$ as reported in this tissue by Nicholls *et al.* (1992). The relaxant effects by ATP observed in this study might be due to Adn produced by hydrolysis of ATP as previously reported (Ribeiro & Lima, 1985). Actually, we did not observe any relaxant effect of a nonhydrolyzable analogue of ATP, α,β-MeATP. Since both Adn and ATP did not induce relaxation of dog bladder strips, we consider that mechanisms which induce relaxation via $P_1$- and $P_{2y}$-purinoceptors do not exist in dog urinary bladder smooth muscle.

Previous studies indicate that ATP induces contraction via $P_{2x}$-purinoceptors in rat urinary bladder, which is dependent on extracellular calcium (Iacovou *et al.*, 1988; Bhat *et al.*, 1989). The rank order of agonist potency was proposed to be α,β-MeATP > ATP > ATP, which was the same as that obtained in rat and dog urinary bladder smooth muscles in our study. Therefore, the existence of $P_{2x}$-purinoceptors was revealed not only in rat but also in dog urinary bladders.

Another important finding concerning $P_2$-purinoceptors obtained in our study was that the contractile response of rat urinary bladder smooth muscles induced by α,β-MeATP was not observed in dog. The contractile response of the rat bladder to this drug was also observed after desensitization of $P_{2x}$-purinoceptors by α,β-MeATP. These findings suggest that a subtype of $P_2$-purinoceptor other than $P_{2x}$ exists in rat bladder.

$P_{2y}$-purinoceptors in smooth muscles have been thought to mediate relaxation (Burnstock & Kennedy, 1985). However, Bailey & Hourani (1990) reported a contraction mediated by $P_{2y}$-purinoceptors. The $P_{2y}$-purinoceptors in other types of cells has been shown to mobilize intracellular calcium by activation of phospholipase C and inositol-triphosphate formation (Forsberg *et al.*, 1987; Okajima *et al.*, 1988; Boyer *et al.*, 1989). In rat cultured aortic smooth muscle cells, ATP was reported to stimulate inositol phosphate accumulation and calcium mobilization, although the type of receptor which mediated the response to ATP was not completely clarified (Phaneuf *et al.*, 1986). Therefore, it seemed possible that α,β-MeADP induced contraction of rat urinary bladder smooth muscle was via $P_{2y}$-purinoceptors. However, after desensitization of $P_{2x}$-purinoceptors, 2-MeSATP induced a contraction the magnitude of which was less than one third

of that in the control. Furthermore, the effects of cibacron blue 3GA (100 μM), a putative $P_{2y}$-selective antagonist (Burnstock & Warland, 1987) on the contraction induced by 2-MeSATP were similar to those on the contraction induced by the $P_{2x}$-purinoceptor selective agonist, α,β-MeATP (Kasakov & Burnstock, 1983). These findings indicated that a contractile response via $P_{2y}$-purinoceptors hardly existed in this tissue. Since 2-MeSATP is known to be roughly equipotent with ATP on $P_{2x}$-purinoceptors (Burnstock & Kennedy, 1985), 2-MeSATP may contract rat urinary bladder smooth muscles predominantly via $P_{2x}$-purinoceptors.

Cibacron blue 3GA at high concentrations, (100 μM) was reported to inhibit the contraction produced by ATP in guinea-pig and rat urinary bladders (Choo, 1981). This was also the case in our study. The drug antagonized the effects of α,β-MeATP at concentrations lower than 3 μM. The effects of 2-MeSATP lower than 30 μM were also antagonized by cibacron blue 3GA. However, the drug significantly enhanced the contractile response to relatively high concentrations of both α,β-MeATP and 2-MeSATP. Therefore, cibacron blue 3GA was considered not to be an effective inhibitor for $P_{2x}$-purinoceptors in the rat urinary bladder smooth muscle.

As cibacron blue 3GA did not significantly inhibit the contraction induced by ADPβS at high concentrations, the possibility of stimulating $P_{2x}$-purinoceptor by the drug was considered. In fact, the contractile responses to ADPβS at 300 μM and 1 mM were significantly reduced by desensitization of $P_{2x}$-purinoceptors, and the inhibition by cibacron blue 3GA (100 μM) became significant under these conditions. This suggested that after desensitizaton of $P_{2x}$-purinoceptors, ADPβS induced contraction via receptors which were sensitive to cibacron blue 3GA. According to the classical nomenclature of Burnstock & Kennedy (1985), these receptors in rat urinary bladder may not be $P_{2y}$-purinoceptors, since they show little sensitivity to 2-MeSATP. Further studies are needed to clarify the nature of these receptors.

In conclusion, the present study indicated that three subtypes of purinoceptors, $P_1$ (mediating relaxation), $P_{2x}$ and another type of $P_2$ (mediating contraction), exist in rat urinary bladder smooth muscles, whereas there is a single subtype of the receptor, $P_{2x}$ (mediating contraction), in dog urinary bladder. The physiological role of ATP in dog bladder is simple: ATP released from the nerve terminal induces solely contraction. In rat bladder it is rather complicated: ATP induces either contraction or relaxation. However, assuming that ATP is usually released with ACh as previously reported (Fujii, 1988), the major physiological role of ATP may be the inhibition of the contraction induced by ACh, as shown in Figure 1.

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# Evoked noradrenaline release in the rabbit ear artery: enhancement by purines, attenuation by neuropeptide Y and lack of effect of calcitonin gene-related peptide

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**1** Adenosine (30 μM) and its analogues 5'-N-ethylcarboxaminoadenosine (5 and 30 μM) and L-phenylisopropyladenosine (5 and 30 μM), potentiated the evoked but not spontaneous release of tritiated noradrenaline in the rabbit central ear artery.

**2** Prejunctional inhibition of the evoked but not spontaneous release of tritiated noradrenaline by 100 nM neuropeptide Y is greater at 2 min than at 10 min after superfusion of the peptide.

**3** Calcitonin gene-related peptide (2.63 to 263 nM) did not affect the evoked or spontaneous release of tritiated noradrenaline in this preparation.

**4** These results are discussed in terms of prejunctional modulation of sympathetic transmission in the rabbit central ear artery.

**Keywords:** Rabbit ear artery; prejunctional modulation; adenosine; sympathetic transmission; neuropeptide Y; calcitonin gene-related peptide

## Introduction

Purines are known to be inhibitors of adrenergic neurotransmission and act to inhibit the release of noradrenaline (NA) prejunctionally via $P_1$-purinoceptors activated by adenosine (ADO) following the breakdown of adenosine 5'-triphosphate (ATP) which is released with NA (Starke, 1977; Wallin, 1981; Burnstock, 1990). This phenomenon is known to occur in many blood vessels and is thought to be mediated by the $A_1$-subtype, but not $A_2$-subtype of the $P_1$-purinoceptor (see Burnstock, 1990; Rivilla *et al.*, 1992). In contrast, in the rabbit ear artery (REA) (Zhang *et al.*, 1989) and guinea-pig pulmonary artery (Wiklund *et al.*, 1989), ADO has been reported to enhance the release of NA, although the subtype of $P_1$-purinoceptor involved is not known.

Neuropeptide Y (NPY) is reported to inhibit the release of NA prejunctionally (Pernow *et al.*, 1986; Wong-Dusting & Rand, 1988), but to enhance sympathetically-mediated vasoconstriction (Glover, 1985; Daly & Hieble, 1987; Budai *et al.*, 1989; Saville *et al.*, 1990) in the REA. It has been suggested that the modulatory actions of NPY conserves sympathetic cotransmitters NA and ATP, whilst maintaining or enhancing postjunctional excitator actions (Stjärne *et al.*, 1986; Wong-Dusting & Rand, 1988).

Calcitonin gene-related peptide (CGRP) is known to inhibit the vasoconstriction elicited by electrical field stimulation (EFS) in the REA (Hanko *et al.*, 1985; Maynard *et al.*, 1990; Moritoki *et al.*, 1990). It is therefore possible that in addition to its potent postjunctional inhibitory action on NA- and ATP-induced contractions and its endothelium-independent relaxant action (Maynard *et al.*, 1990), CGRP may also be acting prejunctionally to inhibit the release of the sympathetic cotransmitter(s) NA and/or ATP as it does in the guinea-pig vas deferens (Ellis & Burnstock, 1989).

In this study we have examined the effect of ADO, a mixed $A_1/A_2$ receptor agonist, its analogues 5'-N-ethylcarboxaminoadenosine (NECA), a selective $A_2$-receptor agonist and L-phenylisopropyladenosine (L-PIA), a selective $A_1$-receptor agonist (see Burnstock *et al.*, 1984) on the spontaneous and evoked release of tritiated noradrenaline ([$^3$H]-NA) from the REA in order to try to identify the excitatory, prejunctional $P_1$-purinoceptor subclass involved. We compare these findings with those previously presented (Maynard & Burnstock, 1989) on the effect of CGRP on the spontaneous and evoked release of [$^3$H]-NA in the REA, in order to clarify whether it acts prejunctionally to inhibit the release of NA. In addition, the prejunctional, inhibitory action of NPY was examined after two different incubation times to investigate whether the pre- and postjunctional modulatory actions of NPY occur simultaneously. The findings are combined with previous reports to discuss the modulation of sympathetic transmission in the REA.

## Methods

Isolated REA segments were dissected out from New Zealand White male rabbits (2.5–3.0 kg) and prepared as previously described (Maynard *et al.*, 1991).

Briefly, once cleaned of excess connective and fatty tissue the 4 mm, endothelium-intact, REA segments were loaded with [$^3$H]-NA. The tissues were then mounted under an initial 0.5–1.0 g tension and superfused (1 ml min$^{-1}$) with oxygenated (95% $O_2$ and 5% $CO_2$), physiological solution (composition (mM): NaCl 133, KCl 4.7, $NaHPO_4$ 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, $CaCl_2$ 2.52 and glucose 7.8). The preparations were then stimulated twice at 6 ($S_1$) and 32 min ($S_2$), with trains of monophasic square wave pulses using 30 V, 0.3 ms, 5 Hz for 90 s by Grass SD9 stimulators.

Superfusate samples were collected before, during and after the stimulation periods. In addition, a further collection was made halfway between $S_1$ and $S_2$, and 14 min after $S_2$ to ensure that the prestimulation, spontaneous, radioactive overflow had returned to its initial level at the beginning of the experiment before the addition of a test drug or peptide, and after each stimulation period.

Superfusion times for the test substances varied in order to test the most effective time of action for each substance. CGRP was superfused for 2 min before electrical stimulation ($S_2$) which is known to be its optimal incubation time (Maynard *et al.*, 1990), whilst superfusion with NPY was

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begun 2 or 10 min before electrical stimulation ($S_2$) (Saville *et al.*, 1990). ADO was superfused for 20 min before electrical stimulation ($S_2$), and a pilot study showed no difference in the evoked or spontaneous release of [$^3$H]-NA between 5 min and 20 min of superfusion with ADO prior to electrical stimulation ($S_2$). Both L-PIA and NECA were superfused for 5 min; the vehicle/control containing dimethyl sulphoxide (DMSO), the solvent used for L-PIA (50 µl in 100 ml Krebs solution) was superfused for 5 min and tetrodotoxin (TTX) for 10 min prior to electrical stimulation ($S_2$).

Concentrations used for test substances were near the $EC_{50}$ value for each drug/peptide: ADO (30 µM, Kennedy & Burnstock, 1985; Zhang *et al.*, 1989); NECA and L-PIA (5, 30 µM, Burnstock *et al.*, 1984; Rivilla *et al.*, 1992); CGRP (2.63–263 nM, Hanko *et al.*, 1985; Moritoki *et al.*, 1990); NPY (100 nM, Glover, 1985; Daly & Hieble, 1987; Wong-Dusting & Rand, 1988).

Results were calculated as previously detailed (Maynard *et al.*, 1991) and analysed with Student's *t* test for paired and unpaired observations as necessary. They are expressed as mean ± s.e.mean, and a probability of 0.05 or less was considered significant.

All drugs were obtained from the Sigma Chemical Company and peptides were purchased from Cambridge Research Biochemicals. [$^3$H]-NA was purchased from New England Nuclear, West Germany.

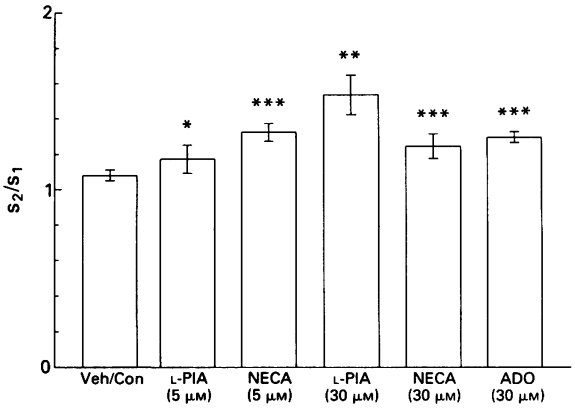


**Figure 1** Histograms showing the potentiating action of adenosine (ADO) and its analogues 5'-N-ethylcarboxaminoadenosine (NECA) and L-phenylisopropyladenosine (L-PIA) on the evoked release of [$^3$H]-noradrenaline in the rabbit central ear artery. The results were calculated as the ratio of the second stimulation ($S_2$), after superfusion of the drug or vehicle/control (Veh/Con, 50 µl dimethylsulphoxide in 100 ml Krebs solution), to the first ($S_1$) stimulation period i.e. ($S_2/S_1$), and are expressed as the mean ± s.e.mean. The level of significance was calculated with Student's unpaired *t* test. $*P<0.05$; $**P<0.01$ and $***P<0.001$. $n>4$ for each data point (see text).

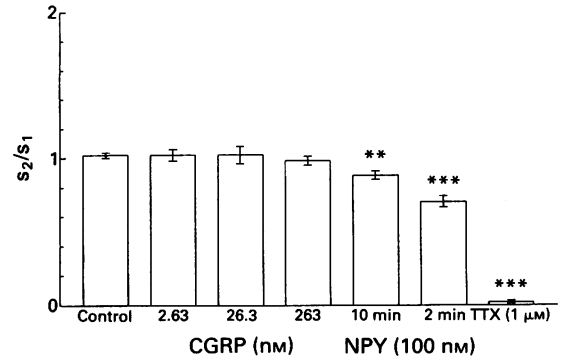


**Figure 2** Histograms showing the effect of calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY) and tetrodotoxin (TTX) on the evoked release of [$^3$H]-noradrenaline in the rabbit central ear artery. The results were calculated as the ratio of the second stimulation ($S_2$), after superfusion of the drug or no drug (Control), to the first ($S_1$) stimulation period i.e. ($S_2/S_1$), and are expressed as the mean ± s.e.mean. The level of significance was calculated with Student's unpaired *t* test. $**P<0.01$ and $***P<0.001$. $n>4$ for each data point (see text).

## Results

Throughout all experiments the spontaneous overflow of [$^3$H]-NA was not significantly different before or 14 min after the $S_1$ and $S_2$ periods, i.e. none of the peptides or drugs investigated affected the spontaneous overflow of [$^3$H]-NA, and the background levels returned to normal after the evoked-release stimulation periods ($S_1$ and $S_2$).

In control ($n = 10$) (no drug or peptide in superfusate) and vehicle/control ($n = 4$) experiments the evoked release of [$^3$H]-NA was stable such that the $S_2/S_1$ ratio was not significantly different from 1.0 (Figures 1 and 2). Tetrodotoxin (TTX, 1 µM) blocked the evoked-release of [$^3$H]-NA ($n = 6$), indicating that the parameters of stimulation were neurogenic (Figure 2).

ADO (30 µM, $n = 6$) and its analogues L-PIA (5 µM, $n = 4$; 30 µM, $n = 6$) and NECA (5 µM, $n = 6$; 30 µM, $n = 5$) significantly potentiated the evoked-release of [$^3$H]-NA such that the $S_2/S_1$ ratio was increased (Figure 1). L-PIA, but not NECA, exhibited a concentration-dependent enhancement of the evoked release of [$^3$H]-NA.

After 10 min of superfusion ($n = 11$), NPY (100 nM) significantly ($P<0.01$) inhibited the evoked-release of [$^3$H]-NA. This inhibition was more pronounced ($P<0.001$) after 2 min superfusion ($n = 6$) (Figure 2).

CGRP (2.63 nM, $n = 4$; 26.3 nM, $n = 4$; 263 nM, $n = 6$) had no effect on the evoked-release of [$^3$H]-NA (Figure 2). Experiments using superfusion times of 30 s to 5 min showed no difference in the effect of 263 nM CGRP on the evoked-release of [$^3$H]-NA. Longer superfusion times were not examined as the action of CGRP on neurogenic contractions is reversed after 5 min (Maynard *et al.*, 1990).

## Discussion

In most tissues, the prejunctional $P_1$-purinoceptor has been shown to be of the $A_1$ subtype (see Burnstock, 1990), and L-PIA is known to be a more potent $A_1$-receptor agonist than is NECA (Londos *et al.*, 1980; Snyder, 1985; Rivilla *et al.*, 1990). In light of these reports, our results suggest the likelihood of the $A_1$-subtype of excitatory, prejunctional $P_1$-purinoceptor in the REA. This conclusion is based on the observations that L-PIA, but not NECA, potentiated the evoked release of [$^3$H]-NA in a concentration-dependent manner. The presence of $A_2$-subtype receptors in the REA, however, cannot be ruled out since NECA also significantly enhanced the evoked release of [$^3$H]-NA, albeit not in a concentration-dependent manner. NECA is also known to act at both $A_1$- and $A_2$-receptor subtypes (Nicholls *et al.*, 1992). Experiments using recently available selective $P_1$-purinoceptor agonists and antagonists (Nicholls *et al.*, 1992) are necessary for further clarification.

Although we have provided additional pharmacological evidence that prejunctional $P_1$-purinoceptor agonists augment the release of NA, it has been reported that ADO, inhibits neurogenic contractile responses in the REA (Zhang *et al.*, 1989). It is likely, however, that the potent postjunctional, endothelium-independent relaxant action of ADO (usually mediated by the $A_2$-subtype of $P_1$-purinoceptor in the REA (Kennedy & Burnstock, 1985; see also Burnstock, 1990)) might overcome the prejunctional enhancement of sympathetic transmitter(s) released. This would explain the reduced neurogenic contractions observed in the REA in the presence of ADO (Zhang *et al.*, 1989).

Both inhibitory prejunctional and augmenting postjunctional modulatory actions of NPY on sympathetic transmission exist in the REA (Glover, 1985; Daly & Hieble, 1987; Potter, 1988; Wong-Dusting & Rand, 1988; Budai *et al.*, 1989; Gustafsson & Nilsson, 1990; Saville *et al.*, 1990; this study). The present results in combination with data from previous studies, puts these observations in a chronological framework. The inhibitory action of NPY on the release of [$^3$H]-NA was significantly more effective at the earlier superfusion time of 2 than at 10 min in these experiments.

In contrast, we and others have shown that the time of maximum potentiation of the sympathetic postjunctional response by NPY observed in this preparation occurs after 5–10 min of incubation and remains enhanced in the presence of exogenous NPY for 20–30 min (Glover, 1985; Budai *et al.*, 1989; Saville *et al.*, 1990). This critical time difference might explain why earlier studies demonstrated only the enhancing post-, but not the inhibitory prejunctional action of NPY in other preparations (Edvinsson & Skarby, 1984; Ekblad *et al.*, 1984; Zukowska-Grojec *et al.*, 1986), resulting in the suggestion that it is difficult to demonstrate both pre- and postjunctional actions of NPY in a given sympathetically-innervated tissue (Wahlestedt *et al.*, 1990). Since in this study, NPY was applied exogenously, it is reasonable to assume that NPY encountered both pre- and postjunctional receptors simultaneously. Consequently, the time difference between the maximum inhibitory, prejunctional and enhancing postjunctional actions may have a physiological basis. Whether the explanation is due to: (a) heterogeneity of NPY receptors and their actions (e.g. $Y_2$-receptors can be found both pre- and postjunctionally in vascular smooth muscle, and it is possible that these may desensitize faster than $Y_1$ postjunctional receptors); (b) interactions amongst $\alpha_1$- and $\alpha_2$-adrenoceptors and $Y_1$ and $Y_2$ receptors during 'cross-talk' or reciprocal regulation, and (c) differences between and/or regulation at the second-messenger level as yet unknown (Burnstock, 1990; Wahlestedt *et al.*, 1990).

The present results also show that CGRP does not act prejunctionally to modulate the release of NA in the REA, even though, like ADO (Zhang *et al.*, 1989), it causes a significant reduction in sympathetically-mediated contractions (Maynard *et al.*, 1990). This strongly suggests, therefore, that like ADO, the attenuation of sympathetically-mediated contractions by CGRP is likely to be due to its potent endothelium-independent relaxant action. Further experiments need to be conducted to elucidate whether CGRP might affect the prejunctional release of ATP in the REA, since both NA and ATP act as cotransmitters in this preparation (Suzuki, 1985; Benham *et al.*, 1987; Saville & Burnstock, 1988). In addition, it has been shown that CGRP may inhibit prejunctionally the release of ATP, but not NA in the guinea-pig vas deferens (Ellis & Burnstock, 1989).

Recently, high levels of receptor binding sites have been reported for somatostatin and substance P (SP), but not for CGRP in the rabbit superior cervical ganglion, which provides the REA with its main sympathetic supply (Manth *et al.*, 1992). These anatomical findings therefore corroborate the pharmacological data that somatostatin (Maynard *et al.*, 1991) and SP (Illes & van Falkenhausen, 1986), but not CGRP (this study) have inhibitory prejunctional actions on the release of NA in the REA.

In conclusion, we have shown that ADO and its analogues, probably via $A_1$-subtype, prejunctional, $P_1$-purinoceptors augment the release of NA in the REA. In contrast, all the peptides localized in perivascular nerves of the REA to date (i.e. NPY (Saville *et al.*, 1990); SP, CGRP (Maynard *et al.*, 1990), and somatostatin (Maynard *et al.*, 1991)), with the exception of CGRP, which has no effect, inhibit the prejunctional release of NA in the REA. In addition, we propose that NPY might initially exert its prejunctional, inhibitory action, before its postjunctional, augmenting action on sympathetically-mediated contractile responses. We also postulate that ADO and CGRP inhibit sympathetically-mediated contractions not by inhibiting the release of NA prejunctionally, but through their potent endothelium-independent relaxant actions.

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# Role of endopeptidase 3.4.24.16 in the catabolism of neurotensin, *in vivo*, in the vascularly perfused dog ileum

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**1** The degradation of tritiated and unlabelled neurotensin (NT) following close intra-arterial infusion of the peptides in ileal segments of anaesthetized dogs was examined.

**2** Intact NT and its catabolites recovered in the venous effluents were purified by chromatography on Sep-Pak columns followed by reverse-phase h.p.l.c. and identified by their retention times or by radioimmunoassay.

**3** The half-life of neurotensin was estimated to be between 2 and 6 min. Four labelled catabolites, corresponding to free tyrosine, neurotensin (1–8), neurotensin (1–10) and neurotensin (1–11), were detected.

**4** Neurotensin (1–11) was mainly generated by a phosphoramidon-sensitive cleavage, probably elicited by endopeptidase 24–11.

**5** Two endopeptidase 3.4.24.16 inhibitors, phosphodiepryl 03 and the dipeptide Pro-Ile, dose-dependently potentiated the recovery of intact neurotensin. Furthermore, both agents inhibited the formation of neurotensin (1–10), the product that results from the hydrolysis of neurotensin by purified endopeptidase 3.4.24.16. In contrast, the endopeptidase 3.4.24.15 inhibitor Cpp-AAY-pAB neither protected neurotensin from degradation nor modifed the production of neurotensin (1–10).

**6** Our study is the first evidence to indicate that endopeptidase 3.4.24.16 contributes to the catabolism of neurotensin, *in vivo*, in the dog intestine.

**Keywords:** Neurotensin; degradation; dog ileum; endopeptidase 24.15; endopeptidase 24.16; peptidases; phosphodiepryl 03; Pro-Ile

## Introduction

Neurotensin (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) functions as a paracrine regulator of intestinal motility. This statement is supported by the presence of neurotensin-like immunoreactivity (NT-LI) in mucosal N cells (Orci *et al.*, 1976) from which the peptide can be released upon various humoral or luminal stimuli (Mashford *et al.*, 1978; Hammer *et al.*, 1982). Furthermore, neurotensin receptor sites have been described in smooth muscle tissues and in enteric nerve synaptosomes of intestine from various species (Kitabgi & Freychet, 1979; Goedert *et al.*, 1984; Ahmad *et al.*, 1987; Ahmad & Daniel, 1991). These binding sites probably modulate the contractility of several gastrointestinal tissue preparations *in vitro* and *in vivo* (for review see Kitabgi, 1982). Thus, in an *ex vivo* model of vascularly perfused ileum of the dog, Sakai *et al.* (1984) demonstrated that intra-arterial administration of neurotensin inhibited the field-stimulated, atropine-sensitive contractile response of the ileum by both neural and myogenic actions.

We have established the distribution of various exo- and endopeptidases in purified membranes from muscular, nervous and mucosal origin (Checler *et al.*, 1987; Barelli *et al.*, 1989; 1993a), prepared from dog ileum. In agreement with the concept of a local regulation that implies a short term action, we have demonstrated that the various layers of the intestinal wall contain peptidases that efficiently catabolize neurotensin. In particular, our *in vitro* studies clearly demonstrated that endopeptidase 3.4.24.16 mainly contributed to neurotensin degradation in these muscular and nervous layers (Checler *et al.*, 1987; Barelli *et al.*, 1989), as previously shown in membrane preparations and cells from central origin (Checler *et al.*, 1988a). However, the assessment of the involvement of endopeptidase 24.16 in the physiological inactivation of neurotensin has awaited the development of selective inhibitors of the enzyme. We have previously described a dipeptide, Pro-Ile (Dauch *et al.*, 1991) and more recently a phosphonamide peptide (Barelli *et al.*, 1992) that behave as potent and selective inhibitors of purified endopeptidase 3.4.24.16. In the present study, we have used these novel tools to examine the contribution of endopeptidase 3.4.24.16 to the inactivation of neurotensin, *in vivo*, in the dog ileum.

## Methods

### *Canine isolated perfused ileum*

All procedures were approved by the McMaster Animal Care Committee. Ileal segments were prepared as previously described (Manaka *et al.*, 1989). Briefly, fasted mongrel dogs of either sex (weighing 15–30 kg) were anaesthetized with α-chlorasole (50 mg $kg^{-1}$; Sigma) and urethane (500 mg $kg^{-1}$; Sigma). Ventilation was set up at approximately 15 strokes $min^{-1}$ with a Harvard animal ventilator pump. The femoral artery and vein were cannulated for monitoring blood pressure and for administration of sodium pentobarbitone (25–50 mg) in order to maintain anaesthesia throughout the experiment. The abdominal cavity was opened with a midline incision and an ileal segment (about 15–20 cm long and 15–35 g weight) was prepared. The largest vein and artery

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irrigating a single ileal segment were cannulated and the venous cannula left open to drain. The arterial cannula was perfused at a flow rate of 12 ml $min^{-1}$ with a peristaltic pump (Harvard apparatus) with Krebs-Ringer bicarbonate solution (warmed to 37°C and gassed with 95% $O_2$, 5% $CO_2$) containing (mM): NaCl 137.4, $MgSO_4$ 1.2, $NaHPO_4$ 1.2, $NaHCO_3$ 21.1, $CaCl_2$ 2.5 and glucose 5.5 The ileal segment was then ligated and the collateral circulation tied off. Five to seven segments, separated from each other by at least one complete vascular arcade, were sequentially prepared for each dog.

### *Experimental procedure*

An equilibration period preceded the start of each experiment until the venous effluent became free of haematies by inspection. Dog ileum segments were pretreated for 10 min by perfusion of Krebs-Ringer bicarbonate buffer either without (control) or with freshly prepared solutions of peptidase inhibitors before neurotensin injection. At zero time, a mixture of tritiated ($10^6$ c.p.m., 107 Ci $mmol^{-1}$) and unlabelled (10 nmol) neurotensin was injected in 1 ml. After 20 s, the peristaltic pump was stopped for 2 min in order to facilitate neurotensin diffusion inside ileal tissues, then the perfusion was continued for a period of 8 min (from injection). Perfusates corresponding to 2 min time intervals were collected.

Venous effluents were passed through ODS-Silica cartridges (C18 Sep-pak, Millipore) that had been previously activated by several washes with acetonitrile (2 × 5 ml) then with 4 × 5 ml of 0.1% (v/v) trifluoroacetic acid (TFA), 0.05% (v/v) triethylamine (TEA) in water. After loading of samples, resin was washed with 0.1% (v/v) TFA, 0.05% (v/v) TEA in water, then peptides were eluted with 4 ml of 80% acetonitrile containing 0.1% (v/v) TFA, 0.05% (v/v) TEA. More than 90% of the radioactivity applied was recovered after the various step-elutions. Eluates were lyophilized and reconstituted in 2 ml of water containing 0.02% bovine serum albumen. Aliquots containing about 6000 c.p.m. were submitted to reverse-phase h.p.l.c.

### *Reverse-phase h.p.l.c. analysis*

The h.p.l.c. procedure has been described previously (Checler *et al.*, 1988a). Briefly, aliquots of reconstituted samples were loaded onto a RP18 lichrosorb column (Merck, France) and eluted at room temperature. Elutions were performed at a flow rate of 1 ml $min^{-1}$ and absorbing material was detected at 230 nm. Intact neurotensin and its catabolites were separated by means of a 42 min linear gradient of 0.1% TFA, 0.05% TEA in water/0.1% TFA, 0.05% TEA in acetonitrile from 90:10 (v/v) to 60:40 (v/v) and 500 µl fractions were collected. Radioactive products were identified by their retention times as compared with synthetic standards run in the same conditions and neurotensin-like immunoreactivity contents were estimated by radioimmunoassay.

### *Radioimmunoassays*

Neurotensin-like immunoreactivity (NT-LI) was determined with an antiserum directed towards the C-terminal end of NT (antiserum 29G) (Cuber *et al.*, 1990). This polyclonal antiserum does not cross-react with neurotensin (1–12) or neurotensin (9–13) (<0.1%) and displays a detection limit and an $EC_{50}$ value of 0.5 and 5 fmol of neurotensin/tube, respectively. Radioimmunoassays were carried out at 4°C in a final volume of 500 µl in phosphate-buffered saline (50 mM $Na_2HPO_4$ and 140 mM NaCl, pH 7.5) containing 0.1% gelatin, 6000–7000 c.p.m. of $^{125}$I-labelled neurotensin (Sadoul *et al.*, 1984) and various concentrations of unlabelled competitor (synthetic neurotensin) or diluted samples (De Nadai *et al.*, 1989). Incubations with a final dilution of antiserum 1:50,000 were allowed to reach equilibrium for 24 h, then separation of bound and free peptide was classically performed by charcoal precipitation.

### *Peptides and drugs*

Synthetic NT was purchased from Neosystem (Strasbourg, France). Tritiated neurotensin [3,11-tyrosyl-3-5-$^3$H(N)] (107 Ci $mmol^{-1}$) was obtained from New England Nuclear (France). Bestatin ((2s,3r)-3 amino 2-hydroxy-4-phenyl-butanoyl leucine), phosphoramidon (N-(α-rhamno-pyranosyloxy-hydroxyphosphynyl)-leucyl-tryptophan) and prolyl-isoleucine were from Sigma Chemicals. Captopril (2-D-methyl-3-mercaptopropanoyl-L-proline) was from the Squibb Institute (Princeton, U.S.A.). CPP-AAY-pAB (N-(1(**R**,**S**)-carboxyl-3-phenyl-propyl)-alanyl-alanyl-tyrosyl-pAB) was kindly given by Dr M. Orlowski (Mount Sinai School of Medicine, New York, U.S.A.). Phosphodiepryl 03 (N-(phenylethylphosphonyl)-glycyl-prolyl-hexanoic acid) was synthesized as described by Dive *et al.* (1990).

## Results

Data presented in Table 1 indicated that 83.6 ± 10.1% of the infused radioactivity was recovered under control conditions. The distribution of the label within the three time-intervals indicated that most of the radioactivity (84.4 ± 1% of total radioactivity recovered) appeared in the 0–4 min interval while the radioactivity recovered in the two following periods (4–6 min and 6–8 min) represented 9.3 ± 1.3 and 6.3 ± 0.4 of total radioactivity, respectively (Table 1). It was noteworthy that pretreatment of ileal segments with various specific peptidase inhibitors neither influence the recovery of radioactivity nor affected the distribution of the label within the sequential time intervals (Table 1).

### *Degradation rate of neurotensin in dog ileum*

The perfusion of tritiated and unlabelled neurotensin in dog ileum led to a linear decrease in the recovery of intact peptide until the 4–6 min time interval. Then, the percentage of native neurotensin remained constant, corresponding to about 30% of intact tritiated peptide (Figure 1). The same values were obtained when recovery of unlabelled neurotensin was estimated by radioimmunoassay (not shown). This plateau value probably reflected a steady state between the rate of peptide diffusion within the ileal tissues and the degradation process taking place inside these tissues. Taking into account these considerations, it was estimated that the half-life of neurotensin in this *ex vivo* model was between 2 and 6 min.

### *Catabolites of neurotensin in dog ileum*

We examined the nature of the tritiated catabolites recovered in the 4–6 min time interval. H.p.l.c. analysis of the fraction corresponding to the Sep-Pak washout (see Methods) indicated that all the radioactivity eluted with the retention time of free tritiated tyrosine (not shown). Accordingly, this fraction did not display any neurotensin-like immunoreactivity. Radioactivity recovered by elution of the Sep-Pak columns with acetonitrile indicated five major radiolabelled products following h.p.l.c. analysis (Figure 2a). Three of these eluted with the retention times of neurotensin(1–8), neurotensin (1–10) and neurotensin(1–11). The main radioactive product exhibited the retention time of standard neurotensin and probably accounted for the remaining intact peptide since it corresponded to immunoreactive material (Figure 2b). In contrast, other peaks did not display neurotensin-like immunoreactivity, thereby confirming that the neurotensin antiserum used in the present study did not cross-react with any of the neurotensin catabolites (Figure 2b). Finally, the small

**Table 1** Effect of various peptidase inhibitors on the recovery and distribution of radioactivity during dog ileum perfusion with tritiated neurotensin

| *Pretreatment* | *Recovery* | *Distribution of radioactivity recovered* 0–4 min | 4–6 min | 6–8 min |
|---|---|---|---|---|
| Control | 83.6 ± 10.1 | 84.4 ± 1.0 | 9.3 ± 1.3 | 6.3 ± 0.4 |
| Bestatin | 90.3 ± 9.7 | 86.7 ± 1.8 | 8.2 ± 0.7 | 5.2 ± 1.1 |
| Captopril | 86.2 ± 2.0 | 84.6 ± 3.0 | 9.9 ± 2.0 | 5.5 ± 1.0 |
| Phosphoramidon | 96.3 ± 0.9 | 89.7 ± 0.3 | 7.5 ± 0.6 | 2.8 ± 0.9 |
| Pro-Ile | 91.4 ± 0.4 | 86.0 ± 2.7 | 8.9 ± 2.5 | 5.0 ± 0.2 |
| Cpp-AAY-pAB | 86.2 ± 1.3 | 82.0 ± 0.6 | 11.5 ± 0.1 | 6.4 ± 0.6 |
| P03 | 87.1 ± 0.7 | 88.3 ± 4.8 | 8.1 ± 3.6 | 3.6 ± 1.0 |

Perfused dog ileum segments were pretreated for 10 min in the absence (control) or in the presence of the indicated peptidase inhibitor. At zero time, tritiated NT ($10^6$ c.p.m., 107 Ci $mmol^{-1}$) was injected and perfusion was continued for a total period of 8 min. Samples collected during the 0–4, 4–6 and 6–8 min time-intervals were counted. Recovery corresponds to the total radioactivity recovered expressed as the percentage of initial radioactivity infused. The distribution of radioactivity between the various time-intervals is expressed as the percentage of total radioactivity recovered. Values are the mean ± s.e.mean of three to six independent experiments carried out with three independent dogs. Peptidase inhibitors were perfused at the following final concentrations: bestatin, 10 μM; captopril, 1 μM; phosphoramidon, 1 μM; Pro-Ile, 10 mM; Cpp-AAY-pAB, 2 μM; P03 (phosphodiepryl 03) 0.5 μM.

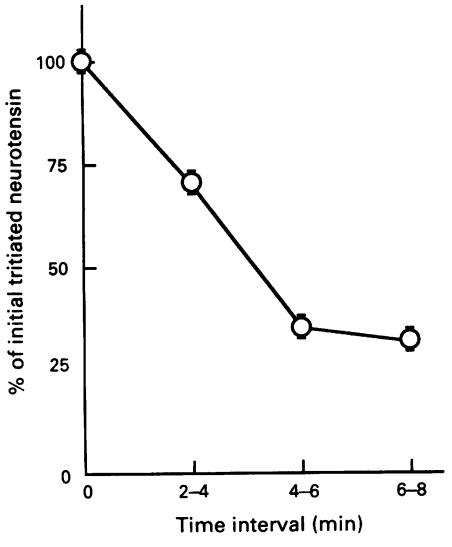


**Figure 1** Kinetics of disappearance of tritiated neurotensin (NT) after infusion in dog ileum. Dog ileum segments were pretreated for 10 min by perfusion with Krebs-Ringer-bicarbonate buffer. Tritiated neurotensin ($10^6$ c.p.m., 107 Ci $mmol^{-1}$) was then infused in a 1 ml bolus. Labelled catabolites and intact tritiated neurotensin recovered in the venous effluent during the three time-intervals were loaded on Sep-Pak cartridges. Samples corresponding to the acetonitrile step elution were lyophilysed, reconstituted in water and an aliquot (about 6000 c.p.m.) was applied to reverse-phase h.p.l.c. as described in Methods. Labelled peptides were identified by comparison of their retention times with those of synthetic standards run in the same conditions. Values represented the radioactivity corresponding to intact tritiated NT recovered after h.p.l.c. analysis and are expressed as the percentage of total radioactivity recovered in the two Sep-Pak elution steps. Values are the mean ± s.e.mean of four separate experiments carried out with four dogs.

amount of radioactivity that behaved as free labelled tyrosine could reflect a small proportion of tyrosine that would have resisted the washing step of the Sep-Pak procedure.

It was noteworthy that the nature of the catabolites generated during the various time intervals appeared identical although they were recovered in lesser amounts (Table 2) in the 0–4 min interval. By contrast, a quantitatively similar production of the different catabolites was observed between 4–6 and 6–8 min. This indicated that the plateau value observed for intact neurotensin recovery (Figure 1) was accompanied by a steady-state level of the catabolites formation (Table 2).

### *Effect of peptidase inhibitors on the recovery of intact neurotensin and on the neurotensin (1–10) formation*

We recently described a potent phosphonamide peptide inhibitor, phosphodiepryl 03, that displayed a $K_i$ value of about 1 nM towards endopeptidase 3.4.24.16 (Barelli *et al.*, 1992). This agent elicited a dose-dependent increase of the neurotensin-like immunoreactivity recovered in the venous effluent (Figure 3). This protecting effect of phosphodiepryl 03 on neurotensin was accompanied by a concomitant decrease of the production of tritiated neurotensin (1–10) estimated after h.p.l.c. (Figure 3). This inhibitor appeared to be without effect on the formation of the other neurotensin catabolites (not shown).

It was crucial to examine the effect of the endopeptidase 3.4.24.15 inhibitor, Cpp-AAY-pAB since we have reported that phosphodiepryl 03 not only blocks endopeptidase 3.4.24.16, but also inhibits endopeptidase 3.4.24.15 although with a lower potency ($K_i$ = 7.5 nM) (Barelli *et al.*, 1992). Figure 3 clearly shows that Cpp-AAY-pAB neither modified neurotensin recovery nor affected neurotensin (1–10) production. Altogether, these data strengthen the hypothesis that the protective effect of phosphodiepryl 03 on neurotensin catabolism is mediated via the inhibition of endopeptidase 3.4. 24.16. Such a conclusion was further confirmed by the examination of the effect of Pro-Ile, a dipeptide which we have previously reported is a selective blocker of endopeptidase 3.4.24.16 (Dauch *et al.*, 1991). Figure 4 indicates that Pro-Ile elicited a drastic increase in the recovery of NT-LI, together with an important decrease in neurotensin (1–10) formation. These data confirm the involvement of endopeptidase 24.16 in the catabolism of neurotensin, *in vivo*, in dog ileum. It should be noted that 10 μM phosphoramidon, a potent and specific inhibitor of endopeptidase 24.11 (Suda *et al.*, 1973), also induced a protection of NT-LI and slowed down the formation of neurotensin(1–10) (Figure 4) and neurotensin(1–11) (data not shown). This was in keeping with our previous data showing that thiorphan (another endopeptidase 24.11 inhibitor, Roques *et al.*, 1980) led to the partial protection of neurotensin in dog ileum (Checler *et al.*, 1988b). Finally, the aminopeptidase M inhibitor, bestatin (Umezawa *et al.*, 1976) slightly affected neurotensin recovery and neurotensin(1–10) formation (Figure 4). This effect could probably be explained by the slight inhibition (12% at 10 μM) elicited by this inhibitor on purified endopeptidase 3.4.24.16 (Barelli *et al.*, 1988b).

Finally, the angiotensin-converting enzyme inhibitor captopril (10 μM) did not modify neurotensin recovery and neurotensin(1–10) production (Figure 4). This is in agreement with our previous work showing that angiotensin-converting enzyme does not contribute to the primary catabolism of neurotensin, in dog ileum (Checler *et al.*, 1988b).

## Discussion

In this study, we have shown that neurotensin is rapidly catabolized in a vascularly perfused isolated ileal segment of anaesthetized dog with an estimated half-life between 2 and 6 min. This agrees with the very short-life of the peptide (about 1 min) after intravenous bolus administration in rat and man (for review see Checler, 1991). It is noteworthy that the experimental procedure that included the flushing of the intestinal segment prior to administration of neurotensin precluded the possibility that plasma or blood cell factors could contribute to peptide degradation.

The major N-terminal degradation products of neurotensin generated during the transit of the peptide across intestinal tissues corresponded to neurotensin(1–8), neurotensin(1–10) and neurotensin(1–11). The formation of these products increased and reached an apparent plateau value at the 4–6 min and 6–8 min time intervals. This probably reflected a resistance of these products to secondary cleavages. This was not the case for their C-terminal counterparts neurotensin(9–13) and neurotensin(11–13) that were recovered in much lower amounts. This explained the recovery of free tyrosine that probably corresponded to the production of the tyrosine 11 resulting from the proteolytic subsequent breakdown of neurotensin(11–13) and neurotensin(9–13).

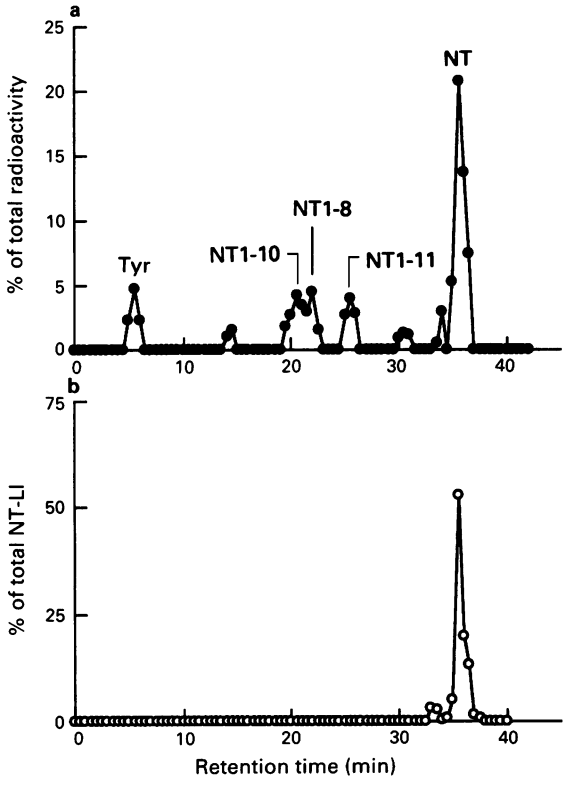


**Figure 2** High performance liquid chromatography (h.p.l.c.) analysis of neurotensin catabolites recovered in 4–6 min time-interval of the venous effluent. Dog ileum segments were pretreated for 10 min by perfusion with Krebs-Ringer bicarbonate buffer. Tritiated neurotensin ($10^6$ c.p.m., 107 Ci mmol$^{-1}$) and unlabelled neurotensin (10 nmol) were then infused in a 1 ml bolus. Peptides recovered in the venous effluent were extracted on Sep-Pak cartridges, lyophilyzed and reconstituted in 2 ml of water as described in Methods. An aliquot (about 6000 c.p.m.) of the sample corresponding to the acetonitrile elution step was applied to reverse-phase h.p.l.c. and eluted in the conditions described in Methods. Fractions corresponding to 0.5 min (500 µl) were collected, counted and tested for their content in neurotensin-LI as described in Methods. Labelled peptides (a) were identified by comparison of their retention times with those of synthetic standards run under the same conditions. The radioactivity (a) and NT-LI (b) contents are expressed as the percentage of total radioactivity or total NT-LI recovered after h.p.l.c. analysis.

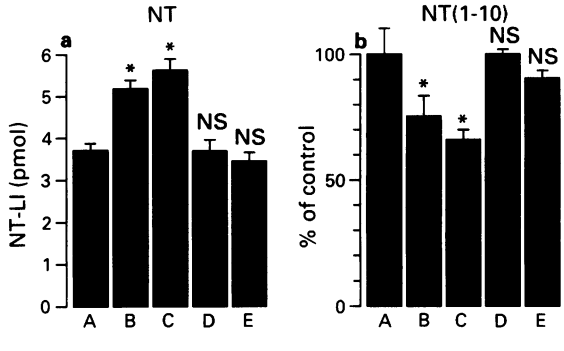


**Figure 3** Effect of phosphodiepryl 03 and Cpp-AAY-pAB on neurotensin recovery and neurotensin (1–10) formation. Dog ileum segments were pretreated for 10 min by perfusion with Krebs-Ringer-bicarbonate buffer in the absence (A) or in the presence of phosphodiepryl 03 0.05 µM (B) or 0.5 µM (C) or with Cpp-AAY-pAB 0.5 µM (D) or 2 µM (E). At zero time, tritiated neurotensin ($10^6$ c.p.m., 107 Ci mmol$^{-1}$) and unlabelled neurotensin (10 nmol) were injected in 1 ml, then the perfusion was continued for a total period (from injection) of 8 min and 2 min-samples were collected. Samples were loaded on Sep-Pak cartridges and the peptides eluted in the acetonitrile step were lyophilyzed, reconstituted in 2 ml of water and an aliquot was h.p.l.c. analyzed as described in Methods. All collected fractions were counted and tested for their content of neurotensin-LI. Values are expressed as pmol of neurotensin-like immunoreactivity (a) or correspond to tritiated neurotensin (1–10) formation (b) expressed as the percentage of neurotensin(1–10) recovered in absence of inhibitor (control). Values are the means ± s.e.mean of ten determinations derived from three independent experiments carried out on three dogs. Asterisks indicate the statistical significance determined by Student's unpaired *t* test of inhibitory versus control conditions: $*P<0.0001$. NS = non-significant.

**Table 2** Identification and quantification of the tritiated catabolites recovered after perfusion of tritiated neurotensin in dog ileal segments

| *Time interval* (min) | *Radioactivity recovered in the Sep-Pak washout* | *Radioactivity recovered in the Sep-Pak acetonitrile step* | | | | | |
|---|---|---|---|---|---|---|---|
| | *Tyr* | *NT(1–8)* | *NT(1–10)* | *NT(1–11)* | *NT(11–13)* | *NT(9–13)* | *NT* |
| 0–4 | 18.0 ± 6.2 | 2.7 ± 0.3 | 2.2 ± 0.3 | 2.7 ± 0.7 | 2.3 ± 0.5 | 2.1 ± 0.4 | 70.0 ± 1.7 |
| 4–6 | 37.4 ± 6.5 | 8.6 ± 1.6 | 7.2 ± 1.5 | 7.4 ± 2.0 | 1.6 ± 0.4 | 2.5 ± 0.8 | 35.3 ± 3.3 |
| 6–8 | 37.3 ± 4.3 | 11.0 ± 1.8 | 7.4 ± 1.2 | 8.9 ± 1.3 | 1.3 ± 0.6 | 1.9 ± 0.1 | 32.2 ± 1.8 |

Dog ileum segments were pretreated for 10 min with Krebs-Ringer Bicarbonate buffer. Tritiated NT ($10^6$ c.p.m., 107 Ci mmol$^{-1}$) was infused as a 1 ml bolus and the labelled products recovered in the various time-intervals were submitted to the Sep-Pak extraction procedure described in the Methods. Samples recovered in the washout and acetonitrile steps were lyophilyzed, reconstituted in water and aliquots were h.p.l.c. analyzed as described. Neurotensin catabolites were identified by comparison of their retention times with those of synthetic standards run in the same conditions. The content of radioactivity under each peak was expressed as the percentage of total radioactivity recovered by the Sep-Pak extraction procedure. Values are the means ± s.e.mean of four independent experiments performed with four dogs.

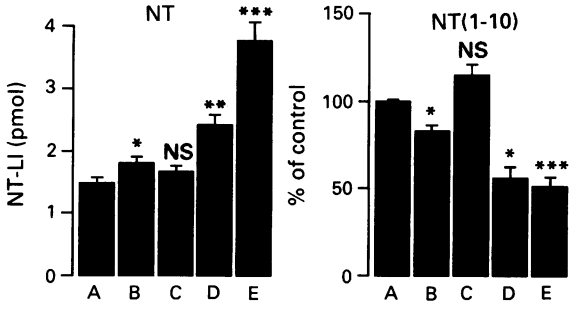


**Figure 4** Effect of specific peptidase inhibitors on the degradation of neurotensin in perfused ileum segment of dog. Dog ileum segments were pretreated for 10 min by perfusion with Krebs-Ringer-bicarbonate buffer in the absence (control conditions) or in the presence of various peptidase inhibitors. At zero time, tritiated neurotensin ($10^6$ c.p.m., 107 Ci mmol$^{-1}$) and unlabelled neurotensin (10 nmol) were injected in a 1 ml bolus then the perfusion was continued for a total period (from injection) of 8 min and 2 min-samples were collected. Samples were loaded on Sep-Pak cartridges, and the peptides recovered in the acetonitrile step were lyophilyzed, reconstituted in 2 ml of water and an aliquot was h.p.l.c. analyzed as described in Methods. Labelled peptides were identified by their retention time as compared with those of synthetic standards run under the same conditions. Values are the mean ± s.e.mean of six determinations (three independent experiments carried out on three dogs). Asterisks indicate the statistical significance determined by Student's unpaired $t$ test of inhibitory versus control conditions: $*P<0.01$, $**P<0.005$, $***P<0.001$. Peptidase inhibitors were used at the following final concentrations in the perfusion buffer: control without inhibitor (A); bestatin, 10 μM (B); captopril, 1 μM (C); phosphoramidon, 1 μM (D); Pro-Ile, 10 mM (E).

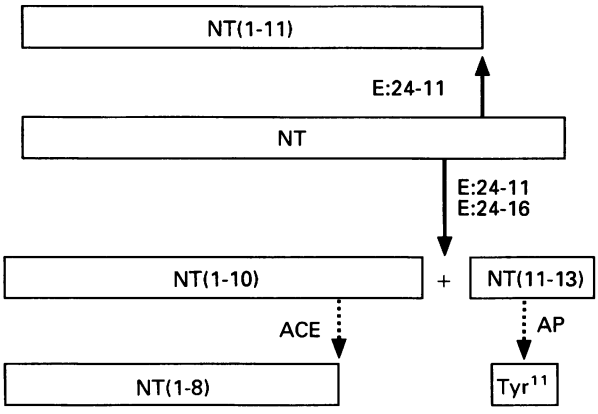


**Figure 5** Model of neurotensin catabolism in vascularly perfused ileum segments of dog. Full arrows indicate primary cleavages taking place on the neurotensin molecule (present study) and dashed arrows indicate secondary cleavages occurring on the neurotensin catabolites (our previous study Checler *et al.*, 1988b). Neurotensin (NT): pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu E:24.11, endopeptidase 3.4.24.11; E:24.16, endopeptidase 3.4.24.16; ACE, angiotensin-converting enzyme; AP, aminopeptidases.

We previously showed that endopeptidase 24.11 is responsible for neurotensin(1-11) formation and also contributes to neurotensin(1-10) production (Checler *et al.*, 1988b) in dog ileum. In agreement with these studies, the present data indicate that the endopeptidase 24.11 inhibitor phosphoramidon fully blocked neurotensin(1-11) formation and partly inhibited neurotensin(1-10) production. However, there is an important phosphoramidon-insensitive generation of neurotensin(1-10). A good candidate for mediating the formation of this peptide could be endopeptidase 3.4.24.16. Thus, endopeptidase 3.4.24.16 was first detected and later purified from central (Checler *et al.*, 1986; Barelli *et al.*, 1988a) and peripheral (Barelli *et al.*, 1988b; 1993a,b) tissue sources on the basis of its ability to degrade neurotensin, leading to neurotensin(1-10) formation. This activity remains the major candidate for the catabolism of neurotensin since we have established that endopeptidase 3.4.24.16 is the only proteolytic activity that was shown to participate ubiquitously in the inactivation of neurotensin in various tissue sources, cells or membrane preparations from central or peripheral origin (Checler *et al.*, 1988a).

We recently described various inhibitors which allowed us to examine the putative contribution of endopeptidase 3.4.24.16 in the formation of neurotensin(1-10). Phosphodiepryl 03 was reported to block endopeptidase 3.4.24.16 with a $K_i$ value of about 1 nM (Barelli *et al.*, 1992). This inhibitor clearly protected neurotensin from degradation in dog ileum and induced a selective inhibition of the expected catabolite i.e. neurotensin(1-10) (Figure 3). Although phosphodiepryl 03 (at a 1000 fold concentration above its $K_i$ value for endopeptidase 24.16) did not inhibit purified endopeptidase 3.4.24.11, angiotensin-converting enzyme, leucine aminopeptidase and carboxypeptidase A, we established that this inhibitor also inhibited endopeptidase 24.15 ($K_i$ ~7.5 nM) (Barelli *et al.*, 1992). We therefore examined the effect of the endopeptidase 24.15 inhibitor, Cpp-AAY-pAB. We demonstrated (Figure 3) that this agent was unable to protect neurotensin from degradation in dog ileum and did not modify neurotensin(1-10) formation. These data indicated that the effect of phosphodiepryl 03 on neurotensin catabolism was clearly mediated via the inhibition of endopeptidase 24.16. This conclusion was further reinforced by the fact that the dipeptide, Pro-Ile, elicited an effect similar to that of phosphodiepryl 03 on neurotensin recovery and neurotensin(1-10) formation (Figure 4). Thus, we previously showed that Pro-Ile, which mimics the Pro-Tyr bond of neurotensin targetted by endopeptidase 24.16, inhibited endopeptidase 24.16 ($K_i = 90$ μM). Furthermore, the inhibitory spectrum of this dipeptide towards other exo- and endopeptidases indicated that it behaved as a fully selective endopeptidase 24.16 inhibitor (Dauch *et al.*, 1991). Altogether, these observations clearly established the participation of endopeptidase 24.16 in the metabolism of neurotensin and represented the first demonstration that neurotensin could behave as a physiological substrate of endopeptidase 24.16. A model that summarizes the proteolytic events taking place on neurotensin, *in vivo*, in dog ileum is presented in the Figure 5.

Phosphodiepryl 03, which displays high potency and solubility, can be regarded as a novel tool with which to assess whether endopeptidase 24.16 is involved in the central mechanisms of neurotensin inactivation. Furthermore, on account of the large specificity of endopeptidase 24.16, which can also hydrolyse a series of other neuropeptides besides neurotensin, phosphodiepryl 03 could prove useful in examining the contribution of endopeptidase 24.16 in the physiological termination of the action of other biologically active peptides.

We wish to acknowledge Dr J.C. Cuber (Hôpital E. Herriot, Lyon, France) for providing us with the neurotensin antiserum. We sincerely thank Dr M. Orlowski (Mount Sinaï School of Medicine, New York, U.S.A.) for giving us the Cpp-AAY-pAB inhibitor. Drs C. Labbé-Jullié and J.N. Bidard kindly provided the labelled neurotensin. We are grateful to F. Aguila for technical assistance and to V. Cheron for secretarial work. This work was supported by the CNRS, INSERM, the Fondation pour la Recherche Médicale and the Medical Research Council of Canada.

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# Characterization of the prostaglandin $E_2$ sensitive (EP)-receptor in the rat isolated trachea

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**1** Using a range of natural and synthetic prostanoid receptor agonists and antagonists, we have shown that the rat isolated trachea contains a heterogeneous population of prostaglandin receptor sub-types mediating both relaxation and contraction of the smooth muscle. Prostaglandin $E_2$ ($PGE_2$) elicits smooth muscle relaxation of pre-contracted preparations, the responses being well defined, with a mean potency ($p[A_{50}]$) of $7.81 \pm 0.05$.

**2** 11-deoxy $PGE_1$ 16,16-dimethyl $PGE_2$ and misoprostol were all full agonists at this receptor, whilst AH13205 was a low potency agonist, and sulprostone was inactive.

**3** The $EP_1$ receptor antagonist, AH6809 (5 μM), and the selective DP receptor antagonist, BW A868C (0.1 μM), had no significant effect on the concentration-effect (E/[A]) curves to $PGE_2$.

**4** The putative $EP_4$-receptor antagonist, AH23848B, produced non-competitive antagonism of the $PGE_2$ response curves; $pA_2$ values of $5.07 \pm 0.15$ and $5.24 \pm 0.19$ were obtained at concentrations of 30 μM and 100 μM respectively.

**5** The synthetic thromboxane $A_2$ mimetic, U46619, caused smooth muscle contractions, with a mean $p[A_{50}]$ of $6.90 \pm 0.11$. This response was antagonized by the TP receptor antagonist, GR32191B, yielding a mean $pA_2$ of 8.31.

**6** At concentrations of 1 μM and above, prostaglandin $D_2$ ($PGD_2$) and the IP-receptor agonist, cicaprost, generally elicited concentration-dependent relaxations of the rat trachea. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was without affinity or efficacy.

**7** These data suggest that the rat isolated trachea contains EP-receptors, TP-receptors, and few, if any, DP, IP or FP-receptors. The inactivity of sulprostone ($EP_3/EP_1$ receptor selective) and the low potency of AH13205 ($EP_2$-receptor selective) suggest that the rat trachea contains an atypical EP-receptor that does not conform to the current classification system.

**Keywords:** Rat trachea; prostaglandin EP-receptors; smooth muscle relaxation; receptor characterization

## Introduction

Prostanoid receptors at which prostaglandin $E_2$ ($PGE_2$) is the most potent natural agonist have been termed EP-receptors (Kennedy *et al.*, 1982; Coleman *et al.*, 1984) and by use of a range of synthetic agonists and antagonists, these can be further classified into at least three distinct subtypes (Coleman *et al.*, 1987a,b). Prostanoid EP-receptors which generally mediate smooth muscle contraction and are susceptible to block by the antagonists AH6809 and SC 19220 have been termed $EP_1$-receptors. Those receptors that are not blocked with the $EP_1$-receptor antagonists have been designated as either $EP_2$ or $EP_3$-receptors, $EP_2$-receptors generally mediating smooth muscle relaxation and $EP_3$-receptors inhibition of neurotransmitter release and smooth muscle contraction (Ahluwalia *et al.*, 1988). Further evidence for the existence of at least three distinct sub-types of EP-receptors is the recent isolation of functional cDNA clones encoding murine $EP_1$, $EP_2$ and $EP_3$ receptors (Sugimoto *et al.*, 1992; Watabe *et al.*, 1993; Honda *et al.*, 1993). Receptors at which thromboxane $A_2$ ($TXA_2$), $PGD_2$, $PGF_{2\alpha}$ and $PGI_2$ are the most potent natural agonists have been correspondingly termed TP, DP, FP and IP-receptors.

This classification is hampered by a lack of selective antagonists, the fact that most prostanoid agonists display activity at a number of these receptors and that most tissues do not contain homogeneous populations of prostanoid receptors. To date, selective antagonists are available only for $EP_1$-receptors (e.g. AH6809 and SC 19220), TP-receptors (e.g. GR32191B and BM 13.177) and DP-receptors (e.g. BW A868C).

The aims of the present study were as follows: firstly, to characterize the prostaglandin EP-receptor mediating smooth muscle relaxation of the rat isolated trachea using agonist potency order information and selective antagonists; and secondly, to characterize any other prostanoid receptors located in this tissue.

## Methods

Male Sprague-Dawley rats (300–500 g) were killed by cervical dislocation, the trachea removed and cleared of fat and connective tissue. The trachea was cut into 6 rings containing approximately 2 cartilage bands which were mounted between parallel wire hooks in 10 ml organ baths containing Krebs solution (composition, mM: NaCl 117.56, KCl 5.36, $CaCl_2$ 2.55, $MgSO_4$ 1.18, $NaH_2PO_4$ 1.15, $NaHCO_3$ 25.00 and glucose 11.10). The buffer was maintained at 37°C and gassed with 95% $O_2$/5% $CO_2$. Indomethacin (2.8 μM) was routinely present to inhibit endogenous prostanoid production. Changes in tension were recorded isometrically by means of an Ormed Beam force displacement transducer connected to Sekonic SS-250 chart recorders.

### *Experimental protocols*

*General* At the beginning of each experiment a force of 1.0 g was applied to each preparation; these were allowed to equilibrate for 60 min during which time the tissues were retensioned and the buffer changed three times. To prevent any possible TP-receptor-mediated contractile effects, 1 μM GR32191B (approximately 200 fold greater than its $K_B$) was

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added to the bathing solution 30 min prior to constructing agonist E/[A] curves.

*Relaxant effects* Tissues were contracted with 5 μM bethanechol (previous experiments having shown that this concentration was approximately equivalent to its $[A_{50}]$ in this preparation; data not shown). A paired curve design was used throughout, first curves were to the standard agonist, $PGE_2$, and second curves either a time control or to one of the test agonists. A 45 min washout period was allowed between curves. In experiments to measure the effects of antagonists on the $PGE_2$ response, antagonists were added to the Krebs solution 30 min before the construction of second curves.

*Contractile effects* To measure the contractile effects of prostanoid agonists, a similar experimental protocol was used; however, the tissues were not pre-contracted with bethanechol and in studies involving U46619, the TP receptor antagonist GR32191B was omitted from the bathing solution.

### *Data analysis*

Individual E/[A] curves were fitted to a logistic function of this form:

$$E = \frac{\alpha[A]^n}{[A_{50}]^n + [A]^n}$$

where α, $[A_{50}]$ and n are the asymptote, location (expressed as $\log_{10}$ $[A_{50}]$) and slope parameters respectively. All agonist E/[A] curves were fitted using the data analysis package 'KaleidaGraph' on a Macintosh IIcx computer whilst statistical differences were assessed using the 'StatWorks' package. Individual relative potencies were calculated as $[A_{50}]$ test agonist/$[A_{50}]$ $PGE_2$ and a geometric mean calculated.

Individual $pA_2$ values from paired curve data were calculated using the equation;

$$\log_{10}(CR-1) = \log_{10}[B] + pA_2$$

where CR = $[A_{50}]$ agonist curve in the presence of the antagonist/$[A_{50}]$ agonist control curve and [B] is the concentration of antagonist.

### *Statistical analysis*

*Agonist data* Differences between the computer generated values of α, p$[A_{50}]$ and n for each paired E/[A] curve data set were calculated. One-way analysis of variance (ANOVA) was subsequently performed using the delta values from all treatment groups; if ANOVA indicated a difference, further comparisons were made using the modified *t* statistic.

*Antagonist data* Differences between the computer generated values of α, p$[A_{50}]$ and n for each paired E/[A] curve data set were calculated. Two-way analysis of variance was subsequently performed using the delta values from all treatment groups; if ANOVA indicated a difference, further comparisons were made using the modified *t* statistic.

Differences were considered significant at the level of $P<0.05$.

### *Drugs*

$PGE_2$, $PGD_2$, $PGF_{2\alpha}$, 11-deoxy $PGE_1$, 16,16-dimethyl $PGE_2$ and U46619 (9, 11-dideoxy-9α, 11α-methanoepoxy-$PGF_{2\alpha}$) were purchased from Cascade Biochemicals, Reading, Berkshire. AH13205 (*trans*-2-[4-(1-hydroxyhexyl)pentyl)phenyl]-5-oxocyclo pentaneheptanoic acid), AH6809 (6-isopropoxy-9-oxaxanthene-2-carboxylic acid), GR32191B ([1α(Z), 2β, 3β, 5α]-(+)-7-[5-[1,1'-biphenyl]-4-ylmethoxy)-3-hydroxy-2-(1-piperidinyl) cyclopentyl]-4-heptenoic acid) and AH23848B ([1α(Z), 2β, 5α]-(±)-7-[5-[[(1,1'-biphenyl)-4-yl]methoxy]-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid) were obtained from Glaxo Group Research, U.K. Misoprostol was a gift from Searle, U.S.A., sulprostone and cicaprost were gifts from Schering A.G., Germany and BW A868C ((±)-3-benzyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2-hydroxyethylamino)-hydantoin) was a gift from Wellcome Research Laboratories, Beckenham, Kent. Bethanechol chloride and indomethacin were purchased from Sigma Chemical Co., Poole, Dorset.

Prostaglandins were stored at −20°C as either ethanolic or methyl acetate stocks and diluted in Krebs buffer; indomethacin was dissolved in 10% w/v $Na_2CO_3$.

## Results

### *Relaxant effects of $PGE_2$ and analogues*

In the presence of 1 μM GR32191B, $PGE_2$ caused a concentration-dependent smooth muscle relaxation in all preparations tested, with a mean p$[A_{50}]$ of 7.81 ± 0.05 (s.e., $n = 16$) and a maximal relaxation of 78.98 ± 3.54% ($n = 16$) of the induced tone. First and second curve data were generally superimposable. Figure 1 shows the relaxant effects of $PGE_2$ and four analogues. 11-deoxy $PGE_1$, 16,16-dimethyl $PGE_2$ (16,16-$dME_2$) and misoprostol were all less potent than $PGE_2$, their respective concentration-effect curves were all well defined and reached similar asymptotes to $PGE_2$. AH13205 was a low potency agonist and complete E/[A] curves were unobtainable. Sulprostone was inactive at concentrations of up to 3 μM. 11-deoxy $PGE_1$ and 16,16-dimethyl $PGE_2$ E/[A] curves were not significantly different from one another. The rank order of potencies of the EP-

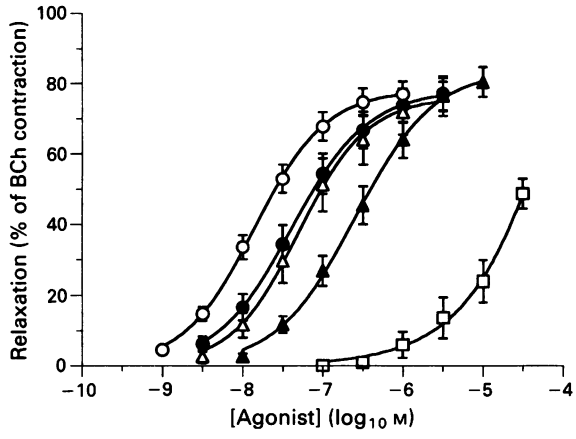


**Figure 1** Concentration-effect curves for relaxation of the bethanechol contracted rat trachea by prostaglandin $E_2$ ($PGE_2$) and related analogues in the presence of 1 μM GR32191B. $PGE_2$ (○, $n = 16$); 11-deoxy $PGE_1$ (●, $n = 7$), 16,16-dimethyl $PGE_2$ (△, $n = 7$); misoprostol (▲, $n = 8$) and AH13205 (□, $n = 6$). Mean values are shown with s.e.mean.

**Table 1** Comparison of potencies of prostanoid EP-receptor agonists in rat isolated trachea

| *Compound* | *p$[A_{50}]$* | *Relative potency* | *95% CL* | n |
|---|---|---|---|---|
| $PGE_2$ | 7.81 ± 0.05 | 1.0 | – | 16 |
| 11-deoxy $PVE_1$ | 7.36 ± 0.11 | 2.4 | 1.3–4.4 | 7 |
| 16,16 $dME_2$ | 7.20 ± 0.13 | 2.8 | 1.3–5.8 | 7 |
| Misoprostol | 6.57 ± 0.08 | 16.1 | 12.2–21.2 | 8 |
| AH13205 | – | 1118 | 339–3684 | 6 |
| Sulprostone | Inactive | – | – | 3 |

Relative potency is the mean of individual tissue results and relative to the standard agonist ($PGE_2 = 1$). p$[A_{50}]$ values are mean ± s.e.mean.

receptor agonists was; $PGE_2$>11-deoxy $PGE_1$ = 16,16-dimethyl $PGE_2$>misoprostol>AH13205>sulprostone = 0. The mean p[$A_{50}$]s and relative potencies are shown in Table 1.

### *Relaxant effects of other prostanoid agonists*

To check for the presence of other prostanoid receptors mediating smooth muscle relaxation, $PGD_2$ and cicaprost were tested in this system following the same protocol as above. Relaxant responses to $PGD_2$ and cicaprost were observed only at concentrations greater than 1 μM giving potencies relative to $PGE_2$, calculated graphically from p[$A_{30}$] values, of >3300 and >200 respectively ($n = 3$).

### *Contractile effects of prostanoid agonists*

$PGE_2$, $PGF_{2\alpha}$ and sulprostone (0.1 μM–10 μM) had no contractile effects on quiescent tissues. In contrast, U46619 produced concentration-related smooth muscle contractions, paired control curve data yielding p[$A_{50}$] values of 6.90 ± 0.11 ($n = 4$) and 6.87 ± 0.22 ($n = 4$) for 1st and 2nd curves respectively (data not shown).

### *Effect of antagonists on the relaxant response of $PGE_2$*

The putative $EP_4$-receptor antagonist, AH23848B (30 μM and 100 μM), caused significant rightward shifts in location, and depression of the asymptotes of the $PGE_2$ response curves, apparent $pA_2$s of 5.07 ± 0.15 ($n = 4$) and 5.24 ± 0.19 ($n = 4$) being obtained at these concentrations respectively (Figure 2). Neither the $EP_1$/DP-receptor antagonist, AH6809 (5 μM), nor the DP receptor antagonist, BW A868C (0.1 μM), had any significant effect on $PGE_2$ E/[A] curves.

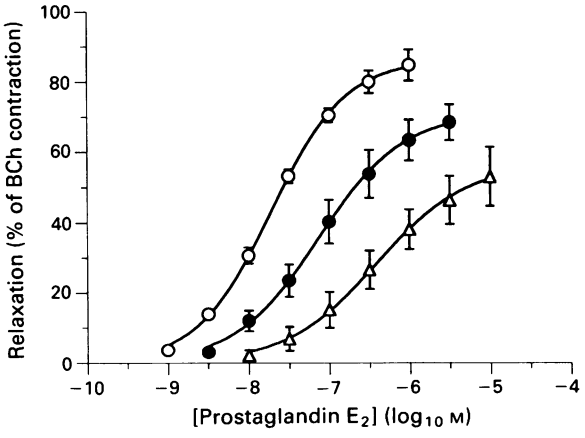


**Figure 2** Antagonism of prostaglandin $E_2$ ($PGE_2$) concentration-response curves (○) in rat isolated trachea by 30 μM (●) and 100 μM (△) AH23848B. Each point is the mean with s.e.mean of 4 determinations.

**Table 2** Comparison of the relative potencies of prostaglandin $E_2$ analogues in various isolated tissue preparations

| *Agonist* | *Rat trachea* | *Rabbit jugular vein* | *Cat trachea* | *Pig saphenous vein* |
|---|---|---|---|---|
| | (Relative potencies, $PGE_2$ = 1) | | | |
| 11-deoxy $PGE_1$ | 2.4 | 2.1† | 13†† | NT |
| 16,16-dM$E_2$ | 2.8 | 2.1† | 20††† | NT |
| Misoprostol | 16.1 | 8.3† | 3.7††† | NT |
| AH13205 | 1118 | NT | 29‡ | 11,040* |
| Butaprost | NT | 685† | 17** | NT |
| Sulprostone | Inactive | >3000† | >10,000‡ | 6,480* |

Published data: †Lawrence & Jones (1992); ††Dong *et al.* (1986); †††Coleman *et al.* (1988); ‡Nials *et al.* (1993); *Louttit *et al.* (1992b); **Gardiner (1986).
NT, not tested.

### *Effect of GR32191B on the contractile response to U46619*

The TP receptor antagonist, GR32191B (30 nM and 0.3 μM, $n = 2$), produced rightward shifts of the U46619 curves, and a $pA_2$ of 8.31 (range, 8.24 to 8.40) was calculated.

## Discussion

The aim of this study was to classify the receptors subserving prostanoid activities in the rat isolated trachea, in particular the prostaglandin EP-receptors mediating smooth muscle relaxation of the preparation.

Due to limited antagonist availability, the classification of prostanoid receptors is based mainly on agonist potency orders in carefully chosen bioassays, although further evidence for this subclassification comes from the recent cloning and expression of cDNA for various prostanoid receptors. The rank order of agonist potencies obtained in the present study is very similar to that reported by Lawrence & Jones (1992) as an $EP_2$-receptor in the histamine-contracted rabbit jugular vein. However, they found the $EP_2$-selective agonist, butaprost, to be 685 times less potent than $PGE_2$ in relaxing this preparation (Table 2), which is not consistent with the result obtained by Gardiner (1986) who found butaprost to be approximately 17 times less potent than $PGE_2$ at the $EP_2$-receptors in cat isolated trachea (Table 2). The low potency of this $EP_2$-receptor ligand in the rabbit jugular vein is a similar result to the one that we obtained using another $EP_2$-selective agonist, AH13205 (Nials *et al.*, 1993), which we found to be more than 1000 times less potent than $PGE_2$ in relaxing the rat trachea. Further similarities between the rabbit jugular vein and rat trachea can be shown using the $EP_3$/$EP_1$ selective agonist, sulprostone, which has a relative potency of >3000 in the rabbit and is inactive in the rat. Coupled with the similarity of the relative potencies of 11-deoxy $PGE_1$, 16,16-dimethyl $PGE_2$ and misoprostol, it would suggest that both preparations contain similar, atypical prostaglandin EP-receptors. These seem to be different receptors from those present in the cat trachea and the rabbit ear artery (Humbles *et al.*, 1991) where misoprostol is more potent than either 11-deoxy $PGE_1$ or 16,16-dimethyl $PGE_2$.

AH23848B, originally identified as a TP-receptor antagonist (Brittain *et al.*, 1985) or partial agonist (Lumley, 1986), has been shown to be a weak antagonist at the putative $EP_4$-receptor in the pig saphenous vein (Louttit *et al.*, 1992a,b), with a $pA_2$ of 5.0 ± 0.1. In the present study, increasing concentrations of the antagonist caused concentration-dependent rightward displacements and reductions in maximal response of the $PGE_2$ curves. The apparent $pA_2$ values obtained at each antagonist concentration are consistent with that obtained by Louttit *et al.* (1992a). The depression in asymptote of the $PGE_2$ response may be due to the relatively high concentrations used, and the lack of selectivity of AH23848B. The low potencies of AH13205 and sulprostone in the pig saphenous vein and rat trachea also suggest that both preparations may contain a similar $EP_4$-receptor. However, clarification of the situation would require antagonist studies using AH23848B in the rabbit jugular vein and agonist potency order information from a range of prostaglandin $E_2$ analogues in the pig saphenous vein.

The rat trachea appears to contain few, if any, DP-receptors because of the previously mentioned finding that $PGD_2$ is weak or inactive. The selective DP-receptor antagonist, BW A868C (Giles *et al.*, 1989) failed to antagonize the response to $PGE_2$ at a concentration 100 times greater than its $pK_B$ at DP-receptors. Similarly,

AH6809 which has some DP (Keery & Lumley, 1988) as well as $EP_1$-receptor blocking activity, failed to antagonize the $PGE_2$ response. In the rabbit jugular vein, relaxant responses can also be mediated by prostanoid IP receptors (Giles *et al.*, 1990) and we found that the IP-receptor agonist, cicaprost did cause relaxations in most tissues. However, due to the low potency of cicaprost, full agonist-response curves could not be obtained. These results suggest that the rat trachea probably contains a small population of IP-receptors.

In addition to atypical EP-receptors mediating smooth muscle relaxation, the rat trachea also contains prostanoid receptors mediating smooth muscle contraction. U46619 was shown to contract the trachea ($p[A_{50}] = 6.90$), and this response was antagonized by GR32191B. A $pA_2$ of 8.31 was calculated, consistent with an action at TP-receptors (Lumley *et al.*, 1989). As the effects of $PGE_2$ were resistant to AH6809, and $PGE_2$, $PGF_{2\alpha}$ and sulprostone are devoid of contractile activity, the rat trachea appears not to contain $EP_1$, $EP_3$ or FP-receptors mediating smooth muscle contraction.

In conclusion, using agonist potency order information and, where available, selective antagonists, we have demonstrated that the rat isolated trachea contains atypical EP-receptors (possibly the same as the putative $EP_4$-receptor in pig saphenous vein), contractile TP-receptors, no FP, $EP_1$, $EP_2$ or $EP_3$-receptors, but possibly small populations of DP and IP-receptors. Because of the low potency of cicaprost and the well defined responses to $PGE_2$ analogues, this tissue represents a useful preparation to examine atypical EP-receptor activity of potential prostanoid EP-receptor agonists.

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# Studies of the nucleoside transporter inhibitor, draflazine, in the human myocardium

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**1** The aim of the present study was to determine the effect of the nucleoside transporter inhibitor, draflazine, on the force of contraction in human myocardium and the affinity of the compound for the nucleoside transporter. Nucleoside transport inhibitors, like draflazine, are of potential importance for cardiopreservation of donor hearts for heart transplantation.

**2** Functional experiments were performed in isolated electrically driven (1 Hz, 1.8 mmol $l^{-1}$ $Ca^{2+}$) human atrial trabeculae and ventricular papillary muscle strips. The affinity of draflazine for the myocardial nucleoside transporter was studied in isolated membranes from human ventricular myocardium and human erythrocytes in radioligand binding experiments using [$^3$H]-nitrobenzylthioinosine ([$^3$H]-NBTI). Dipyridamole was studied for comparison.

**3** In membranes from human myocardium and erythrocytes, [$^3$H]-NTBI labelled 1.18 pmol $mg^{-1}$ protein and 23.0 pmol $mg^{-1}$ protein, respectively, nucleoside transporter molecules with a $K_D$ value of 0.8 nmol $l^{-1}$. Draflazine concentration-dependently inhibited binding of [$^3$H]-NBTI to myocardial and erythrocyte membranes with a $K_i$-value of 4.5 nmol $l^{-1}$. The potency as judged from the $K_i$ values was ten times greater than that of dipyridamole in both myocardial and erythrocyte membranes.

**4** Draflazine, at concentrations up to 100 $\mu$mol $l^{-1}$, did not produce negative inotropic effects in atrial and ventricular myocardium. (−)-$N^6$-phenylisopropyladenosine (**R-PIA**) and carbachol did not reduce force of contraction in ventricular myocardium, but exerted concentration-dependent direct negative inotropic effects in atrial myocardium.

**5** The data provide evidence that draflazine specifically binds to the nucleoside transporter of the human heart and erythrocytes with high affinity. The compound does not produce negative inotropic effects at concentrations as high as 100 $\mu$mol $l^{-1}$.

**6** Draflazine could be a useful agent for cardiopreservation because it does not produce cardiodepressant effects. Thus, it may be possible to perfuse explanted hearts directly with this agent without the hazard of cardiodepression.

**Keywords:** Nucleoside transporter; nucleoside uptake inhibition; adenosine; human myocardium; cardiac transplantation

## Introduction

Adenosine is released from the heart during ischaemia (Fox *et al.*, 1974), increased cardiac workload (Foley *et al.*, 1978; McKenzie *et al.*, 1981) and during excessive catecholamine stimulation (Schrader *et al.*, 1977). Adenosine receptors on the external surface of myocardial, smooth muscle and endothelial cells mediate antiadrenergic effects (Schrader *et al.*, 1977; Böhm *et al.*, 1984; 1985) and produce vasodilator actions (Berne, 1963) thereby limiting oxygen demand and increasing blood flow to the critically challenged myocardium. These effects led investigators to suggest that endogenously formed adenosine is an endogenous feed back inhibitor against catecholamine overstimulation and a retaliatory metabolite for the temporal and local control of cellular functions (Newby, 1984; Bellardinelli *et al.*, 1989). Conceivably, a cardioprotective effect has been observed (for review see Forman *et al.*,1993; van Belle, 1993). Adenosine is rapidly taken up by the nucleoside transporter into endothelial cells and into erythrocytes (Klabunde & Althouse, 1981; Plagemann *et al.*, 1988). As a consequence, the plasma half-life of adenosine is about 10 s when given intravenously in man (Klabunde, 1983). From these data it appeared worthwhile to inhibit the nucleoside transporter so as to enhance the adenosine concentration at its extracellular receptor sites, thereby increasing its cardioprotective effects. Nucleoside transporter inhibitors have been used in cardioplegic solutions to improve myocardial viability (Ledingham *et al.*, 1990). Draflazine is a compound which selectively inhibits the purine nucleoside transporter (Van Belle & Janssen, 1991) and does not possess other effects as does dipyridamole (Blass *et al.*, 1980; Ahn *et al.*, 1989). Draflazine has also been reported to prevent catecholamine-induced myocardial damage (van Belle *et al.*, 1992). The pharmacological profile of draflazine appears to be advantagous as a compound for cardiac protection, and could be useful as an additional compound in cardioplegic solutions (Flameng *et al.*, 1991). In cardiac transplantation, the availability of donor hearts is markedly limited by the time for which the heart can be kept in cardioplegia for transportation. Thus, the availability of pharmacological agents which preserve cardiac function is of great clinical relevance. Perfusion of the hearts during explantation with cardioprotective agents would allow high myocardial drug concentrations to be achieved. However, this practical approach could be hampered by potential cardiodepressant side effects. In order to evaluate this potentially important question, the effects of draflazine on myocardial force of contraction were investigated on human isolated, electrically driven atrial and ventricular cardiac preparations and were compared to the affinity of the agent at the nucleoside transporter in human myocardial and human erythrocyte membranes. The use of isolated, human myocardial preparations is important because inotropic effects can markedly differ between human and animal tissue

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(Böhm et al., 1989a) and because effects on force of contraction can be studied without interference from pre- or afterload changes or chronotropic effects.

## Methods

### *Myocardial tissue*

Experiments were performed on human isolated, electrically stimulated, ventricular papillary muscle strips and right auricular trabeculae or on membrane preparations from human left ventricular myocardium. Tissue was obtained during aortocoronary bypass operations (without heart failure) ($n = 7$, 4 female, 3 male; age:mean 61.4 years, range 41–71) or cardiac transplantation ($n = 3$, 2 female, 1 male; dilated cardiomyopathy). All patients gave written informed consent before surgery. Medical therapy consisted of diuretics, nitrates, ACE-inhibitors and cardiac glycosides. Patients receiving catecholamines, β-adrenoceptor- or $Ca^{2+}$-antagonists were withdrawn from the study. Drugs used for general anaesthesia were flunitrazepam and pancuronium bromide with isoflurane. Cardiac surgery was performed on cardiopulmonary bypass with cardioplegic arrest during hypothermia. The cardioplegic solution (a modified Bretschneider solution) contained (in mmol $l^{-1}$): NaCl 15, KCl 10, $MgCl_2$ 4, histidine 180, tryptophan 2, mannitol 30 and potassium dihydrogen oxoglutarate 1.

### *Contraction experiments*

Immediately after excision, the papillary muscles and atrial trabeculae were placed in ice-cold preaerated modified Tyrode solution (composition see below) and delivered to the laboratory within 10 min. The experiments were performed on isolated, electrically driven (1 Hz) muscle preparations. Muscle strips of uniform size with muscle fibres running parallel to the length of the strips (diameter $< 1.0$ mm, length 5–9 mm) were dissected in aerated bathing solution (composition see below) at room temperature. Connective tissue, if visibly present, was carefully trimmed away. The preparations were attached to a bipolar platinum stimulating electrode and suspended individually in 75 ml glass tissue chambers for recording of isometric contractions. The bathing solution used was a modified Tyrode solution containing in mmol $l^{-1}$: NaCl 119.8, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaH_2PO_4$ 0.42, $NaHCO_3$ 22.6, $Na_2EDTA$ 0.05, ascorbic acid 0.28 and glucose 5.0. It was continuously gassed with 95% $O_2$ and 5% $CO_2$ and maintained at 37°C; its pH was 7.4. Muscle strip preparations were attached to two stainless metal pins, one of which was connected to a force transducer. Isometric force of contraction was measured with an inductive force transducer (W. Fleck, Mainz, Germany) attached to a Hellige Helco Scriptor (Hellige, Freiburg, Germany) or Gould recorder (Gould Inc., Cleveland, Ohio, U.S.A.). Each muscle was stretched to the length at which developed force was maximal and the resting load was kept constant throughout the experiments. The preparations were electrically paced at 1 Hz with rectangular pulses of 5 ms duration (Grass stimulator SD 9), the voltage was 20% above threshold. All preparations were allowed to equilibrate at least 90 min in a drug-free bathing solution until complete mechanical stabilization. After 45 min, the solution was changed. Concentration-dependent mechanical effects of draflazine and other drugs were obtained. To test mechanical performance, after each experiment the positive inotropic effect mediated by elevation of the extracellular $Ca^{2+}$-concentration (15 mmol $l^{-1}$) was measured. All preparations in this experimental series developed an increase in force of contraction after increase in the $Ca^{2+}$-ion-concentration. Control strips in Tyrode solution revealed a maximally 20% reduction of baseline isometric developed tension over the period necessary to complete pharmacological testing. Agents were applied cumulatively to the organ bath. Each muscle was used only once to record a concentration-response curve.

### *Human myocardial membrane preparations*

Left ventricular myocardium was chilled in 30 ml ice-cold homogenization buffer (20 mmol $l^{-1}$ Tris/HCl, 1 mmol $l^{-1}$ EDTA, 1 mmol $l^{-1}$ dithiothreitol, pH 8.0). Connective tissue was trimmed away and myocardial tissue was minced with scissors, disrupted with an Ultraturrax (Janke and Kunkel, Staufenbreisgau, Germany) and homogenized with a motor-driven glass teflon Elvejhem-Potter for 1 min. The homogenate was spun at 480 *g* for 10 min (JA 20, Beckman, Palo Alto, U.S.A.). The supernatant was retained and the pellet discarded. This homogenate was diluted with an equal volume of ice-cold 1 mol $l^{-1}$ KCl and stored on ice for 10 min. The supernatant was centrifuged at 100,000 *g* for 45 min. The pellet was resuspended in 50 volumes of homogenization buffer and recentrifuged at 100,000 *g* for 45 min. The final pellet was resuspended in incubation buffer (50 mmol $l^{-1}$ Tris/HCl, 10 mmol $l^{-1}$ $MgCl_2$, pH 7.4). Assays were performed in a total volume of 250 µl incubation buffer.

### *Human erythrocyte membrane preparation*

Heparinized blood (15 ml) was taken from healthy volunteers and centrifuged at 150 *g* for 20 min at room temperature (Beckman J21B, rotor JA 20). The supernatant was discarded and the sediment was washed twice in 30 ml buffer (50 mmol $l^{-1}$ Tris/HCl, 10 mmol $l^{-1}$ $MgCl_2$, 1 mmol $l^{-1}$ dithiothreitol) and again centrifuged at 150 *g*. The sediment was resuspended in 10 ml buffer and diluted with an equal volume of 1 mol $l^{-1}$ KCl and treated with five strokes of a motor-driven glass teflon Elvejhem-Potter homogenizer. Following an incubation period for 15 min at 40°C, the suspension was centrifuged for 30 min at 100,000 *g*. The sediment was resuspended in hypotonic medium (25 mmol $l^{-1}$ $MgCl_2$, 10 mmol $l^{-1}$ $KHCO_3$, 20 mmol $l^{-1}$ Tris/HCl) and again centrifuged at 100,000 *g* for 30 min. This final pellet was pottered and suspended in buffer without dithiothreitol, frozen in liquid nitrogen and stored at − 80°C.

### *Radioligand binding experiment*

The nucleoside transporter in human erythrocytes and myocardial membranes was investigated with [$^3$H]-nitrobenzylthioinosine ($^3$H-NBTI) as radiolabelled ligand. The incubation was carried out for 60 min at 37°C in a buffer containing 50 mmol $l^{-1}$ Tris/HCl, 10 mmol $l^{-1}$ $MgCl_2$ and 1 mmol $l^{-1}$ dithiothreitol at a final volume of 250 µl. The assay was started with membrane suspension (20 µg for erythrocytes, 150 µl for myocardial membranes). Nonspecific binding was determined with 30 µmol $l^{-1}$ dipyridamole. The conditions used allowed complete equilibration with the nucleoside transporter. The reaction was terminated by rapid vacuum filtration through Whatman GB/C filters. The filters were washed three times immediately with 6 ml incubation buffer. Filters were dried and placed in 10 ml of scintillation fluid (Quickszint 501, Zinsser Analytics, Frankfurt, Germany) and radioactivity determined with a LKB-β-liquid scintillation counter. Each determination was performed in triplicate.

### *Miscellaneous*

Protein was determined according to Lowry *et al.* (1951). Binding data ($B_{max}$ and $K_D$-values) were determined according to Scatchard (1949). $K_i$ values were determined according to Cheng & Prusoff (1973) from the $IC_{50}$-values which were graphically determined in each individual experiment.

### *Materials*

Draflazine CR 75231, 2-(aminocarbonyl)-N-(4 amino-2,6-dichlorophenyl)-4-(55-*bis*-(4-fluorphenylpentyl)-1-piperazine acetamide) was from Janssen Co, Beerse, Belgium. Dipyridamole, **R**-N'-phenyl-isopropyladenosine (**R**-PIA) and carbachol were purchased from Boehringer-Mannheim (Germany). All other chemicals were of analytical grade or the best grade commercially available. For studies with isolated cardiac preparations, stock solutions were prepared and applied to the organ bath. Applied compounds did not change the pH in the bathing solution.

## Results

### *[$^3$H]-NBTI-binding to the nucleoside transporter*

Human erythrocyte membranes were prepared from three samples obtained from human volunteers. A typical experiment to characterize binding of [$^3$H]-NBTI to human erythrocyte membranes is shown in Figure 1. Binding of [$^3$H]-NBTI was monophasic and saturable. Transformation of the data according to Scatchard (1949) revealed a linear plot with a density of 23 pmol mg$^{-1}$ protein [$^3$H]-NBTI bound and an apparent $K_D$-value of 0.8 nmol l$^{-1}$. Nonspecific binding was low amounting to about 1% of total binding at $K_D$ and about 5% at 30 nmol l$^{-1}$ [$^3$H]-NBTI. In order to evaluate the affinity of the nucleoside transporter inhibitors, draflazine and dipyridamole, competition experiments were performed. Figure 2 illustrates the concentration-dependent antagonism of [$^3$H]-NBTI-binding by dipyridamole and draflazine. Both compounds completely antagonized binding of [$^3$H]-NBTI to the erythrocyte nucleoside transporter. Analysis of binding data revealed $K_i$ values of 4.5 nmol l$^{-1}$ and 48 nmol l$^{-1}$ for drafalzine and dipyridamole, respectively. Figure 3 demonstrates a representative radioligand binding experiment with [$^3$H]-NBTI on membranes from the human left ventricle. As in erythrocyte membranes, [$^3$H]-NBTI concentration-dependently bound to the membranes with a $K_D$ value of 0.8 nmol l$^{-1}$ and a $B_{max}$ value of 1.18 nmol [$^3$H]-NBTI bound mg$^{-1}$ protein. Figure 4 shows concentration-dependent inhibition of [$^3$H]-NBTI-binding by draflazine and dipyridamole in human ventricular membranes. As in erythrocyte membranes, the potency of draflazine in inhibiting binding of [$^3$H]-NBTI to membranes was about 10 times higher than that of dipyridamole. Taken together, in radioligand binding studies with [$^3$H]-NBTI, the density of the nucleoside transporter in erythrocytes was determined to be about 19 times higher than in human ventricular membranes. In both systems, the potency of draflazine was about 10 times greater than that of dipyridamole in binding to the nucleoside transporter.

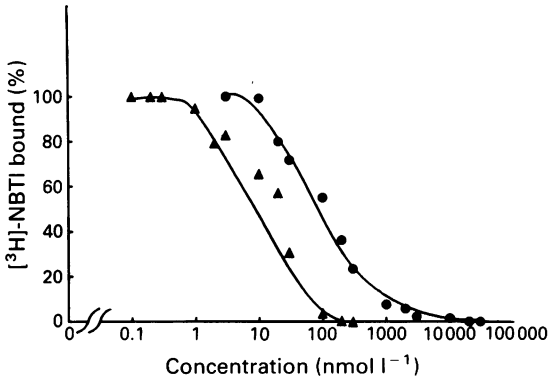


**Figure 2** Inhibition of [$^3$H]-nitrobenzylthioinosine ([$^3$H]-NBTI) (0.8 nmol l$^{-1}$) binding to the nucleoside transporter of human erythrocyte membranes by the nucleoside transporter inhibitors, draflazine (▲) and dipyridamole (●). Ordinate scale: specific binding as a percentage of maximal bound [$^3$H]-NBTI. Abscissa scale: concentration of competitors.

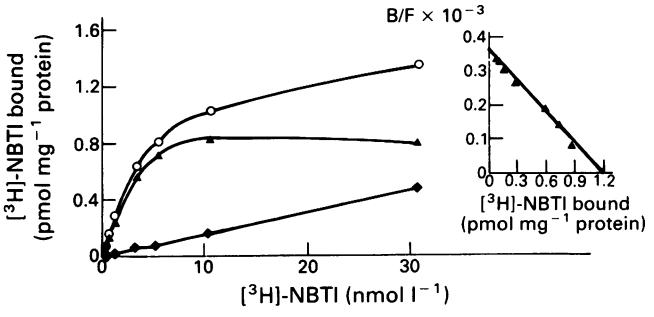


**Figure 3** Binding of [$^3$H]-nitrobenzylthioinosine ([$^3$H]-NBTI) (0.1–30 nmol l$^{-1}$) to the nucleoside transporter of human ventricular membranes. The inset shows the linear transformation of the data obtained in the saturation experiment. Bound [$^3$H]-NBTI (pmol mg$^{-1}$ protein) is plotted as a function of the ratio of bound to free [$^3$H]-NBTI (B/F × 10$^3$). The intercept with the abscissa scale is the number of binding sites ($B_{max}$); the slope is the apparent affinity. Each point represents the mean of triplicate observations: (○) total binding; (▲) specific binding; (◆) nonspecific binding.

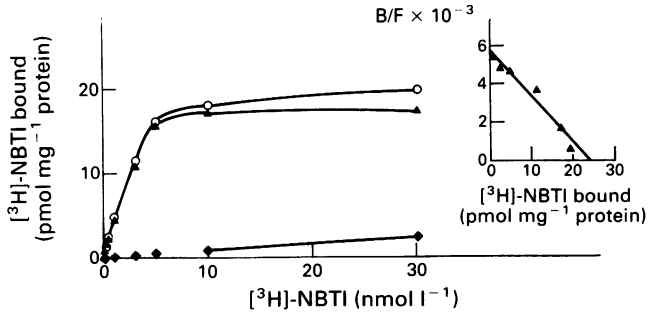


**Figure 1** Binding of [$^3$H]-nitrobenzylthioinosine ([$^3$H]-NBTI) (0.1–30 nmol l$^{-1}$) to the nucleoside transporter of human erythrocyte membranes. The inset shows the linear transformation of the data obtained in the saturation experiment. Bound [$^3$H]-NBTI (pmol mg$^{-1}$ protein) is plotted as a function of the ratio of bound to free [$^3$H]-NBTI (B/F × 10$^{-3}$). The intercept with the abscissa scale is the number of binding sites ($B_{max}$); the slope is the apparent affinity. Each point represents the mean of triplicate observations: (○) total binding; (▲) specific binding; (◆) nonspecific binding.

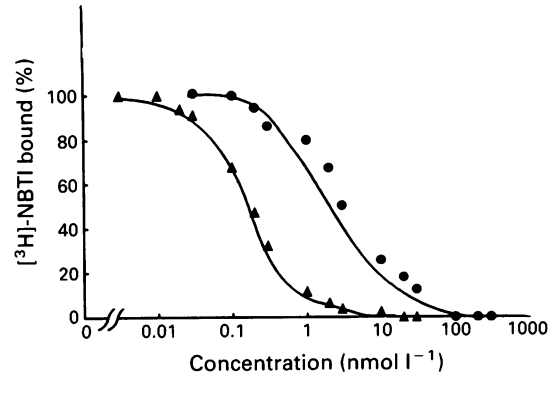


**Figure 4** Inhibition of [$^3$H]-nitrobenzylthioinosine ([$^3$H]-NBTI) (0.8 mmol l$^{-1}$) binding to the nucleoside transporter of human ventricular membranes by the nucleoside inhibitors, draflazine (▲) and dipyridamole (●). Ordinate scale: specific binding in percentage of maximal bound [$^3$H]-NBTI. Abscissa scale: concentration of competitors.

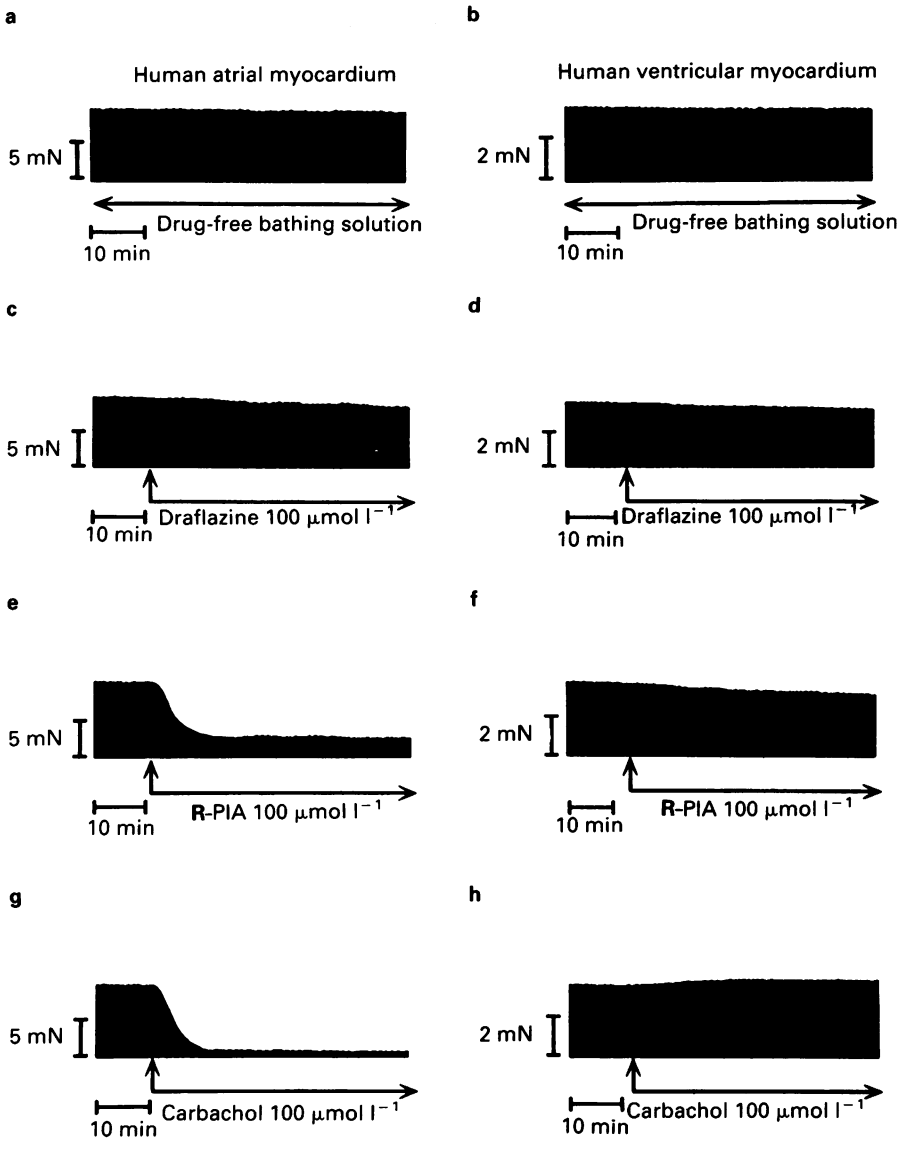


**Figure 5** Original recordings illustrating the effects on force of contraction in isolated cardiac preparations from human atrial (left tracings) and human ventricular (right tracings) myocardium of drug-free bathing solution (a,b), draflazine (c,d), the adenosine receptor agonist, **R-PIA** (e,f) and carbachol (g,h).

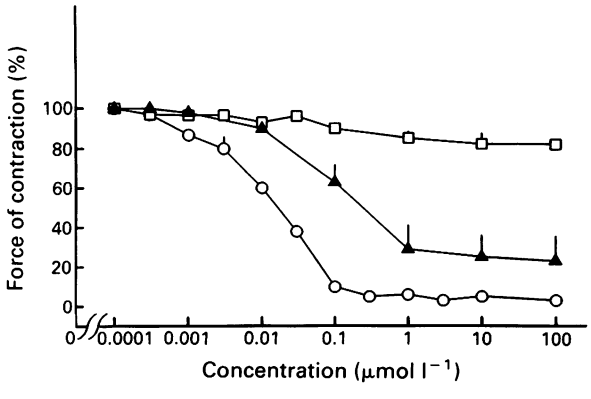


**Figure 6** Concentration-response curves for the effects on contraction in human isolated, electrically driven atrial trabeculae of draflazine (□), **R-PIA** (▲) and carbachol (○) (0.0001–100 μmol $l^{-1}$). Basal force of contraction was 11.6 ± 0.6 mN ($n = 20$). Ordinate scale: force of contraction in % of predrug values. Abscissa scale: concentration of studied drugs in μmol $l^{-1}$.

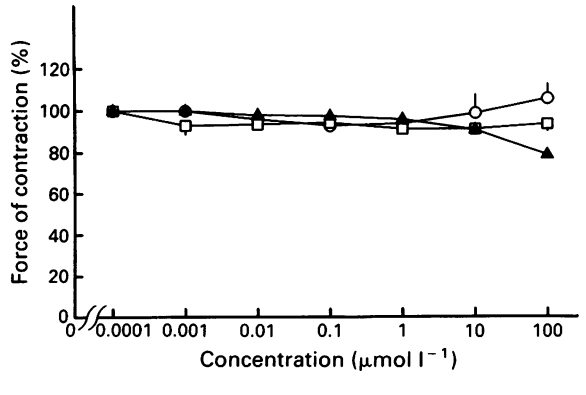


**Figure 7** Concentration-response curves for the effects on force of contraction in human isolated, electrically driven papillary muscle strips of draflazine (□), **R-PIA** (▲) and carbachol (○) (0.0001–100 μmol $l^{-1}$). Basal force of contraction was 3.0 ± 0.5 mN ($n = 31$). Ordinate scale: force of contraction in % of predrug values. Abscissa scale: concentration of studied drugs in μmol $l^{-1}$.

*Effects of draflazine on force of contraction*

Original recordings in Figure 5 demonstrate the effect of draflazine on isometric force of contraction in isolated, electrically driven right atrial and left ventricular cardiac preparations. The effects of the $A_1$-adenosine receptor agonist, **R-PIA** and the muscarinic cholinoceptor agonist, carbachol, as well as the time course of force of contraction in drug-free bathing solution are shown for comparison. Similar to force generation in drug-free bathing solution, draflazine did not produce negative inotropic responses up to 100 $\mu$mol $l^{-1}$ in atrial or in ventricular cardiac preparations. In contrast, the $A_1$-adenosine receptor agonist, **R-PIA**, and carbachol produced concentration-dependent negative inotropic effects in atrial heart muscle without affecting force development in ventricular heart muscle. Figure 6 shows the concentration-response curves for effects of draflazine, **R-PIA** and carbachol in atrial preparations. Draflazine had no effects on developed tension over the concentration range 0.001–100 $\mu$mol $l^{-1}$. **R-PIA** and carbachol concentration-dependently reduced force of contraction. Carbachol was about ten times more potent than **R-PIA**. In ventricular preparations, **R-PIA** and carbachol did not reduce developed force of contraction (Figure 7).

## Discussion

Adenosine is an endogenous nucleoside which is released from the myocardium in situations where an increased ATP-breakdown occurs. In addition, adenosine acts as an anti-platelet aggregating agent (Cronstein *et al.*, 1985) and potently inhibits leucocyte activation (van Belle, 1985). Therapeutic applications of adenosine include the treatment of supraventricular tachycardias (Di Marco *et al.*, 1983) and controlled hypothermia in neurosurgery (Sollevi *et al.*, 1984). From this profile of actions it has been suggested that adenosine is a local endogenous regulator of cellular metabolism and function (Newby, 1984). Consistently, adenosine has been reported to protect the heart from ischaemia and stunning (Lasley *et al.*, 1990; Bunch *et al.*, 1992). On the contrary, uncoupling of adenosine $A_1$-receptors by pertussis toxin treatment was observed to produce myocardial lesions comparable to those observed following catecholamine-treatment (Böhm *et al.*, 1988). One possible physiological intervention to increase the concentration of the nucleoside at its extracellular receptor, is the application of nucleoside transport inhibitors like dipyridamole or lidoflazine which could result in an improved cardiac function following situations like catecholamine stress, ischaemia or long term storage in cardioplegic solutions (Schubert *et al.*, 1989).

The limited time for conservation of ischaemic donor organs is still one important problem in heart transplantation. Cardiac function following cardiac transplantation is inversely related to the time of ischaemic storage of the organ and in general limited to 4–6 h post explanation (Heck *et al.*, 1989; Pflugfelder *et al.*, 1989). Thus, nucleoside transporter inhibition appears to be an attractive mechanism for increasing the adenosine concentration at its extracellular receptor sites thereby preserving mechanical function following storage in cardioplegic solutions. Indeed lidoflazine was observed to improve cardiac function during intermittent aortic cross clamping in dogs (Chang-Chun *et al.*, 1992). The most pronounced effects of the nucleoside transporter inhibitor would be expected at a 100% receptor occupation. In this study, we determined the concentration by radioligand binding studies using competition experiments with dipyridamole or draflazine. Complete antagonism by draflazine or dipyridamole to [$^3$H]-NBTI binding was observed at 30 $\mu$mol $l^{-1}$ and 300 $\mu$mol $l^{-1}$ draflazine and dipyridamole, respectively, in human myocardial membranes under the used assay conditions. In the erythrocyte membranes, these concentrations were even higher. Thus, it would be advantagous to achieve these rather high concentrations in the myocardium to obtain an efficient nucleoside uptake inhibition. These concentrations could be reached during cardiac transplantation by perfusing the hearts directly following explantation with cardioplegic solution and the nucleoside uptake inhibitor. However, the effects of these compounds are not specific for the inhibition of nucleoside transporter. Dilazep, for instance, possesses $Ca^{2+}$ antagonistic properties (Wainwright *et al.*, 1993) and thus, might elicit cardiodepressant effects. Therefore, these unwanted cardiodepressant effects were studied with draflazine in human isolated cardiac preparations. The use of human myocardium is important because response to inotropic agents can differ between human and laboratory animal myocardium (Böhm *et al.*, 1989a). Draflazine, at concentrations up to 100 $\mu$mol $l^{-1}$ did not produce negative inotropic effects. Thus, direct negative inotropic effects do not have to be expected when the explanted heart is directly perfused with draflazine. However, one might argue that adenosine could directly produce antiadrenergic effects, when its uptake into cells is maximally inhibited. As shown by the metabolically stable $A_1$-adenosine receptor agonist, **R-PIA**, stimulation of the receptor does not produce any inhibitory direct effect on force of contraction in human ventricular myocardium as previously also reported in rodent myocardium (Böhm *et al.*, 1984; Dobson *et al.*, 1986). Only in atria is adenosine known to produce direct negative inotropic effects (Böhm *et al.*, 1989b). In preparations with oxygenated Tyrode solution, draflazine did not reduce atrial force of contraction. This observation is in agreement with the suggestion that the preparations are well oxygenated and do not release adenosine in amounts able to exert negative inotropic effects even in human atrial myocardium.

Draflazine is a lidoflazine analogue which has been described as a specific inhibitor of nucleoside uptake (van Belle & Janssen, 1991). Its affinity for the nucleoside transporter of membranes from human erythrocytes and human myocardium was about ten times higher than that of dipyridamol as judged from the $K_i$ values for inhibition of [$^3$H]-NBTI-binding (this study). In addition, the compound elicited antiarrhythmic effects in anaesthetized pigs (Wainwright *et al.*, 1993). From this pharmacological profile it appears that it might be a useful drug for cardiopreservation and long term storage of explanted donor hearts. The first evidence in favour of this suggestion was provided by experiments in dog hearts which were kept in hyperkalaemic cardioplegic solution without or with draflazine. The hearts with the nucleoside inhibitor exhibited almost complete mechanical activity after 24 h, whereas no mechanical activity was observed in the control group (Masuda *et al.*, 1992).

From the present findings and from observations reported in the literature, it is suggested that the nucleoside transporter inhibitor, draflazine, may be a suitable agent for cardiopreservation. Its lack of cardiopressant effects in human myocardium, even at high concentrations could offer the opportunity to perfuse the explanted heart to obtain a complete or at least high degree of nucleoside transporter inhibition.

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# Block of ATP-sensitive $K^+$ channels in isolated mouse pancreatic β-cells by 2,3-butanedione monoxime

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**1** The patch-clamp technique has been used to examine the action of the chemical phosphatase 2,3-butanedione monoxime (BDM) on ATP-sensitive $K^+$ channels ($K_{ATP}$-channels) from mouse isolated pancreatic β-cells in the absence of ATP and $Mg^{2+}$.

**2** BDM reversibly inhibited whole-cell $K_{ATP}$-currents with a concentration for half maximal inhibition ($K_i$) of 15 ± 1 mM and a Hill coefficient (n) of 2.5 ± 0.2 ($n = 4$).

**3** In outside-out patches, external BDM reversibly reduced the activity of single $K_{ATP}$-channels with an affinity similar to that observed in whole-cell recordings ($K_i = 11 \pm 3$ mM, $n = 2.0 \pm 0.3$, $n = 7$). In inside-out patches, internally applied BDM also reversibly blocked the activity of $K_{ATP}$-channels ($K_i = 31 \pm 2$ mM, $n = 2.2 \pm 0.4$, $n = 8$). In both excised patch configurations, BDM decreased the mean open life-time and the burst duration, thereby producing a decrease in the channel open probability. The drug had no effect on the short intraburst closed times.

**4** BDM had no effect on the single-channel current amplitude.

**5** The results suggest that BDM blocks the $K_{ATP}$-channel directly, by mechanisms independent of channel dephosphorylation.

**Keywords:** ATP-sensitive $K^+$ channel; β-cell; 2,3-butanedione monoxime

## Introduction

2,3-Butanedione monoxime (diacetyl monoxime, BDM) is a well established chemical phosphatase. In cardiac myocytes and dorsal root ganglion neurones, extracellularly applied BDM has been shown to inhibit L-type $Ca^{2+}$-channels ($IC_{50} = 5.8$ mM, Chapman, 1993; $IC_{50} \approx 20$ mM, Huang & McArdle, 1992). Inhibition was reversed by agents that are thought to promote phosphorylation of the L-type calcium channel, such as 8-bromo-cyclic AMP and isoprenaline. These data are consistent with the idea that BDM can behave as a chemical phosphatase and can decrease L-type $Ca^{2+}$-channel activity by dephosphorylation of the channel protein (Huang & McArdle, 1992; Chapman, 1993). In ventricular myocytes, BDM reduces both the slow inward calcium current and the transient outward potassium current, effects which have again been attributed to a phosphatase action of BDM (Couloumbe *et al.*, 1990). BDM has also been shown to inhibit both ATP-sensitive $K^+$-channels ($K_{ATP}$-channels) in cardiac myocytes, $K_i \approx 21$ mM, and voltage-dependent $K^+$-currents expressed in oocytes (Hardin *et al.*, 1993; Lopatin & Nichols, 1993). In these cases, however, inhibition was reversible in the absence of $Mg^{2+}$ ions and ATP, conditions which do not support protein phosphorylation. This suggests that BDM may also block channel activity by a mechanism that does not involve protein dephosphorylation. We have used standard patch-clamp techniques to investigate whether BDM acts as a direct blocker of $K_{ATP}$-channels in mouse pancreatic β-cells.

## Methods

### *Cells*

Patch-clamp studies were made on primary cultured β-cells isolated from mouse islets of Langerhans, as previously described (Rorsman & Trube, 1985; Bokvist *et al.*, 1990).

### *Solutions*

In whole-cell and outside-out patch experiments the extracellular face of the membrane was bathed in a solution containing (in mM): NaCl 138, KCl 5.6, $CaCl_2$ 2.6, $MgCl_2$ 1.1, Na-HEPES 10 (pH 7.4). For the inside-out experiments the extracellular, pipette, solution contained (in mM): KCl 140, $CaCl_2$ 2.6, $MgCl_2$ 1.2, K-HEPES 10 (pH 7.4). In all experiments the intracellular face of the membrane was bathed in a solution which contained (in mM): KCl 140, $CaCl_2$ 4.6, EDTA 10, K-HEPES 10 (pH 7.2; free $[Mg^{2+}] < 6$ nM; free $[Ca^{2+}] < 30$ nM). This solution does not support protein phosphorylation since it does not contain $Mg^{2+}$ or ATP. It also reduces rundown of $K_{ATP}$-channels in excised patches and standard whole-cell recordings from pancreatic β-cells (Kozlowski & Ashford, 1990; Williams, 1992). All chemicals were from Sigma. 2,3-Butanedione monoxime (BDM) was dissolved directly into the appropriate solution.

All experiments were carried out at room temperature (23–26°C).

### *Electrophysiological recording*

Membrane-currents were recorded with a List EPC-7 amplifier (List Electronik, Darmstadt, Germany), filtered at 2–5 kHz (−3dB) with an 8 pole Bessel filter (Frequency devices, Burlingame, MA, U.S.A.) and subsequently digitized at 5–10 kHz using AXOLAB hardware (Axon Instruments, Foster City, CA, U.S.A.) and analysed using in-house software.

Whole-cell currents flowing through $K_{ATP}$-channels were measured according to the protocol of Trube *et al.* (1986), that is as the current elicited by alternate ±10 mV voltage steps (200 ms duration at 0.5 Hz) from a holding potential of −70 mV.

In inside-out patch experiments single $K_{ATP}$-channel currents were recorded at a membrane potential of −70 mV. For outside-out patch studies the membrane potential was held at 0 mV; although this potential lies above the threshold

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for activation of both the delayed-rectifier and calcium-activated K-channels ($K_{Ca}$-channels), these channels will be substantially inactivated by the maintained depolarization. Furthermore, the free $Ca^{2+}$ concentration in our intracellular solution is below that required for activation of $K_{Ca}$-channels (Smith *et al.*, 1990).

### *Data analysis*

To determine the degree of $K_{ATP}$-current inhibition, the whole-cell $K_{ATP}$-current ($I$) measured in the test solution was normalized to the average ($I_c$) of its value in the control solutions preceding, and following, exposure to the test solution. The data were fitted with the Hill equation:

$$\frac{I}{I_c} = \frac{1}{(1 + ([BDM]/K_i)^n)} \quad (1)$$

where $K_i$ is the concentration at which half maximal inhibition occurs and n is the Hill coefficient.

When measuring channel activity (NP) we did not attempt to discriminate between changes in the number of available channels, N, and changes in the channel open probability, P. NP was defined by $(\Sigma_{j=1}^{n} jt_j)/T$, where T is the total time of the recording, $t_j$ is the total time for which the jth channel is open and $n$ is the maximum number of channels observed in the patch. The relationship between BDM concentration and channel activity was determined by normalizing the channel activity in the test solution (NP) to the average of its value in the control solution before and after exposure to BDM (NPc). The data were then fitted by the Hill equation (Equation 1).

Single-channel currents were analysed using the half-amplitude threshold technique following the methods detailed by Colquhoun & Sigworth (1983). Lifetime distributions were log-binned using the method of McManus *et al.* (1987) where:

$$\text{bin} = 1 + \text{integer}(25 \times \log_{10}(\text{event duration in samples})) \quad (2)$$

When the square root of the number of events in a bin is plotted against the open or closed lifetime, the components of the distribution appear as clear peaks with their respective time constants falling in the vicinity of the distribution peaks (Sigworth & Sine, 1987). Conditional probability density functions (PDF) were fitted to the open, closed and burst lifetime distributions by the method of maximum likelihood. We used the following PDF:

$$f(t) = \frac{\sum_{i=1}^{k} a_i \tau_i^{-1} \exp(-t/\tau_i)}{P(t_{min} < t < t_{max})} \quad t_{min} < t < t_{max} \quad (3)$$

where:

$$P(t_{min} < t < t_{max}) = \sum_{i=1}^{k} a_i \, (\exp(-t_{min}/\tau_i) - \exp(-t_{max}/\tau_i)) \quad (4)$$

and $a_i$ is the relative area, and $\tau_i$ the time constant, of the ith component. $t_{min}$ and $t_{max}$ define the shortest and longest observations used in fitting the PDF. No correction was made for missed events.

In patches where multiple channel openings were present, we have limited our kinetic analysis to that of the open and closed times within bursts of channel openings during periods when only a single-channel was active. In patches in which channel activity was low or was dramatically reduced by BDM, we were able to perform a kinetic analysis of the burst of channel openings. A burst was defined as one or more openings which were separated by closures which were less than the burst criteria time, $t_{crit}$. $t_{crit}$ was determined from:

$$a_{c,i} \, [\exp(-t_{min}/\tau_{c,i}) - \exp(-t_{crit}/\tau_{c,i})] = a_{c,f} \exp(-t_{crit}/\tau_{c,f}) \quad (5)$$

where $a_{c,f}$ and $\tau_{c,f}$ are the relative area and time constant of the fastest component of the closed times, and $a_{c,i}$ and $\tau_{c,i}$ are the relative area and time constant of the intermediate component of the closed times (Jackson *et al.*, 1983). Newton's method was used to find the implicit variable, $t_{crit}$. Long closed times composing the $\tau_{c,s}$ component were excluded from this calculation as they occurred too infrequently to substantially affect $t_{crit}$.

Values are quoted as mean ± s.e.mean, $n$ = number of cells.

## Results

### *Effects of BDM on whole-cell $K_{ATP}$ currents*

Following establishment of the whole-cell configuration, the $K_{ATP}$-current rapidly increased, due to washout of ATP from the cell into the pipette (Trube *et al.*, 1986), reaching a maximum within 2–3 min. The subsequent decline (run-down) of $K_{ATP}$-currents is markedly reduced when divalent cations in the intracellular solution are buffered to nanomolar concentrations (Kozlowski & Ashford, 1990; Williams, 1992) and with our intracellular solution we found that the $K_{ATP}$-current remained stable for periods in excess of 30 min. The effect of BDM was tested only after the whole-cell $K_{ATP}$

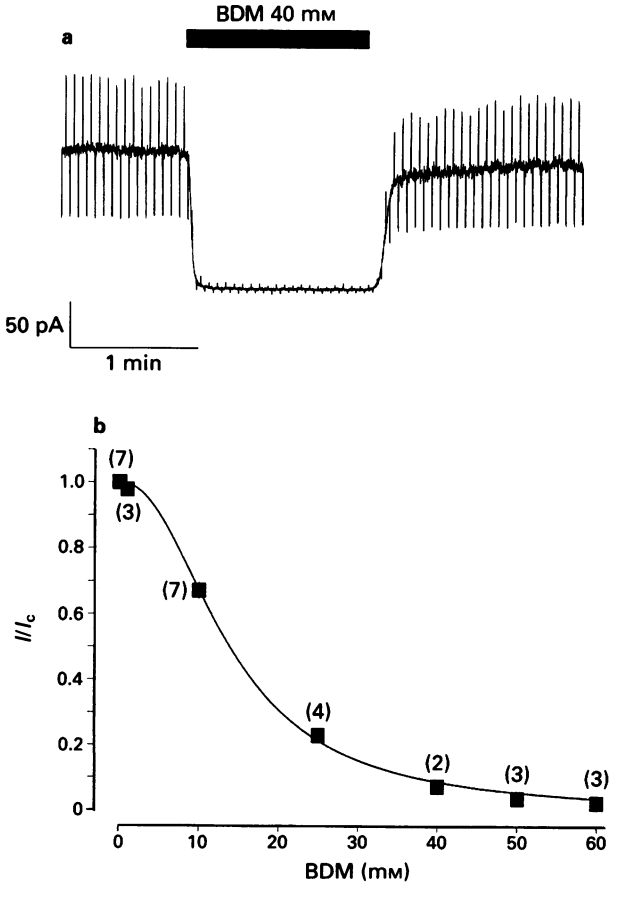


**Figure 1** Effect of BDM on whole-cell $K_{ATP}$-channel currents. (a) Whole-cell currents recorded in response to ±10 mV pulses from a holding potential of −70 mV. Current responses appear as vertical lines due to the compressed time scale. BDM 40 mM was added to the bath during the time indicated by the bar. (b) Relationship between BDM concentration and the whole-cell $K_{ATP}$-channel current ($I$) expressed as a fraction of that in the absence of the drug ($I_c$). The data come from eight experiments and are expressed as mean ± s.e.mean. The latter was within the size of the symbol. The number of observations at each BDM concentration are given within the parentheses. The solid line is a fit to the data to equation 1 of the text with $K_i$ = 13 mM and n = 2.2.

currents had reached a stable level. As shown in Figure 1a the addition of BDM to the extracellular solution caused a rapid and reversible reduction of both the whole-cell $K_{ATP}$-current and the holding current. The relationship between BDM concentration and the whole-cell $K_{ATP}$-current (Figure 1b) was well fitted by the Hill equation (Equation 1) with a $K_i$ of 14 mM and a Hill coefficient of 2.2. Individual fits to four complete dose-response curves gave mean values for $K_i$ of 15 ± 1 mM and of 2.5 ± 0.2 for the Hill coefficient. Whole-cell $K_{ATP}$-currents were unaffected by the addition of 60 mM sucrose to the bath, indicating that the block by high BDM concentrations does not result from the increase in osmotic strength.

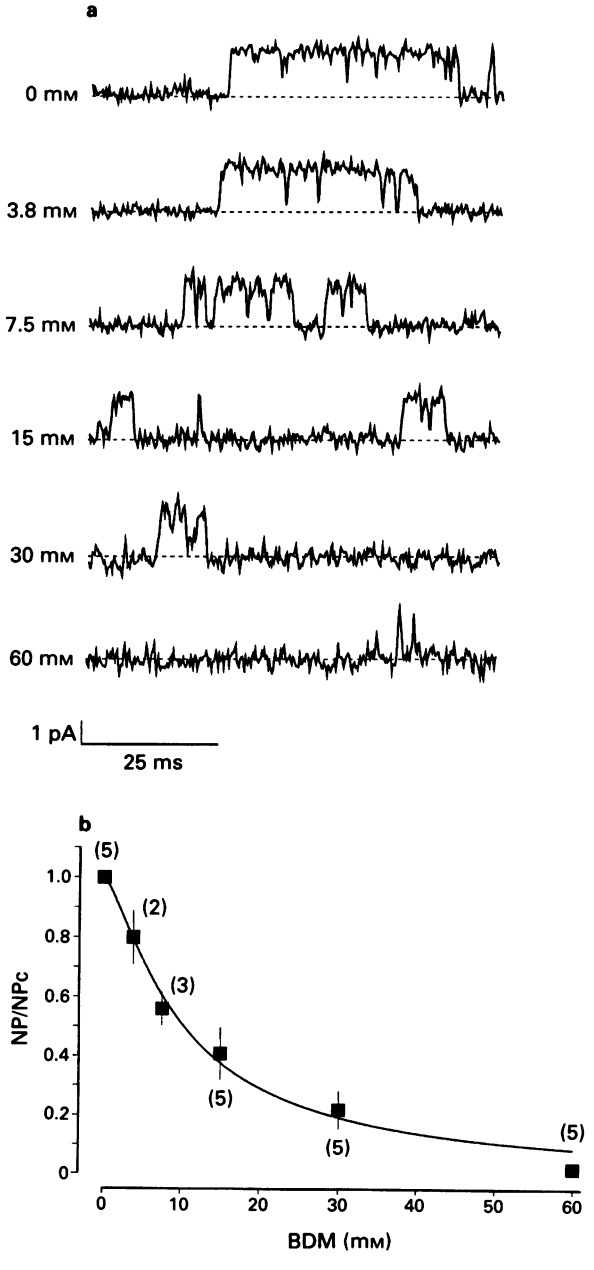


**Figure 2** Effect of BDM on single $K_{ATP}$-channel currents in an outside-out patch held at 0 mV. (a) Single $K_{ATP}$-channel currents recorded at the indicated concentrations of external BDM. The dashed line indicates the channel closed level. Data filtered at 1 kHz for display. (b) Dose-response relationship of external BDM on the channel activity (NP) normalized to that in the absence of the drug (NPc). Each point is the mean from the number of experiments indicated in parentheses. Vertical bars indicate s.e.mean except when this is no larger than the symbol. The solid line is a fit to the Hill equation with a $K_i$ of 10 and a Hill coefficient of 1.4.

### *Effect of external BDM on single $K_{ATP}$-channel currents in outside-out patches*

*Channel activity* In order to study the mechanism of block in more detail, we examined the action of BDM on single $K_{ATP}$-channel currents in outside-out patches held at 0 mV. Increasing the concentration of external BDM reduced the activity of the $K_{ATP}$-channel but had little effect on the single-channel current amplitude (Figure 2a). Figure 2b shows the relationship between BDM concentration and channel activity. The fractional channel activity (NP/NPc) was fitted by the Hill equation (Equation 1) with a $K_i$ of 11 ± 3 mM and a Hill coefficient of 2.0 ± 0.3 ($n = 7$). Inhibition was almost complete at 60 mM BDM with only 2.0 ± 1% ($n = 5$) of channel activity remaining. Sucrose (30 mM) had no effect on channel activity, single-channel current amplitude or single-channel kinetics, supporting the idea that the action of external BDM is not osmotic in origin.

*Kinetics of block* Openings of the $K_{ATP}$-channel are grouped into bursts, which consist of a series of consecutive channel openings separated by fast closures. BDM reduced the burst duration in a dose-dependent manner (Figures 2a and 4b). It also decreased both the number of the openings within a burst and the mean open lifetimes (Figures 3 and 4a).

The distribution of open lifetimes of the $K_{ATP}$-channel at 0 mV (Figure 3) was best described by a PDF consisting of the sum of two exponentials (Equation 3, $k = 2$). This is indicated by the solid line in Figure 3a. In control solution, the time constant of the fast component ($\tau_{o,f}$) illustrated was 0.21 ms (mean 0.19 ± 0.01 ms, $n = 5$) and that of the slower component ($\tau_{o,s}$) was 3.1 ms (mean 3.7 ± 0.4 ms, $n = 5$). BDM had little effect on $\tau_{o,f}$ but greatly reduced $\tau_{o,s}$; in this particular patch, 30 mM BDM reduced $\tau_{o,s}$ from 3.1 to 1.9 ms (Figure 3c). In five patches 30 mM BDM reduced $\tau_{o,s}$ by 70% to 1.1 ± 0.3 ms, but we could not detect a difference in $\tau_{o,f}$, which remained at 0.24 ± 0.1 ms. Although the relative areas (see Methods) of the two open lifetime components differed between patches they were unchanged by BDM (Figure 3a,c).

The decrease in $\tau_{o,s}$ by BDM is reminiscent of the block of $K_{ATP}$-channels produced by external $Ba^{2+}$ (Quayle *et al.*, 1988) and suggests the following reaction scheme for the action of external BDM:

$$\underset{\text{closed}}{C} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{open}}{O} \underset{k_{-b,1}}{\overset{k_{b,1}[BDM]^n}{\rightleftharpoons}} \underset{\text{blocked}}{B} \qquad \text{(Scheme 1)}$$

where O represents the open state associated with $\tau_{o,s}$, the long open time component, C is the sum of the adjacent closed states and B is the blocked state produced by BDM. This scheme predicts that the reciprocal of $\tau_{o,s}$ will be given by:

$$\frac{1}{\tau_{o,s}} = k_{-1} + k_{b,1}\,[BDM]^n \qquad (6)$$

where $k_{-1} = 1/\tau_{o,s}$ in the absence of BDM. Figure 4a shows a representative plot of $1/\tau_{o,s}$ against BDM concentration. The solid line is the best fit of Equation 6 to the data with $k_{b,1} = 8.4\ mM^{-1}\ s^{-1}$ and $n = 1.6$, taking $\tau_{o,s}$ to be 4.6 ms in the absence of BDM (the value measured in the patch illustrated). Mean values for $k_{b,1}$ of 9.0 ± 0.5 $mM^{-1}s^{-1}$ and of 1.5 ± 0.1 for n were obtained in three patches.

The distribution of closed times was described by a PDF consisting of the sum of three exponentials (Equation 3, $k = 3$), representing the short, intermediate and long closures and having time constants $\tau_{c,f}$, $\tau_{c,i}$ and $\tau_{c,s}$ respectively. The fast closures separating openings within bursts are defined by $\tau_{c,f}$, while $\tau_{c,i}$ and $\tau_{c,s}$ represent the closures that separate bursts of openings. The presence of two components of closures between bursts suggest that the bursts are grouped together in clusters. In control solution (Figure 3b), time constants of 0.2 ms for $\tau_{c,f}$, 2.8 ms for $\tau_{c,i}$ and 13 ms for $\tau_{c,s}$

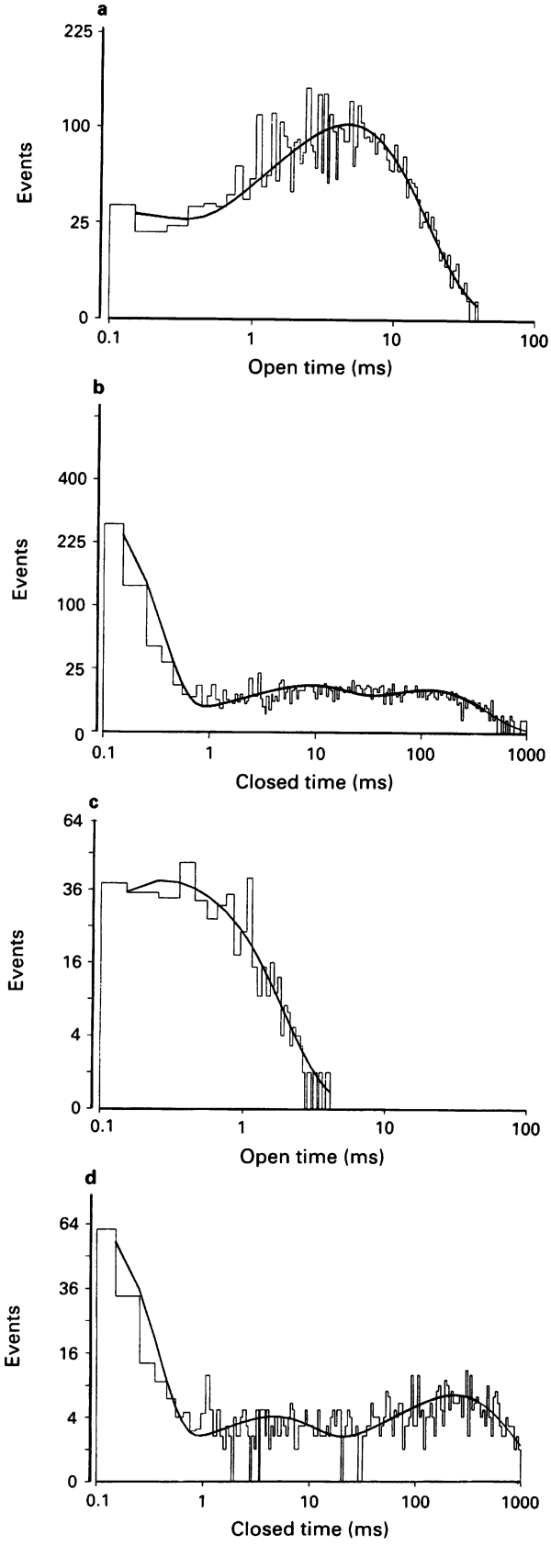


**Figure 3** Effect of external BDM on $K_{ATP}$-channel kinetics. Open times (a and c) and closed times (b and d) are log-binned according to equation 2. The solid lines are fits to the appropriate PDF using equation 3 as described in the text. (a) and (b) Open time and closed time distributions in the absence of BDM; (c) and (d) open time and closed time distributions in the presence of 30 mM BDM. All data come from the same patch. The data were filtered at 2.5 kHz for analysis. See text for fitted parameters.

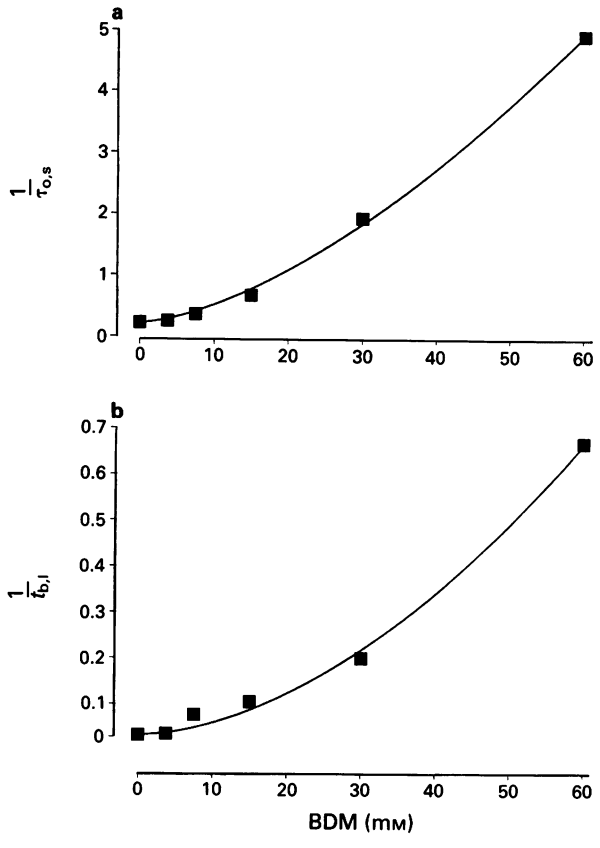


**Figure 4** Effect of external BDM on the mean open time, $\tau_{o,s}$, and mean burst, $t_{b,l}$. (a) Plot of $1/\tau_{o,s}$ against BDM concentration. The solid line is a fit of equation 6 to the data with $k_{b,l} = 8.4\ mM^{-1}\ s^{-1}$ and n = 1.6. (b) Plot of $1/\tau_{b,l}$ against BDM concentration. The solid line is a fit of equation 6 to the data with $k_{b,2} = 0.44\ mM^{-1}\ s^{-1}$ and n = 1.8. Data are from the same patch as (a).

were obtained at 0 mV. BDM (30 mM) had no effect on $\tau_{c,f}$ (mean value 0.20 ± 0.05 ms in the absence and 0.19 ± 0.04 ms in the presence of 30 mM BDM, $n = 5$). BDM, however, greatly prolonged the two slower time constants, $\tau_{c,i}$ and $\tau_{c,s}$; in this particular patch these increased to 5.5 ms and 40 ms respectively (Figure 3d).

Burst of $K_{ATP}$-channel openings were analysed using a burst criteria time, $t_{crit}$, calculated for each experimental condition from Equation 5. The distribution of the burst lengths were fitted with a PDF consisting of the sum of two exponential components, comprising the short and long bursts (Equation 3, $k = 2$), with mean lengths, $t_{b,s}$ and $t_{b,l}$ respectively. In both the presence and absence of BDM, the mean lifetime of the short burst, $t_{b,s}$, was almost identical to that of the mean fast open time, $\tau_{o,f}$ ($t_{b,s} = 0.22 \pm 0.04$ ms, $\tau_{o,f} = 0.19 \pm 0.01$ ms, $n = 5$ in control solution). Furthermore, the short bursts possessed a mean of one opening per burst (1.03 ± 0.02, $n = 5$) which suggest that the short bursts are simply single openings of the $K_{ATP}$-channel to the short open state ($\tau_{o,f}$). A similar phenomena has been described in skeletal muscle (Spruce *et al.*, 1985). The lifetime of the short bursts did not appear to be affected by 30 mM BDM (mean 0.23 ± 0.07 ms, $n = 5$), consistent with the lack of effect of the drug on $\tau_{o,f}$.

The longer component of burst lengths consisted of several openings per burst. Both the mean burst length, $t_{b,l}$ and the number of openings per burst decreased with increasing BDM concentration. In five patches the mean burst length was 19 ± 5 ms in control and 4.0 ± 0.9 ms in the presence of 30 mM BDM. The mean number of openings per long burst was 5.0 ± 0.7 in the absence of BDM and was reduced to 3.9 ± 0.4 in the presence of the drug ($n = 7$, $P = 0.016$).

Figure 4b illustrates a plot of $1/t_{b,l}$ versus BDM concentration. The solid line is a fit to the data of:

$$\frac{1}{t_{b,l}} = k_{-2} + k_{b,2}\,[\mathrm{BDM}]^n \qquad (7)$$

with $k_{b,2} = 0.44\ \mathrm{mM}^{-1}\,\mathrm{s}^{-1}$ and $n = 1.8$ (mean $k_{b,2} = 0.66 \pm 0.12\ \mathrm{mM}^{-1}\,\mathrm{s}^{-1}$ and $n = 1.9 \pm 0.1$, $n = 3$). Equation 7 is derived from the following reaction scheme:

$$\underset{\text{closed}}{C} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{\text{burst}}{\text{O-O}} \underset{k_{-b,2}}{\overset{k_{b,2}[\mathrm{BDM}]^n}{\rightleftharpoons}} \underset{\text{blocked}}{B} \quad \text{(Scheme 2)}$$

where O-O represents openings that collectively form a burst, C is the sum of the interburst closed states and B is the blocked state produced by BDM. In the absence of BDM, $k_{-2} = 1/t_{b,l}$, where $t_{b,l}$ was 30 ms in the example illustrated in Figure 4b.

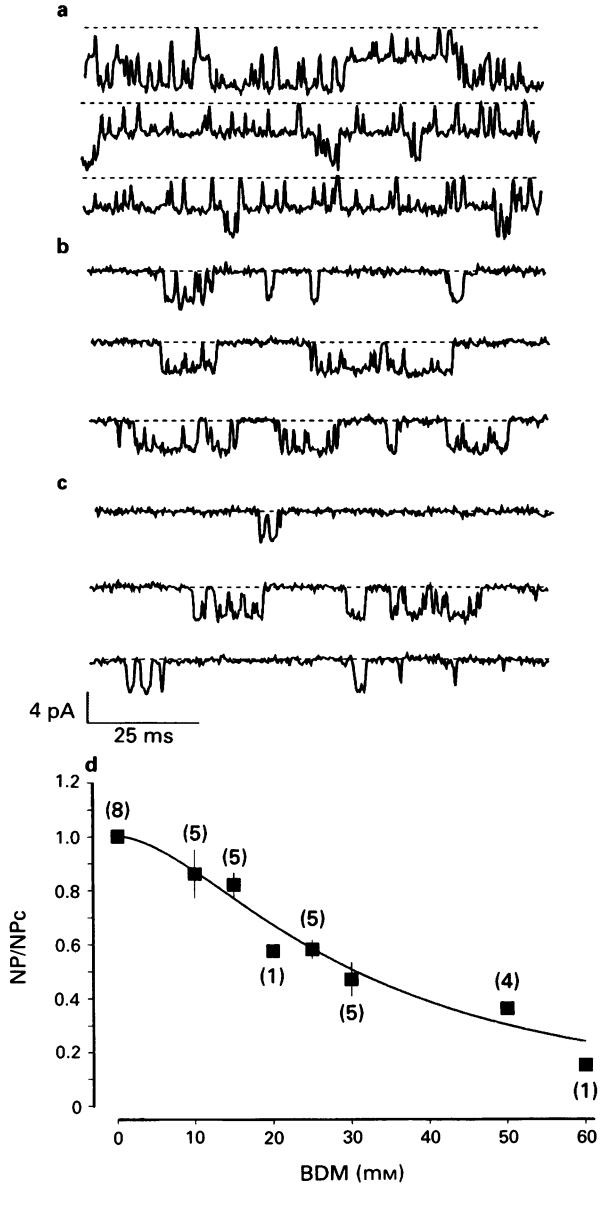


**Figure 5** Effect of internal BDM on single $K_{ATP}$-channel currents recorded from an inside-out patch held at −70 mV. The dashed line indicates the channel closed level. (a) Control; (b) 30 mM BDM; (c) 60 mM BDM; (d) channel activity, NP, normalized to that in the absence of the drug, NPc, plotted against the internal BDM concentration. Each point is the mean from the number of experiments indicated in parentheses. Vertical bars indicate s.e.mean except when this is no larger than the symbol. The solid line is a fit to equation 1 of the text with $K_i = 31$ mM and $n = 1.7$.

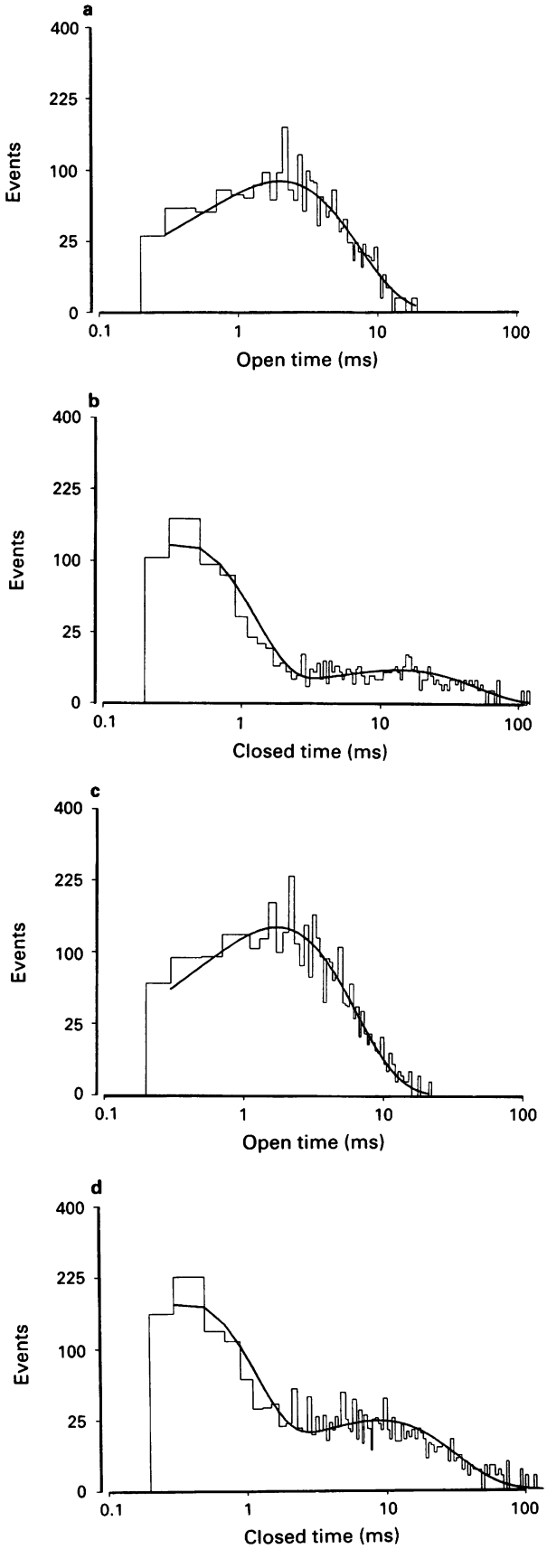


**Figure 6** Effect of internal BDM on $K_{ATP}$-channel kinetics. Open times (a and c) and closed times (b and d) are log-binned according to equation 2. The solid lines are fits to the appropriate PDF using equation 3, as described in the text. (a) and (b) are the open time and closed time distributions in the absence of BDM; (c) and (d) are the open time and closed time distributions from the same patch in the presence of 30 mM internal BDM. See text for fitted parameters.

*Voltage-dependence of block* The block by BDM was voltage-independent, since neither the reduction in channel activity (NP/NPc = 0.17 at 0 mV compared with NP/NPc = 0.15 at −30 mV) or the changes in channel kinetics caused by 30 mM BDM varied with the holding potential. Furthermore, 30 mM BDM had little effect on the single-channel current amplitude or the variance of the open-channel current at potentials between −30 mV and +30 mV. The similarity between the $K_i$ for channel inhibition determined in whole-cell (15 mM) and outside-out patch (11 mM) recordings further supports the idea that the block by external BDM is voltage-independent since the holding potentials were −70 mV and 0 mV respectively in these configurations.

### *Effect of intracellular BDM on single $K_{ATP}$-channels in inside-out patches*

*Channel activity* When applied to the cytosolic face of an inside-out patch, BDM rapidly and reversibly reduced the activity of the $K_{ATP}$-channel. Figure 5a–c shows single-channel currents recorded at different BDM concentrations and Figure 5d the relationship between channel activity and BDM concentration. The data were fitted to Equation 1 with a mean $K_i$ of 31 ± 2 mM and a Hill coefficient of 2.0 ± 0.4 ($n = 8$). The effect of internal BDM on $K_{ATP}$-channels was thus less potent than that of externally applied BDM where the $K_i$ was 11 mM.

Internal BDM did not affect the single-channel current amplitude (Figure 5a, b and c).

*Kinetics* The kinetics of the $K_{ATP}$-channel in the inside-out patch differed from those in the outside-out patch. This difference may be explained by the different holding potentials used (Spruce *et al.*, 1985; Davies *et al.*, 1989), by the different $K^+$ gradients used or by both, that is by differences in the electromotive force (Zilberter *et al.*, 1988). At −70 mV, the distribution of open lifetimes of the $K_{ATP}$-channel in the absence of BDM was described by a PDF that consisted of only one exponential component (Equation 2, $k = 1$) with a single time constant, $\tau_o$. A representative open time distribution in control solution is shown in Figure 6a; 30 mM BDM had only a minor effect on the open channel lifetime, reducing $\tau_o$ by 14% from 1.8 ± 0.1 ms ($n = 8$, Figure 6a) to 1.6 ± 0.1 ms ($n = 4$, Figure 6b).

Although channel openings were grouped into bursts, the bursts did not appear to exhibit clustering behaviour, the distribution of closed times being best described by a PDF consisting of the sum of two exponentials (Equation 3, $k = 2$). Representative examples are shown in Figure 6b and d. The short closures (gaps) within bursts and the long closures between bursts are represented by time constants, $\tau_{c,g}$ and $\tau_{c,l}$ respectively.

BDM had no effect on $\tau_{c,g}$, which had a mean value of 0.41 ± 0.01 ms ($n = 8$) in control solution and of 0.40 ± 0.02 ms in the presence of 50 mM BDM ($n = 5$). Due to the high level of channel activity in the majority of inside-out patches it was not possible to obtain an accurate measure of the long closed time constant, $\tau_{c,l}$ in control solution, but it was apparent from the records that BDM caused a graded reduction in burst length (Figure 5a–c).

## Discussion

We show here that the block of $K_{ATP}$-channel activity by BDM is fully reversible under non-phosphorylating conditions in both whole-cell and excised patch recordings. This indicates that BDM blocks channel activity by a mechanism unrelated to its action as a phosphatase and instead functions as a direct blocker of the $K_{ATP}$-channel. Similar findings have been reported for ventricular myocytes, where BDM causes a rapid and reversible block of the $K_{ATP}$-channel in the inside-out patch configuration with a $K_i$ of 21.3 mM and a Hill coefficient of 1.8 (Hardin & Nichols, 1992). These values are very close to those that we observe ($K_i = 31$ mM and $n = 2$) in the same patch configuration. The higher potency of BDM in blocking ventricular calcium currents ($IC_{50} = 5.8$ mM, Chapman, 1993) may reflect a phosphatase action, as a shift in the potency of block by BDM occurs with conditions that would be expected to affect both the degree and stability of calcium channel phosphorylation (Chapman, 1993). We have not explored any possible actions of BDM under phosphorylating conditions.

The ability of BDM to act as a chemical phosphatase has often been exploited to investigate the role of protein phosphorylation in the regulation of ionic channel activity. Our results imply, however, that it is essential to exclude the possibility that the drug also acts as a direct channel blocker in such studies.

The ability of BDM to reduce the open-channel lifetime, the burst duration and the channel open probability without affecting either the single-channel current amplitude or the fast closed time is reminiscent of the actions of barium (Quayle *et al.*, 1988) and tolbutamide (Gillis *et al.*, 1989) on this channel and suggests that like these agents BDM behaves as a 'slow' blocker according to the nomenclature of Hille (1992).

Although BDM is moderately lipid soluble, with a water/octanol partition coefficient of 16 (Leo *et al.*, 1971), our data suggest that the drug may have both an external and internal site of action. The $K_i$ for channel inhibition by external BDM was 11 mM in outside-out patches and 15 mM in whole-cell recordings suggesting that BDM acts at an extracellular site in the latter case. Internal BDM was less effective at inhibiting channel activity, the $K_i$ being 31 mM. This lower sensitivity to internal BDM cannot be explained by the difference in holding potential between inside-out (−70 mV) and outside-out patches (0 mV) since the whole-cell currents were also recorded at a holding potential of −70 mV. Furthermore, the effects of both internal and external BDM are voltage-independent. The effect of BDM on the channel kinetics was also dependent on whether the drug was applied to the external or internal face of the channel. Thus extracellular applied BDM markedly reduced the longer of the two channel open lifetimes, whereas BDM had only a minor effect on $\tau_o$ when applied intracellularly.

An alternative explanation of the lower efficacy of BDM when applied intracellularly, is that there is a single site of action for BDM the affinity of which varies with the extracellular $K^+$ concentration. In this model external $K^+$ ions would allosterically reduce the binding of BDM, either by acting at an extracellular binding site or within the pore. This would explain why the $K_i$ for BDM inhibition is lower in inside-out patches (where the concentration of $K^+$ is 140 mM) than in outside-out or whole-cell recordings, where the concentration of $K^+$ is only 5 mM.

The Hill coefficient for both external and internal block was greater than one and suggests the cooperative action of more than one BDM molecule is required to inhibit the channel.

Our analysis of the kinetics of the $K_{ATP}$-channel in outside-out patches from β-cells is the first to be documented at a high temporal resolution. We observed that the channel possesses both a short and a long lived open state with mean lifetimes of 0.19 ms and 3.7 ms respectively. These openings occur in bursts, with a mean intraburst closed time of 0.2 ms, and there is an excess of bursts in which only a single opening is present. Similar kinetics have been described for inward $K_{ATP}$-channel currents in inside-out patches from frog skeletal muscle by Spruce *et al.* (1985).

We attempted to quantify the effects of BDM on channel kinetics using an empirical model in which the blocked state represents a new closed state entered directly from the open state. A model in which BDM facilitates entry into an existing closed state can also explain our results and would yield the same rate constants. Theoretically these two models may be distinguished because the former predicts the existence of

an additionally closed state, that produced by BDM block. No additional closed state was observed in the presence of BDM in our experiments. In practice however, a new closed state may not be resolved if it is of a duration similar to that of the closed states existing in the absence of BDM. Therefore it is not possible to distinguish between the two models.

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# Comparison of tachykinin $NK_1$ and $NK_2$ receptors in the circular muscle of the guinea-pig ileum and proximal colon

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**1** The aim of this study was the pharmacological characterization of tachykinin $NK_1$ and $NK_2$ receptors mediating contraction in the circular muscle of the guinea-pig ileum and proximal colon. The action of substance P (SP), neurokinin A (NKA) and of the synthetic agonists [Sar$^9$]SP sulphone, [Glp$^6$,Pro$^9$]SP(6-11) (septide) and [βAla$^8$]NKA(4-10) was investigated. The affinities of various peptide and nonpeptide antagonists for the $NK_1$ and $NK_2$ receptor was estimated by use of receptor selective agonists.

**2** The natural agonists, SP and NKA, produced concentration-dependent contraction in both preparations. $EC_{50}$ values were 100 pM and 5 nM for SP, 1.2 nM and 19 nM for NKA in the ileum and colon, respectively. The action of SP and NKA was not significantly modified by peptidase inhibitors (bestatin, captopril and thiorphan, 1 μM each).

**3** Synthetic $NK_1$ and $NK_2$ receptor agonists produced concentration-dependent contraction of the circular muscle of the ileum and proximal colon. $EC_{50}$ values were 83 pM, 36 pM and 10 nM in the ileum, 8 nM, 0.7 nM and 12 nM in the colon for [Sar$^9$]SP sulphone, septide and [βAla$^8$]NKA(4-10), respectively. The pseudopeptide derivative of NKA(4-10), MDL 28,564 behaved as a full or near-to-full agonist in both preparations, its $EC_{50}$s being 474 nM and 55 nM in the ileum and colon, respectively.

**4** Nifedipine (1 μM) abolished the response to septide and [Sar$^9$]SP sulphone in the ileum and produced a rightward shift and large depression of the response in the colon. The response to [βAla$^8$]NKA(4-10) was abolished in the ileum and largely unaffected in the colon.

**5** The $NK_1$ receptor antagonists, (±)-CP 96,34, FK 888 and GR 82,334 competitively antagonized the response to septide and [Sar$^9$]SP sulphone in both preparations without affecting that to [βAla$^8$]NKA(4-10). In general, the $NK_1$ receptor antagonists were significantly more potent toward septide than [Sar$^9$]SP sulphone in both preparations.

**6** The $NK_2$ receptor antagonists, GR 94,800 and SR 48,968 selectively antagonized the response to [βAla$^8$]NKA(4-10) without affecting that to [Sar$^9$]SP sulphone or septide in the ileum and colon. SR 48,968 produced noncompetitive antagonism of the response to the $NK_2$ receptor agonist in the ileum and competitive antagonism in the colon.

**7** MEN 10,376 and the cyclic pseudopeptide MEN 10,573 antagonized in a competitive manner the response to [βAla$^8$]NKA(4-10) in the ileum and colon. While MEN 10,573 was equipotent in both preparations, MEN 10,376 was significantly more potent in the colon than in the ileum. MEN 10,376 was also effective against septide in both preparations, without affecting the response to [Sar$^9$]SP sulphone. MEN 10,573 antagonized the response to [Sar$^9$]SP sulphone and septide in both preparations, $pK_B$ values against septide being intermediate, and significantly different from, those measured against [βAla$^8$]NKA(4-10) and [Sar$^9$]SP sulphone.

**8** These findings show that tachykinin $NK_1$ and $NK_2$ receptors mediate contraction of the circular muscle of the guinea-pig ileum and colon. In both preparations $NK_1$ receptor antagonists display higher apparent affinity when tested against septide than [Sar$^9$]SP sulphone. These findings are compatible with the proposed existence of $NK_1$ receptor subtypes in guinea-pig, although alternative explanations (e.g. agonist binding to different epitopes of the same receptor protein) cannot be excluded at present. Furthermore, an intraspecies heterogeneity of the $NK_2$ receptor in the circular muscle of the guinea-pig ileum and colon is suggested.

**Keywords:** Tachykinins; $NK_1$ receptor; $NK_2$ receptor; guinea-pig ileum; guinea-pig colon; circular muscle; tachykinin receptor antagonist

## Introduction

Three main types of receptors, $NK_1$, $NK_2$ and $NK_3$, mediate the actions of tachykinins which are encoded by their common, C-terminal sequence (Maggi *et al.*, 1993a for review). In the circular muscle of the guinea-pig ileum, the contractile response to $NK_1$ or $NK_2$ receptor stimulation occurs through a direct excitation of smooth muscle cells, while the $NK_3$ receptor-mediated contraction is indirect, being produced through activation of intramural neurones/nerves (Maggi *et al.*, 1990a).

The lack of a potent and selective $NK_3$ antagonist still hampers the full pharmacological and physiological characterization of this receptor in the intestine. On the other hand, the use of selective antagonists has delineated the hierarchical role of substance P (SP) and neurokinin A (NKA), via $NK_1$ and $NK_2$ receptors, respectively, as enteric excitatory transmitters in the guinea-pig ileum and colon (Bartho *et al.*, 1992; Zagorodnyuk *et al.*, 1993a). The recent developments in the pharmacology of tachykinin receptors, suggesting the possible existence of receptor subtypes (Maggi *et al.*, 1993a for review), make it important to perform a systematic inves-

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tigation of the apparent affinities of the various antagonists toward various tachykinin receptor agonists.

Species-related pharmacological differences, detected by variable affinities of competitive receptor antagonists, have emerged for both $NK_1$ and $NK_2$ receptors (Maggi *et al.*, 1993a for review). The nonpeptide antagonist, (±)-CP 96,345 (Snider *et al.*, 1991) is about 100 times more potent at $NK_1$ receptor expressed in e.g. man and guinea-pigs than rats or mice (Gitter *et al.*, 1991; Patacchini *et al.*, 1992; Barr & Watson, 1993), while the nonpeptide antagonist, RP 67,580 (Garret *et al.*, 1991) is more potent at rat than guinea-pig or human $NK_1$ receptors. For $NK_2$-receptors, certain peptide (e.g. MEN 10,376, Maggi *et al.*, 1991a) and nonpeptide antagonists (SR 48,968; Emonds-Alt *et al.*, 1992; Maggi *et al.*, 1993b) are more potent at human, bovine, guinea-pig and rabbit $NK_2$ receptors than at rat or hamster $NK_2$ receptors, while the cyclic peptide antagonist, L 659,877 (Williams *et al.*, 1988) or the cyclic pseudopeptide MEN 10,573 (Quartara *et al.*, 1992a) present the converse pattern of affinities (Maggi *et al.*, 1990b; Van Giersbergen *et al.*, 1991).

These species-related variations in antagonist potencies probably reflect the existence of species-related changes in the primary sequence of the $NK_1$ and $NK_2$ receptor protein: point mutation studies have established that species-related changes in aminoacid sequence at two discrete positions of the $NK_1$ receptor are responsible for the species-dependent variations in the affinities of CP 96,345 and RP 67,580 (Fong *et al.*, 1992b). The molecular bases for species-related variations in antagonists potencies at the $NK_2$ receptor have not been similarly established.

In addition, pharmacological evidence supports the idea that receptor subtypes (intraspecies heterogeneity) may exist for tachykinin $NK_1$ and $NK_2$ receptors. For example, septide or [$Glp^6$,$Pro^9$]SP(6-11), a synthetic ligand originally developed as a selective $NK_1$ receptor agonist, displays a contractile activity in the guinea-pig ileum which cannot easily be explained by an interaction with the 'classical' $NK_1$ receptor (Petitet *et al.*, 1992). Petitet *et al.* (1992) proposed that a novel, 'septide-sensitive', tachykinin receptor exists in the guinea-pig ileum. A 'septide-sensitive' receptor was also detected in the rat urinary bladder, which is recognized by $NK_1$ receptor antagonists with higher affinity than that observed toward a 'classical' $NK_1$ receptor agonist like [$Sar^9$]SP sulphone (Meini *et al.*, 1994). Likewise, (±)-CP 96,345 was found to be significantly more potent in antagonizing septide than [$Sar^9$]SP sulphone in the circular muscle of the guinea-pig ileum (Maggi *et al.*, 1993c). Thus, the 'septide-sensitive' receptor may be an $NK_1$ receptor subtype.

An intraspecies heterogeneity of the $NK_2$ receptor has also been evidenced by pharmacological studies (Xu *et al.*, 1991; Brunelleschi *et al.*, 1992; Nimmo *et al.*, 1992); it is at present uncertain whether or not these examples fit with the criteria used to define species-related differences in the $NK_2$ receptor (Maggi *et al.*, 1993a for review).

The aim of this study was to characterize the tachykinin $NK_1$ and $NK_2$ receptors mediating contraction in the circular muscle of two distinct intestinal segments (ileum and colon) from the same species (guinea-pig) by use of synthetic receptor selective agonists to stimulate the receptors and a panel of antagonists of both peptide and non peptide nature to block them. Some of these results were presented at the Third meeting of the European Neuropeptide Club, Cambridge, April 5–7, 1993.

## Methods

Male albino guinea-pigs weighing 200–250 g were stunned and bled. A 10–15 cm length of ileum and 2–3 cm segment of proximal colon were excised and placed in warmed (37°C) and oxygenated (96% $O_2$ and 4% $CO_2$, pH 7.4) Krebs solution of the following composition (mM): NaCl 119, $NaHCO_3$ 25, $KH_2PO_4$ 1.2, $MgSO_4$ 1.5, KCl 4.7, $CaCl_2$ 2.5 and glucose 11.

### *Guinea-pig ileum*

The longitudinal muscle and attached myenteric plexus were removed from the ileum as described by Paton & Zar (1968) and discarded. All experiments were performed on longitudinal muscle-myenteric plexus-free ileal rings (2–3 mm wide) in the presence of 10 μM indomethacin. The ileal rings were suspended in 5 ml baths for isolated organs by means of two stainless steel hooks and connected to an isotonic transducer (load 5 mN) to record the mechanical activity of the circular muscle. The preparations were allowed to equilibrate for 90 min with renewal of the bathing solution every 15 min. The rings were then exposed to 10 μM carbachol at 15 min intervals until two reproducible responses were observed. This usually occurred at 150–180 min from setup. The response to 1 μM [βAla$^8$]NKA(4-10) was also determined in some experiments before addition of various tachykinin receptor agonists. The addition of tachykinin receptor agonists produced an increase in both phasic activity and a tonic contraction of the circular muscle of the ileum (Figure 1). To enable a more accurate quantitative evaluation of the effect of agonists, the signal recorded from the isotonic transducer was delivered to a Basile 7083 integrator and the contractile activity of the rings was integrated every 10 s. The level of integration was set at about 10% of the spontaneous mechanical activity of the rings. The overall effect produced by the

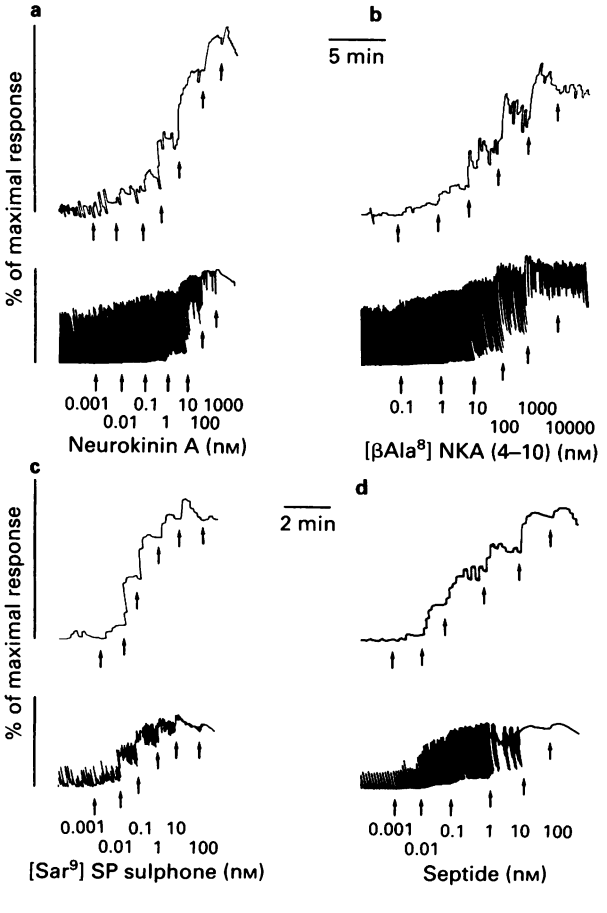


**Figure 1** Typical tracings illustrating the contractile response of the circular muscle of the guinea-pig ileum to cumulative addition of neurokinin A (a), [βAla$^8$]NKA(4-10) (b), [$Sar^9$]SP sulphone (c) and septide (d). For each panel the lower tracing shows the mechanical activity recorded from isotonic transducers and the upper tracing the integrated mechanical activity.

various concentrations of agonists was calculated from the integrated mechanical activity.

### *Guinea-pig colon*

Strips of mucosa-free circular muscle from the proximal colon were prepared as described in a previous study (Giuliani *et al.*, 1993). All experiments were performed in the presence of 10 μM indomethacin. The strips were suspended in 5 ml organ baths and connected to isotonic transducers (load 10 mN). After setup, the strips were allowed to equilibrate for 90 min with renewal of the bathing solution every 15 min. The strips were then exposed to 80 mM KCl at 15 min intervals until two reproducible responses were observed. This usually occurred at 120 min from setup. The response to 1 μM [βAla$^8$]NKA(4-10) was also determined in some experiments before addition of various tachykinin receptor agonists.

### *Experimental protocol*

In both preparations, concentration-response curves to the agonists were constructed in a cumulative manner. The concentration of the agonist was increased by a factor of 10 for each successive dose of the cumulative curve, the next dose being added when the effect of the preceding one had reached its maximum. At the end of the concentration-response curve, the agonist was removed by repeated washing. Preliminary experiments showed that a second concentration-response curve to the various agonists produced at 20 min interval from the first one was fully reproducible in both ileum and colon. For each antagonist, contact time before application of the agonist was 15 min. In some experiments, performed with SR 48,968 in the ileum, contact time was 45 min (see results). In some experiments performed with the natural agonists, SP and NKA, a control curve was established first, then a mixture of peptidase inhibitors (bestatin, captopril and thiorphan 1 μM each) was added and a new curve obtained 15 min later.

### *Experiments with nifedipine*

In a previous electrophysiological study (Zagorodnyuk *et al.*, 1993b) we showed that electrical and contractile responses to [Sar$^9$]SP sulphone and [βAla$^8$]NKA(4-10) in the circular muscle of guinea-pig proximal colon differ in a number of characteristics, including sensitivity to nifedipine. Owing to the possibility that septide stimulates a novel type of tachykinin receptor (Petitet *et al.*, 1992), we assessed the effect of nifedipine (1 μM) on the concentration-response curve to [Sar$^9$]SP sulphone, septide and [βAla$^8$]NKA(4-10). Responses are expressed as % of the maximal response produced by the agonist in the control curve. Contact time of nifedipine (45 min) was determined in preliminary experiments showing full blockade of the contractile response to KCl (80 mM).

### *Data evaluation and statistical analysis*

The increase in integrated mechanical activity (ileum) or tone (colon) produced by the various agonists was expressed as % of the response to the internal standard, [βAla$^8$]NKA(4-10), for experiments aiming to compare the maximal effects ($E_{max}$) produced by different agonists. $EC_{50}$ and 95% CL were calculated by the least square method. Dose-ratios were calculated and the Schild plots constructed for each agonist/antagonist pair tested. When the results of this analysis were consistent with competitive antagonism (slopes of Schild plot not significantly different from unity) $pK_B$ values were calculated by the constrained Schild plot method; $pK_B$ values are presented in Tables 2 and 3 with the corresponding 95% CL. In the ileum, SR 48,968 caused nonparallel rightward shifts of the concentration-response curves and decreased the $E_{max}$ to [βAla$^8$]NKA(4-10). The method described by Kenakin (1987a) for noncompetitive and/or pseudoirreversible antagonist was used to evaluate the equilibrium dissociation constant ($K_B$) of SR 48,968. In practice, a double-reciprocal plot of equieffective concentrations of agonist (A) in the absence (1/A) and in the presence (1/A') of SR 48,968 (B) was constructed, and $K_B$ derived from the equation: $K_B = [B]/\text{slope} - 1$ (Kenakin, 1987a). In order to obtain more accurate estimates of $K_B$ we selected the experiments in which $E_{max}$ to the agonist was depressed to 50% or less than 50% of control by SR 48,968. For the same reason, equieffective concentrations of [βAla$^8$]NKA(4-10) were selected from the upper region of the depressed dose-response curve, as suggested by Kenakin (1987a).

The statistical significance of differences in $pK_B$ values obtained for a given antagonist toward different agonists was evaluated by comparing the Schild plot regression lines by means of analysis of covariance to detect differences in the elevation (position) and slopes of regression lines, as described by Kenakin (1987b).

### *Drugs*

Drugs used were: bestatin, substance P, [Sar$^9$]SP sulphone and septide ([pGlu$^6$,Pro$^9$]SP(6-11)) (Peninsula), GR 82,334 ([D-Pro$^9$,(spiro-γ-lactam)Leu$^{10}$,Trp$^{11}$]physalaemin(1-11)) (Hagan *et al.*, 1991), (Neosystem), amastatin, indomethacin and captopril (Sigma), thiorphan (Bachem), carbachol (Merck).

RP 67,580 {(3α**R**,7α**R**)-7, 7-diphenyl-2-[1-imino-2-(2-methoxyphenyl)ethyl] perhydroisoindol-4-one} (Garret *et al.*, 1991) was a kind gift of Dr C. Garret, Rhone Poulenc, Vitry, France.

SR 48,968 ((S)-N-methyl-*N*[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl] benzamide) (Emonds-Alt *et al.*, 1992) or Dr X. Emonds-Alt, Sanofi Recherche, Montpellier, France. GR 94,800 or PhCO-Ala-Ala-D-Trp-Phe-D-Pro-Pro-NleNH$_2$ (McElroy *et al.*, 1992) was a kind gift from Dr R.M. Hagan, GGR, Ware England.

(±)-CP 96,345 or ((2S,3S) -*cis*-2-(diphenylmethyl)-*N*-[ (2-methoxyphenyl)-methyl]-1-azabicyclo [2.2.2] octan-3-amine] (Snider *et al.*, 1991), neurokinin A, MEN 10,376 ([Tyr$^5$,D-Trp$^{6,8,9}$,Lys$^{10}$]NKA(4-10), MEN 10,573 (cyclo(Leu$\Psi$[CH$_2$NMe] Leu-Gln-Trp-Phe-Gly)), MDL 28,564 ((Leu$^9\Psi$(CH$_2$NH)Leu$^{10}$] NKA(4-10)), FK 888 ((2-(N-Me)indolil)-CO-Hyp-Nal-NMe-Bzl) and [βAla$^8$]NKA(4-10) were synthesized in the Chemistry Department of Menarini Pharmaceuticals.

## Results

### *Circular muscle of the ileum*

*Effect of agonists* In the presence of 10 μM indomethacin, longitudinal muscle-myenteric plexus-free ileal rings developed regular spontaneous phasic contractions (4–10 min$^{-1}$), their amplitude ranging between 20–60% of the maximal response to 10 μM carbachol. SP and NKA produced a concentration-dependent contraction (Figure 1 and 2, Table 1), SP being about 10 times more potent than NKA. The action of SP and NKA was not modified by bestatin, captopril and thiorphan (1 μM each). In fact, the $EC_{50}$s of SP were 100 pM (63–174 pM are 95% CL) and 99 pM (48–263 pM) in the absence and presence of peptidase inhibitors, respectively ($n = 6$ in each group). The corresponding values for NKA were 1.16 nM (0.85–1.38 nM) and 0.83 nM (0.70–1.11 nM), respectively ($n = 6$). When SP or NKA (both 1 μM) were added to the bath as a single concentration, $E_{max}$ was not different from that produced by [βAla$^8$]NKA(4-10), inducing a total closure of the ileal lumen. The $E_{max}$ to NKA or [βAla$^8$]NKA(4-10) during the cumulative concentration-response curve equalled that observed in response to single administration of 1 μM concentration of each agonist. The $E_{max}$ to SP during the cumulative concentration-response curve was slightly less than that produced by [βAla$^8$]NKA(4-

**Table 1** $EC_{50}$s (95% confidence limits in brackets) and $E_{max}$ (expressed as % of the maximal response to [βAla$^8$]NKA(4-10), 1 μM) of natural tachykinins and receptor-selective synthetic agonists-induced contraction in the circular muscle of the guinea-pig ileum and colon

| *Agonist* | *Ileum* | | *Colon* | |
|---|---|---|---|---|
| | $EC_{50}$ | $E_{max}$ | $EC_{50}$ | $E_{max}$ |
| Substance P | 100 pM (63–174) | 69 ± 4* | 5 nM (4–6) | 73 ± 4* |
| Neurokinin A | 1.2 nM (1.1–1.4) | 90 ± 5 | 19 nM (10–55) | 96 ± 4 |
| [Sar$^9$]SP sulphone | 83 pM (54–139) | 60 ± 4* | 8 nM (4–15) | 81 ± 4* |
| Septide | 36 pM (20–86) | 68 ± 6* | 0.7 nM (0.3–1.2) | 88 ± 3 |
| [βAla$^8$]NKA(4-10) | 10 nM (6–24) | 100 | 12 nM (6–28) | 100 |
| MDL 28,564 | 474 nM (400–650) | 86 ± 4 | 55 nM (35–72) | 97 ± 2 |

Each value is from 6–12 experiments. *Significantly different ($P<0.05$) from $E_{max}$ of [βAla$^8$]NKA(4-10)

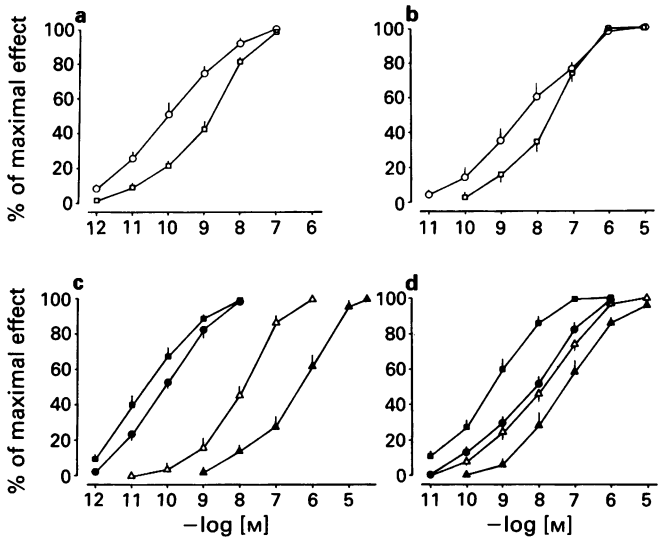


**Figure 2** Concentration-dependent contraction of the circular muscle of the guinea-pig ileum (left a, c) and colon (b, d) to natural tachykinins, SP (○) and NKA (□) and to synthetic tachykinin receptor agonists septide (■), [Sar$^9$]SP sulphone (●), [βAla$^8$]NKA(4-10) (△) and MDL 28,564 (▲). The contractile response was expressed as % of the maximal response produced by each agonist. Each value is mean ± s.e.mean of 6–12 experiments.

10) or NKA (Table 1). The synthetic agonists [Sar$^9$]SP sulphone, septide and [βAla$^8$]NKA(4-10) all produced concentration-dependent contractions of ileal rings. Septide and [Sar$^9$]SP sulphone were distinctly (about 2 orders of magnitude) more potent than [βAla$^8$]NKA(4-10) (Figure 1 and 2, Table 1). The $E_{max}$ produced by septide or [βAla$^9$]SP sulphone during the cumulative concentration-response curve was significantly less than that produced by [βAla$^8$]NKA(4-10) and was similar to the $E_{max}$ produced by SP (Table 1). The pseudopeptide derivative of NKA (4-10), MDL 28,564 was less potent than NKA and [βAla$^8$]NKA(4-10) (400 and 47 times, respectively), but its $E_{max}$ was not significantly different from that produced by NKA or [βAla$^8$]NKA(4-10) (Table 1, Figure 2). The response to tachykinin receptor agonists developed quite rapidly in the circular muscle of the ileum (Figure 1). For SP and [Sar$^9$]SP sulphone the maximal response produced by each dose developed within 30–45 s from agonist application; for [βAla$^8$]NKA(4-10), MDL 28,564 and NKA within 60–120 s, the time course of the response to septide was intermediate and the effect of each dose required 45–90 s to develop maximal effects. In the presence of antagonists (see below), a slower time course of the response to the agonists was observed.

### *Effect of $NK_1$ receptor antagonists*

(±)-CP 96,345, FK 888, GR 82,334 or RP 67,580 displayed no significant agonist activity, and did not affect the response to [βAla$^8$]NKA(4-10) ($n=4$ for each antagonist, Table 2). (±)-CP 96,345 (1–300 nmM, Figure 3), GR 82,334 (30 nM–10 μM) and FK 888 (30 nM–30 μM) produced concentration-dependent rightward shifts of the concentration-response curve to both [Sar$^9$]SP sulphone and septide with slopes of Schild plots not significantly different from unity (Figures 4 and 5, Table 2). The affinity of (±)-CP 96,345, GR 82,334 and FK 888 toward septide-induced response was greater (Table 2) than that toward [Sar$^9$]SP sulphone. At 1 μM, RP 67,580 was inactive toward septide and [Sar$^9$]SP sulphone (Table 2). At 3 μM RP 67,580 produced a significant depression of the maximal response to the two agonists (40–50% reduction, $n=4$ for each agonist).

### *Effect of $NK_2$ receptor antagonists*

SR 48,968, GR 94,800, MEN 10,376 or MEN 10,573 displayed no significant agonist activity. MEN 10,376 (0.3–10 μM), MEN 10,573 (0.3–10 μM) and GR 94,800 (3–30 nM, Figure 6) produced concentration-dependent rightward shifts of the curve to [βAla$^8$]NKA(4-10) with slopes of Schild plots not significantly different from unity (Figure 7, Table 2). The affinity of MEN 10,376 for $NK_2$ receptors in the circular muscle of the ileum was about 1 log unit lower than that measured in other guinea-pig smooth muscle preparations bearing $NK_2$ receptors (see Discussion). To assess whether this may involve breakdown of MEN 10,376 by peptidases and generation of shorter fragments with lower affinity for $NK_2$ receptors (Quartara *et al.*, 1992b), the effect of MEN 10,376 toward [βAla$^8$]NKA(4-10) was investigated in the presence of captopril, bestatin, thiorphan (1 μM each, 15 min beforehand) and amastatin (10 μM, 45 min beforehand): the $pK_B$ determined in these experiments (6.59 ± 0.14, $n=4$) was not significantly different from that measured in the absence of peptidase inhibitors. GR 94,800 was ineffective (up to 1 μM) against septide or [Sar$^9$]SP sulphone ($n=4$ for each agonist), while MEN 10,376 and MEN 10,573 competitively antagonized the response to septide; MEN 10,573 also antagonized competitively the response to [Sar$^9$]SP sulphone

**Table 2** Effect of various tachykinin receptor antagonists on contractions produced by [Sar$^9$]SP sulphone, septide or [βAla$^8$] NKA(4-10) in the circular muscle of the guinea-pig ileum

| *Antagonist* | *[Sar$^9$]SP sulphone* | *Septide* | *[βAla$^8$]NKA(4-10)* |
|---|---|---|---|
| (±)-CP 96,345 | | | |
| slope | −1.03 (0.59–1.46) | −0.87 (0.63–1.10) | inactive up to 1 μM |
| p$K_B$ | 8.17 (7.95–8.38) | 9.24 (9.07–9.41)* | |
| GR 82,334 | | | |
| slope | −1.23 (0.91–1.54) | −1.15 (0.76–1.54) | inactive up to 10 μM |
| p$K_B$ | 7.17 (6.97–7.37) | 7.52 (7.30–7.74)* | |
| FK 888 | | | |
| slope | −0.95 (0.75–1.15) | −1.21 (0.82–1.51) | inactive up to 3 μM |
| p$K_B$ | 7.53 (7.39–7.67) | 8.30 (8.08–8.52)* | |
| RP 67,580 | inactive up to 1 μM | inactive up to 1 μM | inactive up to 1 μM |
| MEN 10,376 | | | |
| slope | inactive up to 10 μM | −0.93 (0.66–1.19) | −0.90 (0.72–1.09) |
| p$K_B$ | | 6.40 (6.29–6.51) | 6.44 (6.33–6.55) |
| MEN 10,573 | | | |
| slope | −0.97(0.55–1.38) | −1.24(0.91–1.57) | −1.14 (0.75–1.53) |
| p$K_B$ | 5.95 (5.75–6.15) | 6.48(6.31–6.64)* | 7.18 (7.01–7.35)** |
| GR 94,800 | | | |
| slope | inactive up to 1 μM | inactive up to 1 μM | −0.91 (0.63–1.20) |
| p$K_B$ | | | 8.85 (8.72–8.98) |

For each agonist/antagonist combination slopes of Schild plot and p$K_B$ values (with 95% CL) are shown. Schild plots were constructed with at least 3 different concentrations of the test antagonist:each concentration was tested in at least 3 experiments on preparations from different animals. For slopes of Schild plot not significantly different from unity, p$K_B$ values were calculated using the constrained plot method. Statistical significance of differences in p$K_B$ was evaluated by analysis of covariance to detect differences in the position of Schild regression lines and differences in slope. *Position of Schild regression line significantly different ($P<0.05$) from that measured against [Sar$^9$]SP sulphone. Slopes of Schild plots not significantly different from each other. **Position of Schild regression line significantly different ($P<0.05$) from that measured against septide. Slopes of Schild plots not significantly different from each other.

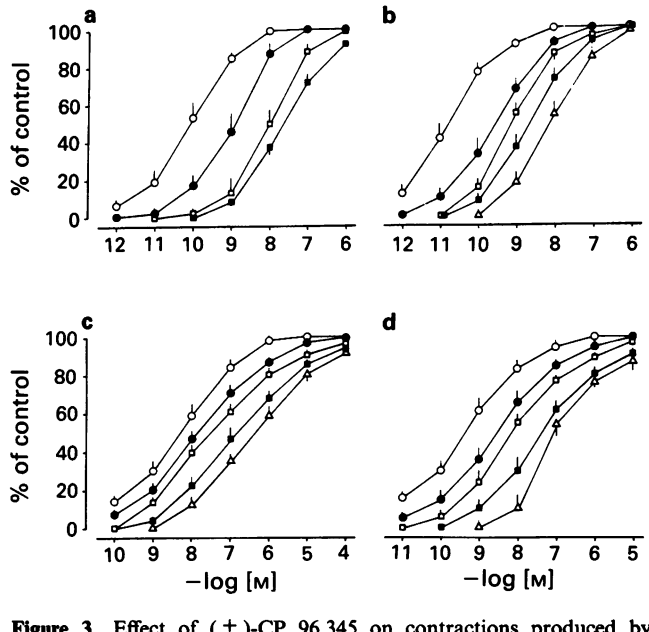


**Figure 3** Effect of (±)-CP 96,345 on contractions produced by [Sar$^9$]SP sulphone in the circular muscle of the guinea-pig ileum (a) and colon (c) and toward septide-induced contraction in the circular muscle of the guinea-pig ileum (b) and colon (d). In each panel (○) represents the control curve. Concentrations of (±)-CP 96,345 tested were: 30 (●) 300 (□) and 500 nM (■) toward [Sar$^9$]SP sulphone in the ileum; 10 (●), 30 (□), 100 (■) and 300 nM (△) toward [Sar$^9$]SP sulphone in the colon; 10 (●), 30 (□), 100 (■) and 300 nM (△) toward septide in ileum; 10 (●), 30 (□), 100 (■) and 300 nM (△) toward septide in the colon. Each value is mean ± s.e.mean of at least 3 determinations.

(Figures 4 and 5), while MEN 10,376 (10 μM, $n=4$) was ineffective (Table 2). As can be seen from Table 2, MEN 10,376 was equipotent against [βAla$^8$]NKA(4-10) and septide (p$K_B$ about 6.4 toward both agonists). Thus MEN 10,376 is at least 30 times more potent toward septide than [Sar$^9$]SP sulphone (Table 2). MEN 10,573 was slightly but significantly more potent towards [βAla$^8$]NKA(4-10) than towards septide and significantly more potent towards septide than towards [Sar$^9$]SP sulphone (Table 2).

SR 48,968 (10 nM–3 μM, $n=28$, Figure 6), displayed a more complex pattern of antagonism: it produced both non-parallel rightward shifts of the curve to [βAla$^8$]NKA(4-10) and depression of $E_{max}$, both effects being concentration-dependent (Figure 6). Schild plot analysis revealed the non-competitive nature of antagonism with a slope of −0.72 (0.48–0.90). To assess whether the depression of $E_{max}$ to [βAla$^8$]NKA(4-10) by SR 48,968 could be reversed, ileal rings were exposed to 3 μM [βAla$^8$]NKA(4-10) at 30 min intervals until reproducible responses were obtained (Figure 8). SR 48,968 (3 μM for 30 min) reduced the response to the agonist to 42 ± 6% of controls ($n=4$, $P<0.05$): as shown in Figure 8, a slow, time-dependent, recovery of inhibition by SR 48,968 was observed up to 87 ± 8% of control at 150 min. Having determined that the depression of $E_{max}$ to [βAla$^8$] NKA(4-10) by SR 48,968 is not a consequence of irreversible interaction with the receptors, the p$K_B$ of SR 48,968 was estimated by the double reciprocal plot method described by Kenakin (1987a). For this analysis the experiments obtained with 1 and 3 μM SR 48,968 were used (Figures 6 and 9): the corresponding p$K_B$ value was 7.83 (7.15–8.51, $n=7$). Owing to the lower potency of SR 48,968 in antagonizing responses to [βAla$^8$]NKA(4-10) in the ileum vs. colon (see below), the question was raised as to whether a 15 min contact time was sufficient for this antagonist to reach equilibrium with $NK_2$ receptors in the guinea-pig ileum. To check this point, the contact time of the antagonist was extended to 45 min. For these experiments a concentration of 1 μM SR 48,968 was selected which, after 15 min contact time, produced both a rightward shift of the curve to the agonist (dose-ratio 30 ± 6, $n=9$) and a depression of $E_{max}$ (76 ± 4% of control response). The corresponding values measured after 45 min contact time (dose ratio 32 ± 11, $E_{max}=80\pm5$% of control, $n=4$) were not significantly different from those obtained at 15 min, i.e. no evidence was found for time-dependency of antagonist action. SR 48,968, up to 0.1 μM, was ineffective

toward [Sar$^9$]SP sulphone or septide (Table 2). At 1 μM the $E_{max}$ to these two agonists was reduced by 25 and 32%, respectively ($n = 5$ in each case). This effect was not due to nonspecific depression of contractility because the concentration-response curve to carbachol (10 nM–10 μM, $EC_{50}$ 164 nM, 100–352 nM) was unaffected by 15 min contact time with 1 μM SR 48,968 ($EC_{50}$ 199 nM, 86–411 nM, $n = 4$).

### *Circular muscle of the colon*

*Effect of agonists* In the presence of 10 μM indomethacin, muscle strips of guinea-pig proximal colon developed a low amplitude (<20% of maximal response to KCl) irregular phasic activity. SP and NKA produced concentration-dependent contractions of the strips (Figure 2), SP being about four times more potent than NKA (Table 1). The action of SP and NKA was not significantly modified in the presence of peptidase inhibitors. The $EC_{50}$s of SP were 5 nM (4–6 nM) and 6 nM (4–9 nM) in the absence and presence of peptidase inhibitors, respectively ($n = 6$ in each group). The corresponding values for NKA were 19 nM (10–55 nM) and 15 nM (9–27 nM), respectively ($n = 6$). When 1 μM SP or NKA was added to the bath as a single concentration, the $E_{max}$ was not different from that produced by 1 μM [βAla$^8$]NKA(4-10). The $E_{max}$ produced by NKA or [βAla$^8$]NKA(4-10) during the cumulative concentration-response curve equalled that observed in response to a single administration of 1 μM concentration of each agonist. The $E_{max}$ produced by SP during the cumulative concentration-response curve was slightly less than that produced by [βAla$^8$]NKA(4-10) or NKA (Table 1). The synthetic agonists [Sar$^9$]SP sulphone, septide and [βAla$^8$]NKA(4-10) all produced concentration-dependent contractions of the strips (Figure 2). The $E_{max}$ to [Sar$^9$]SP sulphone, but not that to septide, was slightly less than that produced by [βAla$^8$]NKA(4-10) (Table 1). Septide was the most potent

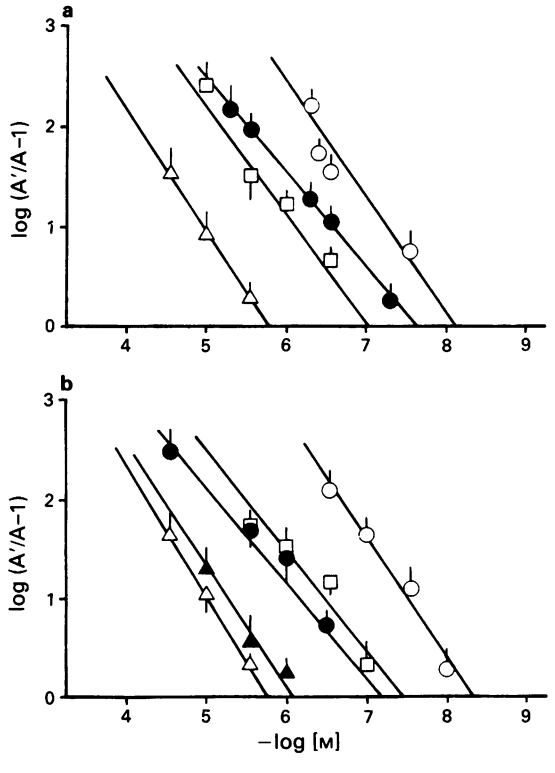


**Figure 4** Schild plots for antagonism of contraction produced by [Sar$^9$]SP sulphone in the circular muscle of the ileum (a) and colon (b) by (±)-CP 96,345 (○), FK 888 (●), GR 82,334 (□), MEN 10.573 (△) and RP 67,580 (▲). Each value is mean ± s.e.mean of at least three determinations. Slopes of Schild plots are given in Tables 1 and 2.

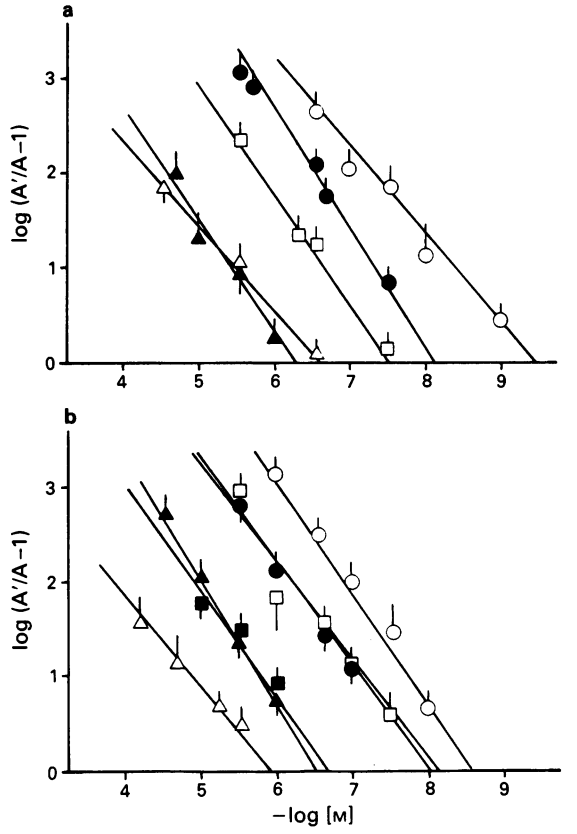


**Figure 5** Schild plots for antagonism of contraction produced by septide in the circular muscle of the ileum (a) and colon (b) by (±)-CP 96,345 (○), FK 888 (●), GR 82,334 (□), RP 67,580 (■), MEN 10,376 (△) and MEN 10,573 (▲). Each value is mean ± s.e. mean of at least three determinations. Slopes of Schild plots are given in Tables 1 and 2.

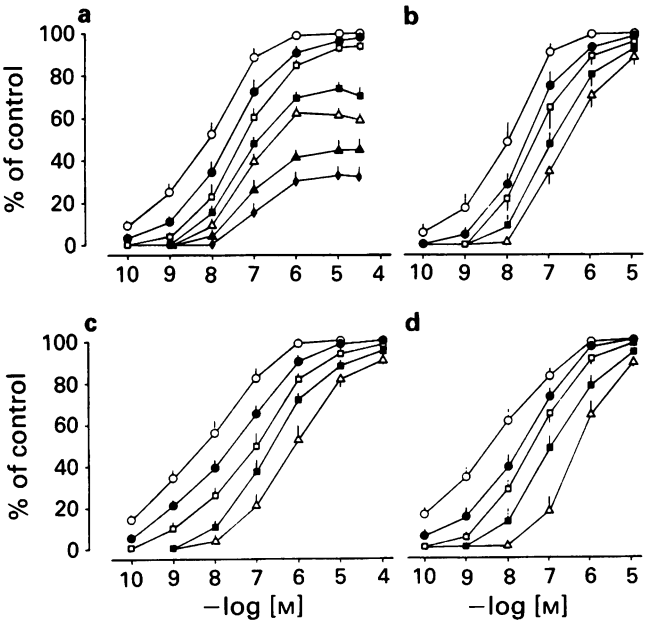


**Figure 6** (a, c) Effect of SR 48,968 on the concentration-response curve to [βAla$^8$]NKA(4-10) in the circular muscle of the guinea-pig ileum (a) and colon (c). (b, d) Effect of GR 94,800 on the concentration-response curve to [βAla$^8$]NKA(4-10) in the circular muscle of the guinea-pig ileum (b) and colon (d). In each panel (○) represents the control curve obtained in the absence of the antagonist. Concentrations of antagonists tested were: 10 (●), 30 (□), 100 (■), 300 (△), 1000 (▲) and 3000 (◆) for SR 48,968 in the ileum; 3 (●), 10 (□), 30 (■), 100 nM (△) for SR 48,968 in the colon; 3 (●), 5 (□), 10 (■), and 30 nM (△) for GR 94,800 in the ileum; 1 (●), 3 (□), 10 (■) and 30 nM (△) for GR 94,800 in the colon. Each value is mean ± s.e.mean of at least 3 determinations.

agonist tested, being about seven times more potent than SP; [Sar$^9$]SP sulphone and [βAla$^8$]NKA(4-10) were equipotent to SP and NKA, respectively (Table 1). The pseudopeptide derivative of NKA (4-10), MDL 28,564 was about two times less potent than NKA and about four times less potent than [βAla$^8$]NKA(4-10) (Figure 2, Table 1). Its $E_{max}$ was not significantly different from that produced by NKA or [βAla$^8$]NKA(4-10) (Table 1). As observed in the ileum, the time course of the contractile response produced by SP or [Sar$^9$[SP sulphone (45–120 s for maximum effect of each concentration) was faster than that of NKA, [βAla$^8$]NKA(4-10) or MDL 28,564 (90–300 s), while the time course of the response to septide was intermediate (90–180 s). In the presence of antagonists (see below), a slower time course of the response to the agonists was observed.

*Effect of $NK_1$ receptor antagonists* (±)-CP 96,345, FK 888, GR 82,334 or RP 67,580 displayed no agonist activity, nor did they affect the concentration-response curve to [βAla$^8$]NKA (4-10) (Table 3, $n = 4$ for each antagonist). (±)-CP 96,345 (10 nM–1 μM, Figure 3), FK 888 (0.1–30 μM), GR 82,334 (0.1–3 μM) or RP 67,580 (1–10 μM) produced concentration-dependent rightward shifts of the curve to septide and [Sar$^9$]SP sulphone with slopes of Schild plot significantly different from unity (Table 3, Figures 4 and 5). As observed in the ileum, the affinity of $NK_1$ receptor antagonists was significantly higher toward septide than toward [Sar$^9$]SP sulphone, the only exception being CP 96,345, for which the difference was not statistically significant (Table 3).

*Effect of $NK_2$ receptor antagonists* SR 48,968, GR 94,800, MEN 10,376 or MEN 10,573 displayed no agonist activity. SR 48,968 (3–100 nM), MEN 10,376 (0.3–10 μM), MEN 10,573 (0.3–10 μM) and GR 94,800 (1–100 nM) produced concentration-dependent rightward shifts of the curve to [βAla$^8$]NKA(4-10): slopes of Schild plots indicated competitive antagonism (Table 3, Figures 6 and 7). SR 48,968 (up to 3 μM) and GR 94,800 (up to 1 μM) were ineffective against [Sar$^9$]SP sulphone or septide (Table 3). MEN 10,376 and MEN 10,573 competitively antagonized the response to septide. MEN 10,573, but not MEN 10,376 (10 μM, $n = 4$) also antagonized the response to [Sar$^9$]SP sulphone (Table 3). MEN 10,376 was about ten times more potent against [βAla$^8$]

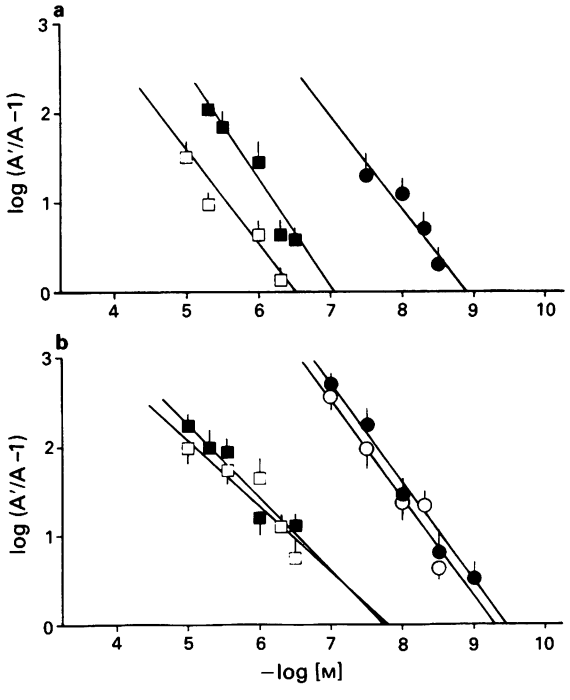


**Figure 7** Schild plots for antagonism of contraction produced by [βAla$^8$]NKA(4-10) in the circular muscle of the ileum (a) and colon (b) by SR 48,968 (○), GR 94,800 (●) MEN 10,376 (□) and MEN 10,573 (■). Each value is mean ± s.e.mean of at least three determinations. Slopes of Schild plots are given in Tables 2 and 3,

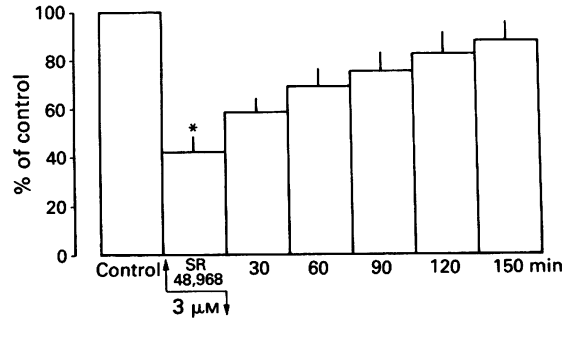


**Figure 8** Reversal, by washing, of the depressant effect of SR 48,968 on the maximal response to [βAla$^8$]NKA(4-10) in the circular muscle of the ileum. Each value is mean ± s.e.mean of 4 determinations. *Significantly different from control, $P < 0.05$.

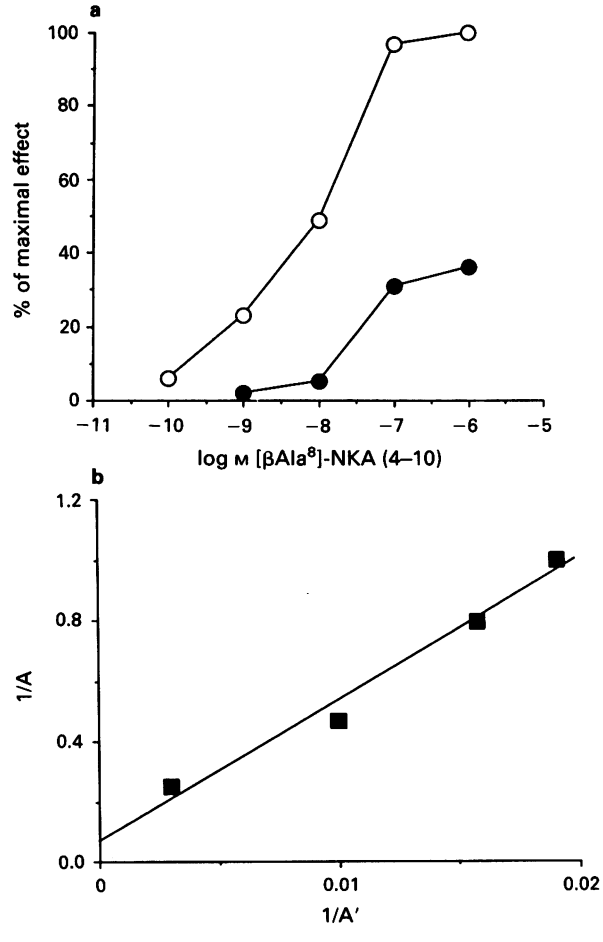


**Figure 9** (a) Example of concentration-response curve to [βAla$^8$]NKA(4-10) in the absence (○) and presence (●) of SR 48,968 (1 μM) in the guinea-pig ileum circular muscle. (b) Double-reciprocal plot of equiactive concentrations of [βAla$^8$]NKA(4-10) from dose-response curve shown in (a) in the absence (1/A) and in the presence (1/A') of SR 48,968 (1 μM) in the guinea-pig ileum circular muscle. Pairs of equieffective concentrations of [βAla$^8$[NKA(4-10) have been chosen producing 35, 31, 25 and 23% of maximal effect. Slope of regression line is 46.5. $pK_B$ value for SR 48,968 in the experiment reported, determined as − log ([SR 48,968] slope − 1), is 7.67.

**Table 3** Effect of various tachykinin receptor antagonists on contractions produced by [Sar$^9$]SP sulphone, septide or [βAla$^8$] NKA(4-10) in the circular muscle of the guinea-pig colon

| *Antagonist* | *[Sar$^9$]SP sulphone* | *Septide* | *[βAla$^8$]NKA(4-10)* |
|---|---|---|---|
| (±)-CP 96,345 | | | |
| slope | −1.16 (0.78–1.53) | −0.95 (0.69–1.22) | inactive up to 1 μM |
| p$K_B$ | 8.41 (8.20–8.62) | 8.89 (8.64–9.13) | |
| GR 82,334 | | | |
| slope | −0.96 (0.58–1.34) | −1.04 (0.80–1.29) | inactive up to 10 μM |
| p$K_B$ | 7.49 (7.29–7.69) | 8.12 (7.94–8.32)* | |
| FK 888 | | | |
| slope | −0.98 (0.64–1.32) | −1.08 (0.90–1.26) | inactive up to 1 μM |
| p$K_B$ | 7.13 (6.95–7.31) | 8.13 (8.03–8.22)* | |
| RP 67,580 | | | |
| slope | −1.25 (0.95–1.52) | −1.18 (0.67–1.69) | inactive up to 1 μM |
| p$K_B$ | 6.25 (6.10–6.40) | 6.82 (6.60–7.04)* | |
| SR 48,968 | | | |
| slope | inactive up to 3 μM | inactive up to 3 μM | −1.15 (0.65–1.64) |
| p$K_B$ | | | 9.41 (9.24–9.58) |
| MEN 10,376 | | | |
| slope | inactive up to 10 μM | −1.04 (0.77–1.31) | −0.86 (0.57–1.14) |
| p$K_B$ | | 5.85 (5.70–6.00) | 7.27 (7.10–7.44)** |
| MEN 10,573 | | | |
| slope | −1.31 (0.85–1.76) | −1.12 (0.79–1.45) | −0.80 (0.38–1.22) |
| p$K_B$ | 5.98 (5.84–6.11) | 6.72 (6.55–6.89)* | 7.28 (7.11–7.45)** |
| GR 94,800 | | | |
| slope | inactive up to 1 μM | inactive up to 1 μM | −1.06 (0.88–1.24) |
| p$K_B$ | | | 9.49 (9.28–9.70) |

For each agonist/antagonist combination slopes of Schild plot and p$K_B$ values (with 95% CL) are shown. Schild plots were constructed with at least 3 different concentrations of the test antagonist:each concentration was tested in at least 3 experiments on preparations from different animals. For slopes of Schild plot not significantly different from unity, p$K_B$ values were calculated using the constrained plot method. Statistical significance of differences of p$K_B$ was evaluated by analysis of covariance to detect differences in the position of Schild regression lines and differences in slope. *Position of Schild regression line significantly different ($P<0.05$) from that measured against [Sar$^9$]SP sulphone. Slopes of Schild plots not significantly different from each other. **Position of Schild regression line significantly different ($P<0.05$) from that measured against septide. Slopes of Schild plots not significantly different from each other.

NKA(4-10) than toward septide (Table 3). MEN 10,573 was slightly but significantly more potent towards [βAla$^8$]NKA(4-10) than towards septide, and significantly more potent towards septide than towards [Sar$^9$]SP sulphone (Table 3).

### *Effect of nifedipine on the response to [Sar$^9$]SP sulphone, septide and [βAla$^8$]NKA(4-10) in the ileum and colon*

Nifedipine (1 μM) abolished spontaneous activity of the strips in both the ileum and colon. In the ileum, the response to septide, [Sar$^9$]SP sulphone or [βAla$^8$]NKA(4-10) (up to 1 μM, for each agonist) was totally abolished by nifedipine ($n=4$ for each agonist). In the colon, the concentration-response curves to [Sar$^9$]SP sulphone and septide were markedly depressed and shifted to the right by nifedipine (Figure 10). In the presence of nifedipine, the $E_{max}$ to [Sar$^9$]SP sulphone and septide (30 μM in each case) averaged 21 ± 4 and 27 ± 3% of controls, respectively ($n=7$) (Figure 10). On the contrary, the response to [βAla$^8$]NKA(4-10) was only depressed but not shifted to the right by nifedipine (Figure 10, $n=5$): in the presence of nifedipine, the $E_{max}$ to [βAla$^8$] NKA(4-10) averaged 80 ± 4% of control (Figure 10). Qualitatively, the contractile response produced by the three tachykinin receptor agonists became slower in the presence of nifedipine, each concentration requiring at least 3–5 min to produce its maximal effect.

## Discussion

The aim of this study was to perform a systematic analysis of the affinities of various peptide and nonpeptide tachykinin antagonists at $NK_1$ and $NK_2$ receptors in the guinea-pig ileum and colon. Since pharmacological evidence has been presented to indicate intraspecies heterogeneity of $NK_1$ and $NK_2$ receptors (see Introduction), a major aim of the study was to unravel possible differences in receptor antagonist potencies in preparations from the same species. Tachykinin $NK_3$ receptors are also present in the circular muscle of the ileum and their stimulation produces an indirect contractile response (Maggi *et al.*, 1990a). No information is available about the possible presence of $NK_3$ receptors in the guinea-pig colon, although in preliminary experiments we found senktide effective in producing concentration-dependent (threshold concentration 1 nM) contractions of this preparation (Maggi, unpublished observations). Since high concentrations of SP and NKA are capable of stimulating $NK_3$ receptors with similar efficiency to neurokinin B, the value of natural tachykinins for characterizing $NK_1$ and $NK_2$ receptors in the intestine, and detect possible intraspecies receptor heterogeneity is limited. For this reason, the receptor-selective agonist, [Sar$^9$]SP sulphone, septide and [βAla$^8$]NKA(4-10) were used for studying the effect of antagonist. The use of these agonists relies on the assumption, supported by previous literature data, of negligible, if any, affinity for $NK_3$ receptors (Lee *et al.*, 1986; Wormser *et al.*, 1986; Dion *et al.*, 1987; Laufer *et al.*, 1988; Rovero *et al.*, 1989).

### *$NK_1$ receptors in the circular muscle of the ileum and colon*

The results of this study indicate that: (i) various $NK_1$ receptor-selective antagonists are significantly more potent against septide than [Sar$^9$]SP sulphone; (ii) MEN 10,376, previously characterized as a selective $NK_2$ receptor antagonist, displays a sizeable affinity toward the septide-induced

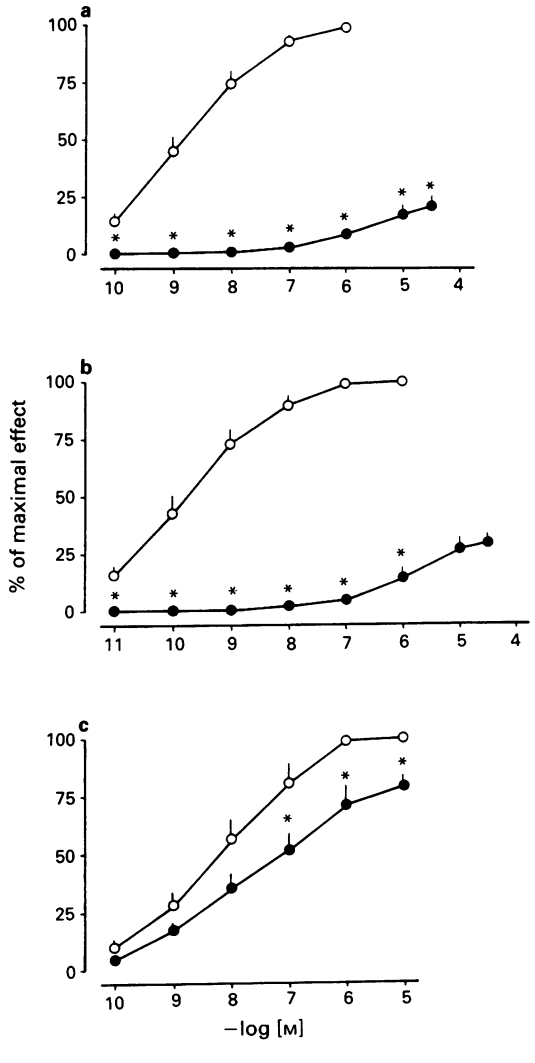


**Figure 10** Effect of nifedipine (1 μM, ●) on the concentration-response curve to [Sar$^9$]SP sulphone (a), septide (b) and [βAla$^8$]NKA(4-10) (c) in the circular muscle of the guinea-pig colon. Each value is mean ± s.e.mean of 5–7 determinations. *Significantly different from control, $P<0.05$.

contraction; (iii) in both preparations (±)-CP 96,345 was markedly more potent than RP 67,580, in keeping with the known species-related pattern of $NK_1$ receptor antagonist affinity.

Petitet *et al.* (1992) raised the issue of the possible existence of a novel, 'septide-sensitive', tachykinin receptor to account for the marked discrepancy between the high potency of septide in stimulating $NK_1$ receptors in the longitudinal muscle of the guinea-pig ileum and its relative low potency in displacing SP from $NK_1$ receptors. The observation that the responses to septide in the guinea-pig ileum and rat urinary bladder are blocked by $NK_1$ receptor antagonists with higher potency than responses produced by SP or 'classical' $NK_1$ receptor agonists (i.e. agonists for which a high binding affinity parallels the high biological activity) (Petitet *et al.*, 1992; Meini *et al.*, 1994; Maggi *et al.*, 1993a,b,c; and present findings), raises the possibility that the 'septide-sensitive' receptor is an $NK_1$ receptor subtype.

The present findings demonstrate that various peptide and nonpeptide $NK_1$ receptor antagonists (with the exception of CP 96,345 in the colon) are significantly more potent in antagonizing the response to septide than that to [Sar$^9$]SP sulphone. The observed differences in $pK_B$ values range between 0.5–1 log unit, and notably on no occasion did we find an $NK_1$ receptor antagonist to be more potent toward [Sar$^9$]SP sulphone than toward septide. Although this latter finding may be incidental, it calls for a word of caution against the existence of a 'septide-sensitive' receptor as a distinct entity from the classical $NK_1$ receptor.

Further discrimination between the responses to septide and [Sar$^9$]SP sulphone was seen with MEN 10,376, a linear NKA(4-10) derivative possessing high affinity for $NK_2$ receptors expressed in the human, bovine, guinea-pig and rabbit species (Maggi *et al.*, 1993a). We reported previously that the potency of MEN 10,376 in antagonizing septide-induced contraction in the guinea-pig isolated bronchus is somewhat intermediate between that displayed toward the $NK_2$ receptor-selective agonist, [βAla$^8$]NKA(4-10), and the $NK_1$-receptor-selective agonist, SP methylester (Maggi *et al.*, 1991b). In the circular muscle of the guinea-pig ileum, the affinity of MEN 10,376 for $NK_2$ receptors is lower than that determined in other guinea-pig smooth muscle preparations (for e.g. in the circular muscle of the colon); in the ileum, MEN 10,376 was equipotent toward [βAla$^8$]NKA(4-10) ($pK_B$ 6.44) and septide ($pK_B$ 6.40). On the other hand, MEN 10,376 is at least 30 times less potent ($pK_B<5$) toward [Sar$^9$]SP sulphone. Therefore, in absolute values, MEN 10,376 is the antagonist which better discriminates between [Sar$^9$]SP sulphone- and septide-induced contraction in the circular muscle of the guinea-pig ileum, although its affinity for the septide-stimulated receptor is about three orders of magnitude lower than that of (±)-CP 96,345.

Assuming that a 'septide-sensitive' $NK_1$ receptor exists, the rank order of potency of natural tachykinins at this receptor is an obvious question of physiological relevance. Our previous observations (Meini *et al.*, 1994) suggest that neurokinin B could be a better ligand than SP at the 'septide-sensitive' receptor in the rat urinary bladder. Owing to the presence of $NK_3$ receptors in the gut and the lack of a suitable $NK_3$ receptor antagonist, functional experiments with neurokinin B at this level would not be informative.

The present findings are compatible with the existence of pharmacologically distinct $NK_1$ receptor subtypes in guinea-pig. On the other hand, other possibilities could be considered to account for these results: the possibility that physicochemical properties of septide determine its unusual pharmacological properties is unconvincing, because other $NK_1$ receptor agonists, including the undecapeptides [Apa$^{9,10}$]SP and [Pro$^{9,10}$]SP show a 'septide-like' profile of action in the guinea-pig ileum (Petitet *et al.*, 1992). Since the recognition epitopes for certain $NK_1$ receptor antagonists by the $NK_1$ receptor protein appear to be distinct from the agonist binding site(s) (Gether *et al.*, 1993), the possibility of an allosteric modulation between agonist and anagonist binding sites should be taken into serious consideration for the $NK_1$ receptor. It may be that the recognition epitopes of the $NK_1$ receptor protein for SP and classical $NK_1$ receptor agonist do not overlap with the epitopes recognizing the 'septide-like' agonists, whereby septide and other agonists with a similar profile are more easily displaced by antagonists. This interpretation may explain why several $NK_1$ antagonists were in general more potent in blocking septide than 'classical' $NK_1$ receptor agonists while no example of the converse pattern has been reported yet. On the other hand, the available data from mutation experiments indicate that septide and SP recognize similar epitopes of the $NK_1$ receptor protein (Fong *et al.*, 1992a). However, a systematic study of the effects of mutations of different regions of the $NK_1$ receptor protein on the binding of septide vs. SP is not available. It is to be noted, finally, that in cell systems expressing the cloned, full length, $NK_1$ receptor protein, septide is distinctly less potent than SP in both binding and functional assays (Fong *et al.*, 1992a see also Hermans *et al.*, 1993), in sharp contrast with the high potency of this hexapeptide in various bioassays (e.g. Laufer *et al.*, 1988; Hall & Morton, 1991; and present data).

The possibility that a 'septide-sensitive' $NK_1$ receptor sub-

type exists as a distinct receptor entity remains a matter for investigation, which cannot be negated or supported from the presently available data on the molecular biology of the $NK_1$ receptor. Although only one gene encoding the $NK_1$ receptor has been isolated thus far (Gerard *et al.*, 1993), the possibility exists that different forms of the receptor protein are generated from the same gene (Fong *et al.*, 1992b; Kage *et al.*, 1993).

### *$NK_2$ receptors in the circular muscle of the ileum and colon*

The present findings substantiate the conclusion that $NK_2$ receptors mediate contraction in the circular muscle of the guinea-pig ileum and colon (Maggi *et al.*, 1990a; Giuliani *et al.*, 1993). In fact, various $NK_1$ antagonists such as (±)-CP 96,345, FK 888 and GR 82,334 failed to affect the response to [βAla$^8$]NKA(4–10) while GR 94,800 and SR 48,968 were effective at concentrations that do not significantly shift the curve to septide or [Sar$^9$]SP sulphone.

In both preparations, the pseudopeptide derivative of NKA(4–10), MDL 28,564 behaves as a full agonist relative to NKA or [βAla$^8$]NKA(4–10). This behaviour has been observed previously in various guinea-pig smooth muscles (Buck *et al.*, 1990; Maggi *et al.*, 1991b; 1992). The agonist activity of MDL 28,564 has been one of the criteria to identify putative species-dependent variants of the $NK_2$ receptor (Maggi *et al.*, 1993a for review) and the two preparations investigated here appear homogeneous in this respect. Two out of the four $NK_2$ receptor antagonists tested, the linear heptapeptide GR 94,800 and the cyclic pseudopeptide MEN 10,573, displayed similar affinities for $NK_2$ receptors in the circular muscle of the ileum and colon. By contrast, the non peptide antagonist, SR 48,968 and the linear heptapeptide, MEN 10,376 were more potent at $NK_2$ receptors in the circular muscle of the colon than ileum. The estimate of the affinity of SR 48,968 for $NK_2$ receptors in the ileum was complicated by the depression of $E_{max}$ to the agonist: up to 0.1 μM SR 48,968 is selective for $NK_2$ receptors. At 1.0 μM a depression of $E_{max}$ to $NK_1$ receptor agonists but not to carbachol was observed indicating that at this concentration SR 48,968 is effective at $NK_1$ receptors as well. $E_{max}$ depression by SR 48,968 is not due to irreversible changes in $NK_2$ receptor function, being reversed, even if slowly, by repeated washings: this depressant effect, detected previously in the rabbit pulmonary artery (Maggi *et al.*, 1993b), may arise from pseudoirreversible antagonism. The estimate of affinity for pseudoirreversible antagonism according to Kenakin (1987) yielded a $pK_B$ value (7.83) which is remarkably lower than the affinity of SR 48,968 measured in the guinea-pig colon ($pK_B$ 9.41) where its profile of action was fully compatible with competitive antagonism. Although the exact mechanism responsible for the very different profile of action of SR 48,968 in the guinea-pig ileum and colon remains to be determined, these observations, along with the higher potency of MEN 10,376 at $NK_2$ receptor in the guinea-pig colon vs the ileum, raise the possibility of an intraspecies heterogeneity of the $NK_2$ receptor.

### *Effect of nifedipine*

In a previous study we showed that the responses to equieffective concentrations of [Sar$^9$]SP sulphone and [βAla$^8$]NKA(4-10) in the guinea-pig colon exhibit a marked difference in their sensitivity to nifedipine (Zagorodnyuk *et al.*, 1993b): the response to the $NK_1$ receptor agonist was greatly diminished (about 80% inhibition) while that to the $NK_2$ receptor agonist was largely unaffected (<20% inhibition) by nifedipine. The present findings demonstrate that: (i) the contraction to $NK_1$ or $NK_2$ receptor agonists is strictly dependent upon influx of extracellular calcium through nifedipine-sensitive calcium channels in the circular muscle of the ileum; (ii) the contraction to $NK_2$ receptor stimulation shows a marked regional difference in the degree of usage of nifedipine-sensitive calcium channels in the guinea-pig intestine. In both preparations, a similar depression of the contractile response to septide and [Sar$^9$]SP sulphone by nifedipine occurs. Thus, if septide were really acting at a tachykinin receptor different from that activated by 'classical' $NK_1$ receptor agonists, like [Sar$^9$]SP sulphone, it would follow that the two receptors have a similar degree of usage of nifedipine-sensitive calcium channels for inducing contraction in the circular muscle of the guinea-pig intestine. In view of the above discussed differences in the action of SR 48,968 and MEN 10,376 at $NK_2$ receptors in the ileum and colon, the remarkable difference in the effectiveness of nifedipine for inhibiting the response to [βAla$^8$]NKA(4-10) suggest that the putative $NK_2$ receptor subtypes couple with different effector systems to produce smooth muscle contraction in the guinea-pig intestine. This working hypothesis needs further evaluation.

### *Conclusions*

In conclusion, the present findings demonstrate, through the use of a series of agonists and antagonists, that both $NK_1$ and $NK_2$ receptors mediate contraction in the circular muscle of the guinea-pig ileum and colon. Our data are compatible with the idea of the existence of a 'septide-sensitive' receptor which could be an $NK_1$ receptor subtype, although other explanations cannot be excluded at the present stage of knowledge. Furthermore, the existence of $NK_2$ receptor subtypes in guinea-pig intestinal smooth muscles is suggested. While pharmacological data suggest heterogeneity, the existence of $NK_1$ and $NK_2$ receptor subtypes should find structural support from the molecular biology approach before being regarded as conclusive.

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# Tachykinin $NK_1$ and $NK_2$ receptor antagonists and atropine-resistant ascending excitatory reflex to the circular muscle of the guinea-pig ileum

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**1** The aim of this study was to investigate the effect of various antagonists, selective for the tachykinin $NK_1$ or $NK_2$ receptor, on the atropine-resistant ascending excitatory reflex (AER) to the circular muscle of the guinea-pig ileum elicited by radial stretch (balloon distension) or electrical field stimulation.

**2** Submaximal and maximal atropine- (1 μM) resistant AER elicited by balloon distension averaged about 40–50% and 70–90% of maximal circular spasm to 80 mM KCl, respectively. The $NK_1$ receptor antagonist, (±)-CP 96,345 (1 μM) inhibited both maximal and submaximal AER. FK 888 (1–3 μM) inhibited submaximal AER only. RP 67,580 (1 μM) was ineffective. The $NK_2$ receptor antagonist, GR 94,800, inhibited both maximal and submaximal AER at all concentrations tested (0.1–3.0 μM), while SR 48,968 was effective only at 1.0 μM. The $NK_2$ receptor antagonists, MEN 10,376 and MEN 10,573 inhibited both submaximal and maximal AER at 10 and 1.0 μM, respectively.

**3** In other experiments, an $NK_1$ receptor antagonist, (±)-CP 96,345 or FK 888 (1.0 μM in each case) was administered first and the effect of GR 94,800 (1.0 μM) on the residual AER response was determined; or GR 94,800 was administered first and the effect of (±)-CP 96,345 or FK 888 was determined. The results of these experiments indicated an additive effect produced by the combined treatment with $NK_1$ and $NK_2$ receptor antagonists.

**4** Electrical field stimulation (10 Hz for 0.5 s, 10–20 V, 0.15–0.3 ms pulse width) with electrodes placed at 1.4–1.8 cm anal to the recording site, produced ascending contractions which were almost abolished by 10 μM hexamethonium (electrically-evoked AER). In the presence of apamin (0.1 μM) and $N^G$-nitro-L-arginine (30 μM) these contractions were reproducible over 10 consecutive stimulation cycles. GR 94,800 (1 μM) and FK 888 (1 μM) both produced a partial inhibition of the electrically-evoked AER and their combined administration produced an inhibitory effect which was larger than that induced by each antagonist alone.

**5** FK 888 (1–3 μM), GR 94,800 (1–3 μM), MEN 10,573 (1 μM) and MEN 10,376 (10 μM) did not significantly affect the atropine-sensitive twitch contractions produced by electrical field stimulation of the guinea-pig ileum longitudinal muscle-myenteric plexus preparation, which were abolished by 10–30 μM procaine, 1 μM tetrodotoxin or 1 μM atropine. (±)-CP 96,345 (1 μM) and SR 48,968 (1 μM) produced 12% and 27% inhibition of cholinergic twitches in the longitudinal muscle of the ileum, respectively.

**6** We conclude that both $NK_1$ and $NK_2$ receptors mediate the atropine-resistant AER to the circular muscle of the ileum. $NK_2$ receptor activation plays a more important role than $NK_1$ receptor activation in the AER evoked by radial stretch. Since a consistent fraction of the distension- and electrically-evoked atropine-resistant AER persists in the presence of combined $NK_1$ and $NK_2$ receptor blockade, the existence of a third excitatory transmitter to the circular muscle of the ileum, in addition to acetylcholine and tachykinins, is suggested.

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

## Introduction

Tachykinins are powerful smooth muscle spasmogens in the guinea-pig ileum and are thought to play a major role as excitatory transmitters to both circular and longitudinal muscle layers (Bartho *et al.*, 1982; Costa *et al.*, 1985; Holzer, 1989; Bartho & Holzer, 1985 for review). A subpopulation of enteric neurones in the myenteric plexus expresses tachykinin-like immunoreactivity: these elements express the preprotachykinin I gene, which encodes the sequence of both substance P and neurokinin A (Sternini *et al.*, 1989) and some of them have appropriate projections for them to be considered the effector motoneurones for reflexly-evoked contraction of the circular muscle (Brookes *et al.*, 1991). Accordingly, both substance P- and neurokinin A-like immunoreactivity have been detected in enteric neurones by immunocytochemistry (e.g. Schmidt *et al.*, 1991; Shuttleworth *et al.*, 1991) and release of both peptides from the mammalian gut has been documented in response to depolarizing stimuli (Theodorsson *et al.*, 1991; Schmidt *et al.*, 1992b). Three main types of tachykinin receptors ($NK_1$, $NK_2$ and $NK_3$) are known to mediate the spasmogenic activity of peptides of this family (Maggi *et al.*, 1993a). In the circular muscle of the guinea-pig ileum, $NK_1$ and $NK_2$ receptors mediate the direct spasmogenic effect of tachykinins on muscle cells, while the $NK_3$ receptor-mediated response is totally indirect and involves the release of endogenous acetylcholine and tachykinins (Maggi *et al.*, 1990; 1994a; Bartho *et al.*, 1992). Since both $NK_1$ (substance P-preferring) and $NK_2$ (neurokinin A-preferring) receptors mediate the direct contraction of circular muscle of the ileum, the question arises as to the relative contribution of endogenous tachykinins in mediating atropine-resistant excitation to the circular muscle.

Earlier studies on the role of tachykinins as mediators of

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the atropine-resistant contractility in the guinea-pig ileum have used first generation peptide antagonists, the best known of which is spantide; these compounds suffer from a number of drawbacks, including low potency and lack of selectivity (see Maggi *et al.*, 1993a for recent review). Especially disturbing is the poor ability of these compounds to discriminate between $NK_1$ and $NK_2$ receptors which does not enable verification of the relative contribution of different tachykinins/different receptors to the overall physiological response.

Recently, the use of the $NK_2$ receptor-selective antagonist, MEN 10,376 ([$Tyr^5$,D-$Trp^{6,8,9}$,$Lys^{10}$]NKA(4–10)) (Maggi *et al.*, 1991) vs. the $NK_1$ receptor selective antagonists, GR 71,251 or GR 82,334 (Hagan *et al.*, 1991) has revealed an important role of $NK_2$ receptors in mediating the ascending excitatory reflex (AER) produced by radial stretch (balloon distension) in the guinea-pig ileum (Bartho *et al.*, 1992; Holzer *et al.*, 1993). From these studies, two basic concepts have emerged: (i) the $NK_2$ receptor prevails over the $NK_1$ receptor in mediating the atropine-resistant contraction of the circular muscle of the ileum, suggesting an important role for neurokinin A in mediating neuromuscular transmission at this level (Bartho *et al.*, 1992) and (ii) tachykinin release and $NK_2$ receptor activation during the AER is not restricted to high degree of stimulation but also occurs in the absence of atropine and when using a submaximal degree of stimulation (Holzer *et al.*, 1993).

In the accompanying paper (Maggi *et al.*, 1994b) we have determined the affinities of a panel of tachykinin receptor antagonists for $NK_1$ and $NK_2$ receptor-induced contraction in the circular muscle of the ileum. From this analysis, it appeared that the affinity of MEN 10,376 for $NK_2$ receptor in the circular muscle of the ileum is lower than that expected on the basis of its potency at $NK_2$ receptors in other guinea-pig smooth muscles. In particular, MEN 10,376 was found to possess very similar affinity for the $NK_2$ receptor and the putative novel type of septide-sensitive receptor described by Petitet *et al.* (1992) in the guinea-pig ileum. On this basis it appeared of interest to re-examine the activity of various $NK_1$ and $NK_2$ receptor antagonists on the atropine-resistant AER.

## Methods

Male albino guinea-pigs weighing 250–350 g were stunned and bled. A 10–15 cm long piece of terminal ileum was excised and placed in warmed (37°C) and oxygenated (96% $O_2$ and 4% $CO_2$, pH 7.4) Krebs solution of the following composition (mM): NaCl 119, $NaHCO_3$ 25, $KH_2PO_4$ 1.2, $MgSO_4$ 1.5, KCl 4.7, $CaCl_2$ 2.5 and glucose 11. The ileum was placed in a thermostated bath (7 ml) and arranged for isotonic (load 5 mN) recording of circular muscle mechanical activity in response to radial stretch produced by distension of a balloon placed at 1–1.2 cm anal to the recording site: the circular muscle contraction evoked in this way is totally hexamethonium- and tetrodotoxin-sensitive (ascending excitatory reflex, AER), as described previously (Holzer, 1989; Bartho *et al.*, 1992). All experiments were performed in the presence of 1 μM atropine which was added to the Krebs solution from the beginning of the experiment.

After a 45 min equilibration time, the intraluminal balloon located anally to the recording site was manually inflated by a syringe with an amount of saline to evoke the AER. In each preparation, maximal AER response was evoked with 0.2–0.3 ml saline; submaximal AER was obtained by distension with 0.1–0.2 ml saline (see Results).

In each preparation, submaximal and maximal AERs were elicited, 5 min apart from each other, at 20 min intervals, until two reproducible responses to both submaximal and maximal stimulation were evoked. At this time the stated concentration of tachykinin receptor antagonists was added to the bath and their effect on submaximal and maximal AER determined 15 min later. This contact time was the same as that used in experiments aiming to determine the affinities of the various antagonists used in this study for $NK_1$ and $NK_2$ receptors in the circular muscle of the ileum (Maggi *et al.*, 1994b). Control experiments showed that submaximal and maximal AER could be elicited for at least seven consecutive stimulation cycles with minimal (10–15% variation) changes in the amplitude of the evoked response.

Control experiments were also performed in which the vehicle dimethylsulphoxide (DMSO) used to dissolve some of the antagonists was added to the preparation after having recorded control responses to submaximal and maximal AER. These experiments ($n = 4$, DMSO 1% final concentration) failed to show any significant effect of the vehicle.

Antagonists tested were: (±)-CP 96,345 (Snider *et al.*, 1991), RP 67,580 (Garret *et al.*, 1991) and FK 888 (Fujii *et al.*, 1992) as $NK_1$ receptor selective antagonists; MEN 10,376 (Maggi *et al.*, 1991), MEN 10,573 (Quartara *et al.*, 1992), SR 48,968 (Emonds-Alt *et al.*, 1992) and GR 94,800 (McElroy *et al.*, 1992) as $NK_2$ receptor antagonists.

At the end of the experiment, KCl (80 mM) was added to the bath. This produced a circular muscle contraction corresponding to total occlusion of the ileal lumen. The response to 80 mM KCl was used as internal standard and the amplitude of AER responses were expressed as a % of the response to KCl.

In a separate set of experiments, the atropine-resistant AER was evoked by electrical field stimulation performed by means of a pair of wire platinum electrodes placed in parallel at 1.4–1.8 cm anally to the site of recording of circular muscle activity, using an approach similar to that described by Allescher *et al.* (1992) for evoking the AER in the rat isolated ileum. The electrodes were connected to a GRASS S88 stimulator: trains of pulses were delivered at a frequency of 10 Hz for 0.5 s every 5 min. Pulse width was 0.1–0.3 ms and voltage was 10–20 V. In each preparation, pulse width and voltage were adjusted to produce maximal circular muscle contraction at the recording site oral to the point of stimulation. This averaged 40–70% of the maximal response to 80 mM KCl. Preliminary experiments (and see results) showed that electrical stimulation with these parameters provided ascending circular muscle contractions which were largely or totally hexamethonium-sensitive. All experiments were performed in the presence of 1 μM atropine.

In a first series of experiments, the electrically-evoked atropine-resistant AER obtained in untreated preparations with ten cycles of stimulation at 5 min intervals was compared to that obtained in the presence of apamin (0.1 μM) and $N^G$-nitro-L-arginine (L-NOARG 30 μM). This was done because preliminary experiments showed a spontaneous fading of the response in untreated preparation while the addition of apamin and L-NOARG improved reproducibility of the response.

In a second series of experiments, in the presence of apamin and L-NOARG, the effect of GR 94,800 (1 μM) or FK 888 (1 μM) alone and in combination was investigated. In these experiments, after having recorded 3–5 control responses at 5 min intervals the drug was added to the bath and its effect recorded over the next 3–4 stimulation cycles. At the end of each experiment, hexamethonium (10 μM) was added to the bath to check the reflex origin of the evoked contractions. All contractile responses were expressed as % of the response to 80 mM KCl.

In other experiments, performed in the absence of atropine, the effects of $NK_1$ and $NK_2$ receptor antagonists on twitch contractions of the longitudinal muscle-myenteric plexus preparation produced by electrical field stimulation (0.1 Hz, 60 V, 0.4 ms pulse width) were determined to assess whether the various drugs may possess nonspecific effects on contractility/local anaesthetic activity. After a 90 min equilibration time, the preparations were electrically stimulated until twitch height reached a steady state. At this stage, the stated concentration of tachykinin receptor antagonists was

added to the bath and its effects recorded for at least 15 min Addition of the vehicle (0.1% DMSO, final concentration in the bath) produced a slight (< 10%) and transient enhancement of twitches. In each preparation atropine (1 μM), tetrodotoxin (1 μM) or procaine (10–30 μM) were tested as positive controls.

### *Data evaluation and statistical analysis*

All values in the text, table and figures are mean ± s.e.mean. Statistical analysis was performed by means of the Student's *t* test for paired data or by means of ANOVA, when applicable.

### *Drugs*

Drugs used were: procaine HCl (Sigma) hexamethonium bromide and atropine HCl (Serva) tetrodotoxin (Sankyo).

RP 67,580 ((3α**R**, 7α**R**)-7,7-diphenyl-2-[1-imino-2-(2-methoxyphenyl)ethyl] perhydroisoindol-4-one) was a kind gift of Dr C. Garret, Rhone Poulenc, Vitry, France; SR 48,968 ((S)-N-methyl-*N* [4- (4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl) butyl]benzamide) of Dr X. Emonds-Alt, Sanofi Recherche, Montpellier, France; GR 94,800 (PhCO-Ala-Ala-D-Trp-Phe-D-Pro-Pro-Nle$NH_2$) was a kind gift from Dr R.M. Hagan, GGR, Ware England. (±)-CP 96,345 ((2S,3S)-*cis*-2-(diphenylmethyl)-*N*-[ (2-methoxyphenyl)-methyl] -1-azabicyclo [2.2.2]octan-3-amine, MEN 10,376 ($Tyr^5$,D-$Trp^{6,8,9}$,$Lys^{10}$] NKA(4–10)), MEN 10,573 (cyclo(Leu$\Psi$[$CH_2$NH]Asp(OBzl)-Gln-Trp-Phe-βAla) and FK 888 ((2-(N-Me)indolil)-CO-Hyp-Nal-NMeBzl) were synthesized in the Chemistry Department of Menarini Pharmaceuticals.

## Results

### *Ascending enteric reflex by balloon distension*

The atropine- (1 μM) resistant ascending enteric reflex (AER) was investigated on 102 preparations. A submaximal AER was elicited at volumes of balloon distension between 0.1–0.2 ml (mean volume 0.13 ± 0.03 ml, $n = 102$) which ranged between 20 and 85% of maximal circular spasm produced by 80 mM KCl (Table 1). Maximal AER was evoked at volumes of 0.2–0.3 ml (mean volume 0.23 ± 0.06 ml, $n = 102$) which ranged between 55 and 100% of maximal circular spasm to KCl.

The effect of various $NK_1$ and $NK_2$ receptor antagonists on the submaximal and maximal atropine-resistant AER is shown in Table 1. (±)-CP 96,345 was ineffective at 0.1 μM while it produced 66% and 46% inhibition of submaximal and maximal AER at 1 μM, respectively. FK 888 was ineffective at 0.1 μM and inhibited (50%) the submaximal AER only at 1 μM. A higher concentration (3 μM) of FK 888 was not more effective than 1 μM (Table 1). RP 67,580 was ineffective at 1.0 μM.

The nonpeptide $NK_2$ receptor antagonist, SR 48,968, inhibited the submaximal and maximal AER (by 63% and 51% respectively) at 1.0 μM while it was ineffective at 0.1 μM. MEN 10,376 was ineffective at 1 μM while at 10 μM it inhibited submaximal and maximal AER by 89% and 60%; MEN 10,573 was ineffective at 0.1 μM, while at 1.0 μM it inhibited submaximal and maximal AER by 75% and 43%, respectively (Table 1).

GR 94,800 inhibited submaximal and maximal AER at both concentrations tested: submaximal AER was inhibited by 48% and 73% at 0.1 and 1.0 μM, respectively; maximal AER was inhibited by 23% and 43% at 0.1 and 1.0 μM, respectively (Table 1). A higher concentration of GR 94,800 (3 μM) was not more effective than 1 μM (Table 1).

**Table 1** Effect of various tachykinin receptor antagonists on the atropine (1.0 μM)-resistant ascending enteric reflex evoked by submaximal or maximal balloon distension in the guinea-pig ileum

| *Antagonist* | | *Concentration* | *Response to distension* (% of response to KCl) Submaximal | % inh. | Maximal | % inh. |
|---|---|---|---|---|---|---|
| (±)-CP 96,345 | ($n = 11$) | Control | 47 ± 8 | | 88 ± 4 | |
| | | 0.1 μM | 41 ± 8 | – | 76 ± 5 | – |
| | | 1.0 μM | 16 ± 6* | 66% | 48 ± 8 * | 46% |
| FK 888 | ($n = 9$) | Control | 48 ± 2 | | 67 ± 3 | |
| | | 0.1 μM | 36 ± 9 | – | 60 ± 4 | – |
| | | 1 μM | 24 ± 10* | 50% | 55 ± 6 | – |
| | ($n = 4$) | Control | 52 ± 6 | | 72 ± 4 | |
| | | 3 μM | 24 ± 6 | 54% | 62 ± 6 | – |
| RP 67,580 | ($n = 7$) | Control | 44 ± 10 | | 71 ± 11 | |
| | | 1.0 μM | 36 ± 12 | – | 58 ± 9 | – |
| GR 94,800 | ($n = 21$) | Control | 44 ± 6 | | 75 ± 3 | |
| | | 0.1 μM | 23 ± 5* | 48% | 58 ± 3* | 23% |
| | | 1.0 μM | 12 ± 4* | 73% | 43 ± 5* | 43% |
| | ($n = 4$) | Control | 59 ± 6 | | 82 ± 5 | |
| | | 3.0 μM | 18 ± 4* | 70% | 44 ± 5* | 47% |
| SR 48,968 | ($n = 11$) | Control | 58 ± 8 | | 89 ± 3 | |
| | | 0.1 μM | 41 ± 9 | – | 70 ± 6 | – |
| | | 1.0 μM | 22 ± 11* | 63% | 44 ± 15* | 51% |
| MEN 10,573 | ($n = 15$) | Control | 71 ± 6 | | 83 ± 4 | |
| | | 0.1 μM | 69 ± 9 | – | 80 ± 7 | – |
| | | 1.0 μM | 18 ± 8* | 75% | 48 ± 6* | 43% |
| MEN 10,376 | ($n = 6$) | Control | 52 ± 7 | | 90 ± 4 | |
| | | 1.0 μM | 42 ± 66 | – | 79 ± 6 | – |
| | | 10 μM | 6 ± 6* | 89% | 36 ± 4* | 60% |

The atropine-resistant AER was evoked in each preparation by submaximal and maximal balloon distension. The amplitude of evoked responses is quantified as % of the control response to KCl. After having established control responses to submaximal balloon distension, the effect of tachykinin antagonists was investigated. For those concentrations of antagonists which produced a statistically significant reduction of evoked AER, the % inhibition values of control response are also presented.
*Significantly different from control value, $P < 0.05$. Inhibitory effects which reached statistical significance are also indicated as % inhibition of control values.

The effect of GR 94,800 (0.1–1.0 μM), administered in the presence of 1 μM (±)-CP 96,345 or FK 888, is shown in Figure 1. In the presence of 1 μM (±)-CP 96,345, GR 94,800 abolished the submaximal AER and inhibited the residual maximal AER by 62% and 85% at 0.1 and 1.0 μM, respectively. In the presence of 1 μM FK 888, GR 94,800 inhibited the submaximal AER by 50% and 79% at 0.1 and 1.0 μM, respectively and the residual maximal AER by 46% and 60% at 0.1 and 1.0 μM, respectively.

The effect of (±)-CP 96,345 (0.1–1.0 μM) or FK 888 (0.1–1.0 μM) in the presence of 1 μM GR 94,800 is shown in Figure 2. In the presence of GR 94,800, (±)-CP 96,345 abolished the residual submaximal AER at 1.0 μM; the maximal AER was further reduced by 36% and 67% at 0.1 and 1.0 μM (±)-CP 96,345, respectively.

In the presence of GR 94,800 (1.0 μM), FK 888 had no further inhibitory effect on either submaximal or maximal AER at 0.1 μM. At 1.0 μM, FK 888 abolished the submaximal AER and inhibited maximal AER by 34% (Figure 2).

### *Ascending enteric reflex by electrical field stimulation*

Electrical field stimulation (10 Hz for 0.5 s, 10–20 V, 0.15–0.3 ms pulse width) evoked atropine (1 μM)-resistant circular muscle contraction at the oral recording site which was placed at 1.6 ± 0.2 cm from the stimulation site ($n = 32$, range 1.4–1.8 cm).

In a first series of experiments, the reproducibility of the evoked response was assessed by delivering trains of stimuli at 5 min intervals and this showed a significant decay (Figure 3). This decay was equally evident when the interstimulus interval was of 10 min (not shown). In the presence of apamin (0.1 μM) and L-NOARG (30 μM) added 45 min before the stimulation, the evoked contraction did not show a significant decay over ten consecutive stimulation cycles, 5 min apart from each other (Figure 3).

In the presence of apamin and L-NOARG, the amplitude of oral contraction evoked by electrical field stimulation averaged 59 ± 5% of maximal circular muscle response to KCl ($n = 18$) and this response was largely >85% inhibited by 1 μM, hexamethonium, indicating its reflex origin. In 13 out of 18 cases tested, including the example shown in Figure 4a, hexamethonium completely abolished the evoked response.

In a second series of experiments, the effect of GR 94,800 (1 μM) and FK 888 (1 μM) on the electrically-evoked AER was determined (Figure 4b, and Figure 5). GR 94,800 produced 36% inhibition of the electrically-evoked AER and, in its presence, FK 888 produced a further 35% reduction of the residual response ($n = 8$, Figure 5). When added first, FK 888 produced 29% inhibition of the electrically evoked AER and, in its presence, GR 94,800 produced 45% inhibition of the residual response ($n = 8$, Figure 5). In both series of experiments, the residual response in the presence of GR 94,800 and FK 888 was almost abolished by 10 μM hexamethonium (Figures 4 and 5).

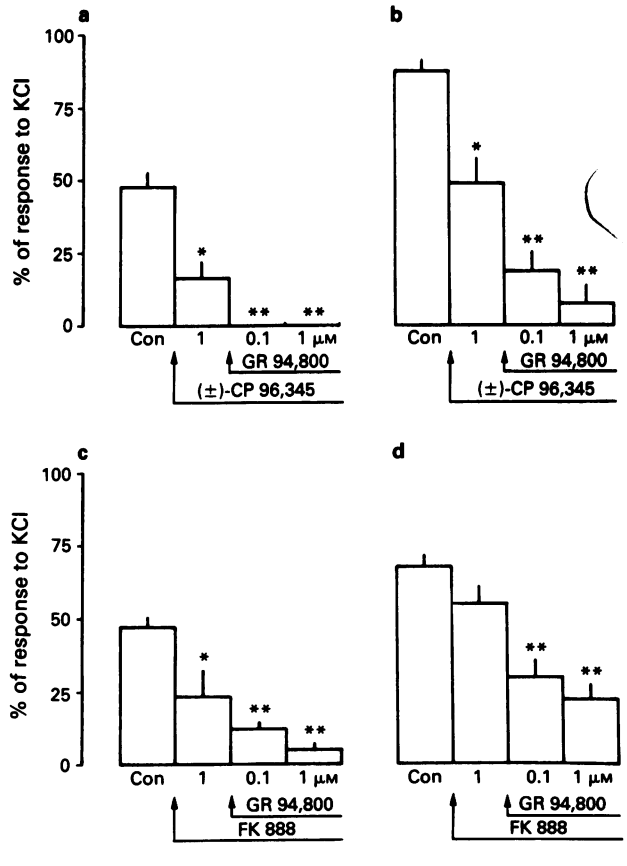


**Figure 1** Effect of tachykinin receptor antagonists on the atropine-resistant AER evoked by submaximal (a,c) or maximal (b,d) balloon distension in the guinea-pig ileum. In the experiments shown in (a) and (b), (±)-CP 96,345 was administered first and, in its presence, GR 94,800 was investigated. In (c) and (d), FK 888 was added first and, in its presence, the effect of GR 94,800 was investigated. Each value is mean ± s.e.mean of at least six determinations. *Significantly different from control (con) response: $P < 0.05$. **Significantly different from the response obtained in the presence of (±)-CP 96,345 (a,b) or FK 888 (c,d), $P < 0.05$.

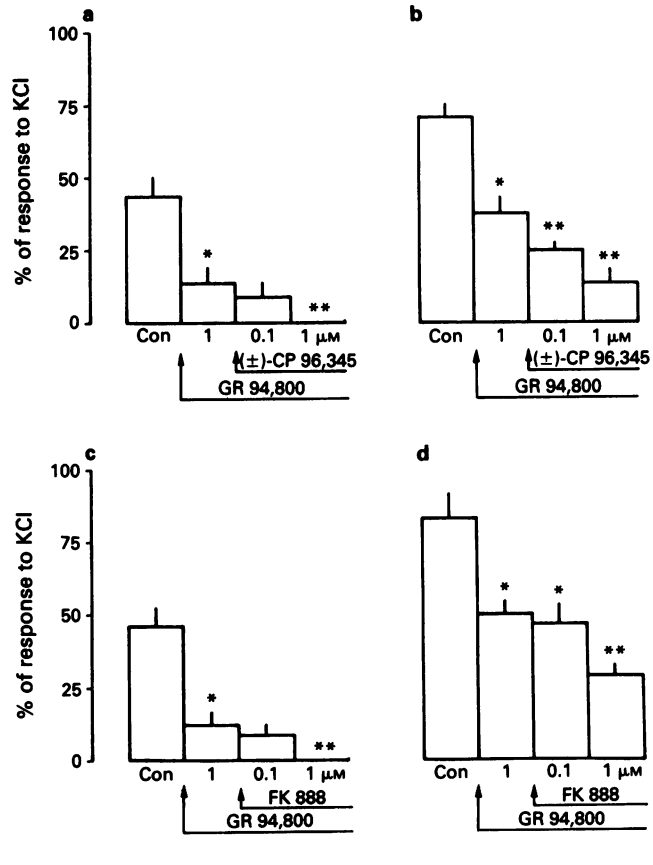


**Figure 2** Effect of tachykinin receptor antagonists on the atropine-resistant AER evoked by submaximal (a,c) or maximal (b,d) balloon distension in the guinea-pig ileum. In the experiments shown in (a) and (b), GR 94,800 was administered first and, in its presence, (±)-CP 96,345 was investigated. In (c) and (d), GR 94,800 was added first and, in its presence, the effect of FK 888 was investigated. Each value is mean ± s.e.mean of at least six determinations. *Significantly different from control (Con) response $P < 0.05$. **Significantly different from the response obtained in the presence of GR 94,800, $P < 0.05$.

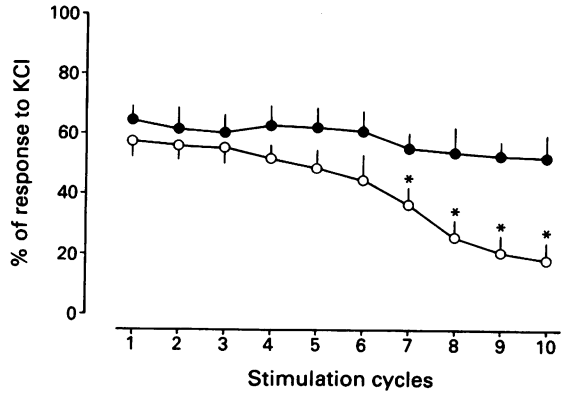


**Figure 3** Effect of apamin (0.1 μM) and $N^G$-nitro-L-arginine (L-NOARG, 30 μM) on time course of the electrically-evoked AER induced by 10 consecutive cycles of stimulation: (○) control; (●) apamin plus L-NOARG. Each value is mean ± s.e.mean of six experiments.

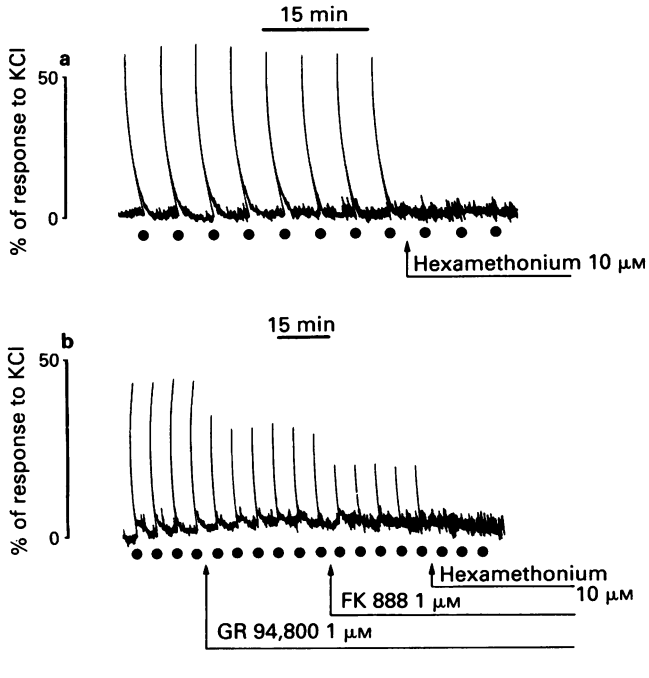


**Figure 4** Electrically-evoked atropine-resistant AER in the guinea-pig ileum in the presence of apamin (0.1 μM) and $N^G$-nitro-L-arginine (L-NOARG, 30 μM): effect of hexamethonium (a); effect of GR 94,800, FK 888 and hexamethonium (b).

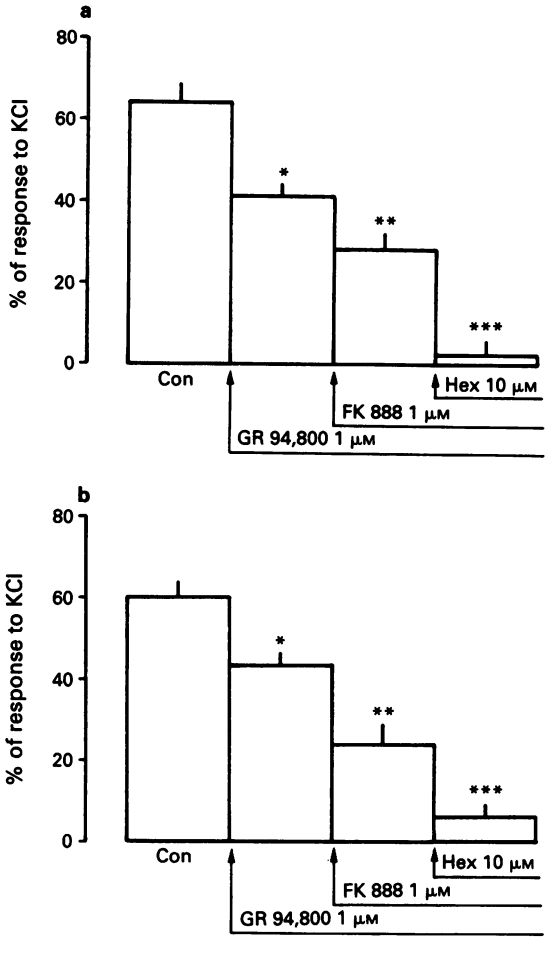


**Figure 5** Effect of GR 94,800 and FK 888 on the atropine-resistant AER evoked by electrical stimulation in the guinea-pig ileum in the presence of apamin (0.1 μM) and $N^G$-nitro-L-arginine (L-NOARG, 30 μM). In experiments shown in (a), GR 94,800 was added first and, in its presence, the effect of FK 888 was determined. In the experiments shown in (b), the effect of FK 888 was determined first and, in its presence, the effect of GR 94,800 was determined. At the end of the experiments hexamethonium (Hex) was added to check the reflex nature of the evoked response. Each value is mean ± s.e.mean of at least six experiments. *Significantly different from control (Con) $P<0.05$; **Significantly different from the value recorded in the presence of GR 94,800 (a) or FK 888 (b), $P<0.05$; ***Significantly different from the value recorded in the presence of FK 888 and GR 94,800, $P<0.05$.

### *Effect of tachykinin receptor antagonists on twitch response of the longitudinal muscle to electrical field stimulation*

FK 888 (1–3 μM), GR 94,800 (1–3 μM), MEN 10,573 (1 μM) and MEN 10,376 (10 μM) ($n = 4$ for each antagonist) had no significant inhibitory effect on the amplitude of twitch contractions of the guinea-pig ileum longitudinal muscle-myenteric plexus preparation evoked by electrical field stimulation (0.4 ms pulse width, maximal voltage delivered at a frequency of 0.1 Hz). (±)-CP 96,345 (1 μM) and SR 48,968 (1 μM) produced 12 ± 3% and 29 ± 7% inhibition of twitches ($n = 4$ for each antagonist). The evoked contractions were suppressed by atropine (1 μM), tetrodotoxin (1 μM) or procaine (10–30 μM, $n = 4$).

## Discussion

Three different inputs have prompted us to re-investigate the role of $NK_1$ and $NK_2$ receptors in the AER of the guinea-pig ileum; first, the availability of novel and more potent antagonists for both receptors, especially the nonpeptide antagonists CP 96,345 and SR 48,968 and the peptide antagonists FK 888 and GR 94,800. Second, Suzuki & Gomi (1992) reported that CP 96,345, at a concentration of 1 μM, inhibits or suppresses the atropine-resistant peristalsis of the guinea-pig isolated ileum. Although this concentration of CP 96,345 is near to those producing nonspecific depressant effects on the contractility of the guinea-pig ileum (Lecci *et al.*, 1991; Legat *et al.*, 1992), these observations were interpreted as evidence of a major role for $NK_1$ receptors (substance P preferring) in mediating intestinal peristalsis. Third, the proposal for the existence of a novel, septide-sensitive receptor in the guinea-pig ileum (Petitet *et al.*, 1992; Maggi *et al.*, 1993b) further complicated the interpretation of results, because the activity of the various antagonists at the putative novel septide-sensitive receptor was unknown.

Both the distension- and the electrically-evoked atropine-resistant circular muscle contraction elicited with the present set up are abolished by hexamethonium, indicating that at least one nicotinic synapse is involved in their genesis. The

simplest arrangement to account for the AER to the circular muscle of the ileum requires the existence of a sensory neurone which detects radial stretch to distension, an interneurone and an effector neurone projecting to circular muscle. Tonini & Costa (1990) provided evidence that acetylcholine, through nicotinic and muscarinic receptors, plays a role at each one of the three transmission sites of this polysynaptic reflex. Tachykinins, via $NK_1$ and $NK_2$ receptors, are thought to play a role as final effectors of the atropine-resistant neuromuscular transmission to the circular muscle and, through a different receptor (putatively $NK_3$), could also play a role in hexamethonium-resistant neuro-neuronal communication (Bartho *et al.*, 1989; 1992; Holzer, 1989; Tonini & Costa, 1990; Holzer *et al.*, 1993). At the present time, there is no evidence to show that $NK_1$ and/or $NK_2$ receptors play a role in neuro-neuronal communication in enteric neural pathways, although such a possibility cannot be ruled out.

As a preliminary step to this study, we assessed the affinities of the various antagonists for $NK_1$ and $NK_2$ receptors mediating contraction of the circular muscle of the guinea-pig ileum (Maggi *et al.*, 1994b). This gave us information on the potency and selectivity of the various ligands used here to block the effect of endogenous tachykinins.

Interpretation of the present results is critically dependent upon: (a) the selectivity and specificity of tachykinin receptor antagonists in blocking $NK_1$ and $NK_2$ and, (b) the assumption that maximal inhibitory effects were produced by individual antagonists used in experiments in which the combined effects of an $NK_1$ and an $NK_2$ antagonist were investigated. With regard to the first point, it appears that because of the combination of their potency, selectivity for only one tachykinin receptor and lack of nonspecific effects, FK 888 and GR 94,800 are the most interesting tools for producing a selective blockade of $NK_1$ and $NK_2$ receptors, respectively, in the circular muscle of the guinea-pig ileum. The interpretation of results obtained with other antagonists is more difficult either because of nonspecific depressant effects on ileal contractility at certain concentrations (e.g. CP 96,345 and SR 48,968 which depress cholinergic twitches of the longitudinal muscle) or because of a more limited ability to discriminate between $NK_1$ and $NK_2$ receptors. Notwithstanding, the results obtained here with several antagonists which belong to different chemical classes are in general agreement with the basic idea that tachykinins are the main transmitters responsible for atropine-resistant excitatory reflex in the ileum. The actions of FK 888 and GR 94,800 were assessed in further detail by studying the effect of their combined administration and by using a different stimulus (electrical stimulation) to evoke the AER: the assumption that 1 μM concentration of these antagonists produced maximally effective inhibitory effects was verified by showing that at 3 μM they did not produce a larger inhibitory effect on the AER.

### *Tachykinin $NK_1$ receptors in the AER to the circular muscle of the guinea-pig ileum*

The present findings disclose a small but sizeable role of $NK_1$ receptors in mediating the atropine-resistant AER, especially when a submaximally effective stimulus is used to produce radial stretch of the ileal wall. CP 96,345 and FK 888 both inhibited the submaximal AER at 1 μM. The nonpeptide antagonist, RP 67,580, which is ineffective at $NK_1$ receptors of the ileum up to 1 μM (higher concentrations produce nonspecific reduction of contractile responses to various agonists) (Maggi *et al.*, 1994b), was ineffective toward the submaximal or maximal AER. At the highest concentrations used in this study, both (±)-CP 96,345 and FK 888 were ineffective toward circular muscle contraction induced by the $NK_2$ receptor agonist, [βAla$^8$]neurokinin A(4–10) (Maggi *et al.*, 1994b): this excludes a nonspecific depressant effect on smooth muscle contractility in the observed AER depression.

(±)-CP 96,345 was more effective than FK 888 in inhibiting the distension-evoked AER, since the maximal response to distension was also inhibited by the nonpeptide antagonist. Since CP 96,345 is more potent than FK 888 in blocking $NK_1$ receptors in the circular muscle of the ileum (Maggi *et al.*, 1994b) this may indicate a larger contribution of $NK_1$ receptors to the AER than that detected through the inhibitory action of FK 888. On the other hand, it is doubtful if the greater effectiveness of (±)-CP 96,345 can be explained in terms of $NK_1$ receptor blockade only: (±)-CP 96,345 interacts, at μM concentrations, with both calcium and sodium channels (Schmidt *et al.*, 1992a; Caeser *et al.*, 1993). Since the activation of the AER involves neuro-neuronal communication in the myenteric plexus, we cannot exclude the possibility that 1.0 μM (±)-CP 96,345 produced some nonspecific depressant effect on neuronal excitability, partly responsible for the observed AER depression. A recent study (Tamura *et al.*, 1993) showed that CP 96,345 exerts a nonselective local anaesthetic effect on the excitability of myenteric neurones in the guinea-pig ileum, the lowest effective concentration being 10 μM. The highest concentration of (±)-CP 96,345 tested in this study, 1 μM produces a slight but significant inhibition of cholinergic twitches in the longitudinal muscle of the ileum (cf. Legat *et al.*, 1992).

Since the contribution of $NK_1$ receptors to the atropine-resistant AER is relatively minor as compared to that exerted by $NK_2$ receptors, it is not easy to dissect out the relative contribution of the putative 'septide-sensitive' receptor, as opposed to the 'classical' $NK_1$ receptor to the AER. (±)-CP 96,345 possesses a very high affinity for the 'septide-sensitive' receptor in the ileum ($pK_B$ 9.24), its dissociation constant measured against septide as agonist being about 1000 times lower than the highest concentration (1 μM) tested here toward the atropine-resistant AER. Because both (±)-CP 96,345 and FK 888 are more potent toward septide than toward a 'classical' $NK_1$ receptor agonist (Maggi *et al.*, 1994b) our conclusion about a sizeable, albeit minor, $NK_1$ receptor-mediated component in the atropine-resistant AER applies equally well to the 'septide-sensitive' and 'classical' $NK_1$ receptor.

In the present study we also used a technique, developed by Allescher *et al.* (1992) for studying the electrically-evoked AER in the rat ileum. The evoked response can be assumed to at least partly overlap with that evoked by balloon distension. The inhibitory effects of FK 888 in this model confirm the results obtained with the balloon distension model, and further imply a certain degree of activation of $NK_1$ receptors in the atropine-resistant AER.

### *Tachykinin $NK_2$ receptors in the AER to the circular muscle of the guinea-pig ileum*

Our results add further weight to the conclusion (Bartho *et al.*, 1992; Holzer *et al.*, 1993) that $NK_2$ receptors, putatively activated by endogenous neurokinin A, play a dominant role in mediating the atropine-resistant AER: the selective $NK_2$ receptor antagonists, GR 94,800 and SR 48,968 inhibited the atropine-resistant AER with relative potency (GR 94,800 > SR 48,968) consistent with their relative potency ($pK_B$ 8.85 and 8.09 for GR 94,800 and SR 48,968, respectively) in antagonizing $NK_2$ receptor-mediated contraction in the circular muscle of the ileum (Maggi *et al.*, 1994b). AER inhibition produced by SR 48,968 at 1.0 μM is not likely to involve blockade of $NK_2$ receptors only for two reasons: (i) 1.0 μM SR 48,968 reduces (by 20–30%) the maximal contractile response to $NK_1$ receptor stimulation in the circular muscle of the ileum (Maggi *et al.*, 1994b); (ii) 1.0 μM SR 48,968 reduces to a similar extent cholinergic twitches of the longitudinal muscle, indicating some nonspecific effect.

The involvement of $NK_2$ receptors in the atropine-resistant AER is further supported by experiments with MEN 10,573 and MEN 10,376, although the affinity of these ligands for $NK_2$ receptors in the circular muscle of the ileum is lower than that of GR 94,800 and SR 48,968: the cyclic pseudopeptide, MEN 10,573 is slightly more potent ($pK_B$ 7.18) than

MEN 10,376 ($pK_B$ 6.44) at $NK_2$ receptors in the circular muscle of the ileum (Maggi *et al.*, 1994b) and is likewise more potent than MEN 10,376 in inhibiting the submaximal and maximal AER evoked by balloon distension. Since MEN 10,376 possesses comparable affinities at $NK_2$ and 'septide-sensitive' receptor in the circular muscle of the ileum (Maggi *et al.*, 1994b) it is quite conceivable that its inhibitory effect on AER involves the blockade of more than one tachykinin receptor.

### *Relative contribution of $NK_1$ and $NK_2$ receptors to the atropine-resistant AER*

The present results indicate that the relative contribution of $NK_2$ receptors to the distension-evoked AER is greater than that of $NK_1$ receptors. Of the various antagonists used in this study, FK 888 and GR 94,800 are, because of their potency and selectivity (Maggi *et al.*, 1994b), and lack of nonspecific effects, the most suitable tools to dissect the relative contribution of different tachykinin receptors in the overall physiological response. FK 888 at concentrations (1.0–3.0 μM) which are about 30–300 times higher than its dissociation constant for $NK_1$ receptors in the circular muscle of the ileum, produced 50% reduction of submaximal atropine-resistant AER without affecting the maximal response to balloon distension. GR 94,800 at a concentration (0.1 μM) which is about 100 times higher than its dissociation constant for $NK_2$ receptors, produced 50% reduction of the submaximal AER and 23% reduction of the maximal AER induced by balloon distension. Higher (1.0–3.0 μM) concentrations of GR 94,800 produced further depression of maximal AER to balloon distension. These results indicate that the submaximal atropine-resistant AER produced by balloon distension is mediated by both $NK_1$ and $NK_2$ receptors while the maximal AER is not significantly blunted by blockade of $NK_1$ receptors only. This interpretation is supported by the results of experiments in which the combined administration of GR 94,800 and FK 888 or of GR 94,800 and CP 96,345 abolished the submaximal AER induced by balloon distension. In these experiments, the addition of FK 888 in the presence of GR 94,800 produced some inhibition of the distension-induced maximal AER indicating that, after occlusion of $NK_2$ receptors, a contribution of $NK_1$ receptors to the maximal AER can be demonstrated. Overall, the results of these experiments indicate an additive effect of the degree of blockade produced by $NK_1$ and $NK_2$ receptor antagonists.

This conclusion is further supported by the additive inhibitory effect produced by FK 888 and GR 94,800 on the electrically-evoked atropine-resistant AER. In these experiments, the degree of blockade of FK 888 was somewhat larger than that expected on the basis of its effectiveness toward the distension-induced AER. However, since apamin and L-NOARG were used to improve the reproducibility of the electrically-evoked response, the results of the two experiments are not strictly comparable.

### *A third excitatory mediator of the atropine-resistant AER?*

The atropine-resistant AER evoked by submaximal distension of an intraluminal balloon is practically abolished by combined $NK_1$ and $NK_2$ receptor blockade. On the other hand, a consistent fraction of the maximal response produced by balloon distension is still evident in the presence of $NK_1$ and $NK_2$ receptor antagonists at concentrations which are at least 100 times higher than their dissociation constant from the respective receptors. Likewise, about 30–40% of the overall atropine-resistant AER evoked by electrical stimulation was resistant to the combined administration of FK 888 and GR 94,800. It appears unlikely that the residual component of the atropine-resistant AER, still observed in the presence of both FK 888 and GR 94,800 may involve $NK_3$ receptors, since the contractile response to $NK_3$ receptor agonists in the circular muscle of the ileum is totally, indirect, being mediated through the release of both acetylcholine and endogenous tachykinins (Maggi *et al.*, 1990; 1994a). The present results raise the possibility that, in addition to acetylcholine and tachykinins, a third excitatory transmitter to the circular muscle of the ileum exists. This hypothesis is indirectly supported by electrophysiological evidence that two distinct types of atropine-resistant excitatory junction potential can be evoked in circular muscle cells of the guinea-pig ileum, and only one of them is sensitive to tachykinin receptor antagonists (Bywater & Taylor, 1986; Crist *et al.*, 1991).

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# Stimulation of chloride secretion by $P_1$ purinoceptor agonists in cystic fibrosis phenotype airway epithelial cell line CFPEo-

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**1** $P_1$ purinoceptor agonists like adenosine have been shown to stimulate $Cl^-$ transport in secretory epithelia. In the present study, we investigated whether $P_1$ agonist-induced $Cl^-$ secretion is preserved in cystic fibrosis airway epithelium and which signalling mechanism is involved. The effects of purinoceptor agonists on $Cl^-$ secretion were examined in a transformed cystic fibrosis airway phenotype epithelial cell line, CFPEo-.

**2** Addition of adenosine (ADO; 0.1–1 mM) markedly increased $^{125}I$ efflux rate. The rank order of potency of purinoceptor agonists in stimulating $^{125}I$ efflux was ADO > AMP > ADP ≃ ATP. A similar order of potency was seen in transformed cystic fibrosis nasal polyp cells, CFNPEo- (ADO > ATP > AMP > ADP). These results are consistent with the activation of $Cl^-$ secretion via a $P_1$ purinoceptor.

**3** The $P_1$ agonists tested (at 0.01 and 0.1 mM) revealed a rank order of potency of 5'-N-ethylcarboxamine adenosine (NECA) > 2-chloro-adenosine (2-Cl-ADO) > **R**-phenylisopropyl adenosine (**R**-PIA).

**4** The known potent $A_2$ adenosine receptor ($A_2AR$) agonist, 5'-(N-cyclopropyl) carboxamidoadenosine (CPCA, 2 μM) but not the $A_1$ adenosine receptor agonist, $N^6$-phenyl adenosine ($N^6$-phenyl ADO, 10 μM) markedly increased $^{125}I$ efflux rate (baseline, 5.9 ± 2.0% $min^{-1}$, + CPCA, 10.9 ± 0.6% $min^{-1}$; $P < 0.01$). The stimulant effect of CPCA (10 μM) was abolished by addition of the $A_2AR$ antagonist 3,7-dimethyl-1-propargylxanthine (DMPX) (100 μM; reported $K_i = 11 \pm 3$ μM). These results favour the involvement of $A_2AR$.

**5** ADO (0.1–1 mM) and CPCA (2 μM) both induced a marked increase in intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$); the effect of the latter was again abolished by pretreatment of the cells with DMPX. By contrast, $N^6$-phenyl ADO did not affect $[Ca^{2+}]_i$.

**6** In patch-clamp experiments, ADO (1 mM) induced an outwardly-rectified whole-cell $Cl^-$ current (baseline, 2.5 ± 0.8 pA $pF^{-1}$, + ADO, 78.4 ± 23.8 pA $pF^{-1}$; $P < 0.02$), which was largely inhibited in cells internally perfused with a selective inhibitory peptide of the multifunctional $Ca^{2+}$/calmodulin-dependent protein kinase, CaMK [273-302] (20 μM), as compared to a control peptide, CaMK [284-302]. Addition of BAPTA (10 mM), a $Ca^{2+}$ chelator, to the perfusion pipette also abolished the ADO-elicited $Cl^-$ current.

**7** In conclusion, our results suggest that $A_2AR$ participates in regulation of airway $Cl^-$ secretion via a $Ca^{2+}$-dependent signalling pathway, which involves CaMK and appears to be at least partially conserved in cystic fibrosis airway epithelial cells.

**Keywords:** $P_1$ purinoceptor; cystic fibrosis; airway epithelium; chloride channel; $Ca^{2+}$; multifunctional calcium/calmodulin-dependent protein kinase

## Introduction

Cystic fibrosis (CF) is the most common inherited fatal disease among Caucasians, with an estimated incidence of 1 in 2000 to 3000 newborns (Boat *et al.*, 1989). The primary physiological defect associated with CF is altered salt and water transport in epithelial cells (Boat *et al.*, 1989; Anderson *et al.*, 1992) which is normally regulated by an adenosine 3':5'-cyclic monophosphate (cyclic AMP) dependent pathway. Considerable efforts have been made in recent years to improve epithelial $Cl^-$ secretion through alternate signal-transduction pathways that are independent of cyclic AMP. $P_2$ purinoceptor agonists, ATP and UTP, have recently been shown to enhance $Cl^-$ secretion in CF nasal epithelial cells (Mason *et al.*, 1991; Stutts *et al.*, 1992), and have been tested in clinical trials (Knowles *et al.*, 1991). Other purine analogues, namely $P_1$ purinoceptor agonists like adenosine (ADO), have also been shown to stimulate $Cl^-$ transport in secretory epithelia (Pratt *et al.*, 1986; Barrett *et al.*, 1990) including airway cells. We questioned whether $P_1$ purinoceptor-mediated $Cl^-$ secretion is preserved in CF airway epithelial cells, and, if so, what signalling mechanism is involved. We investigated the effects of various $P_1$ purinoceptor agonists in a simian virus 40 (SV40)-transformed CF phenotype airway epithelial cell line, CFPEo- (Cozens *et al.*, 1991; 1992).

Purinoceptors have been classified after Burnstock (1990) as $P_1$ and $P_2$ subtypes, based on their preferences for ADO or adenine nucleotides. A $P_1$ receptor typically responds to these agents in the rank order of potency of ADO ≥ AMP ≥ ADP ≥ ATP and is therefore also named adenosine receptor; the responsiveness of the $P_2$ subtype usually shows the reverse order (Stiles, 1991; Olah & Stiles, 1992). $P_1$ receptors are further subclassified into $A_1$ and $A_2$ adenosine receptor subtypes. $A_1$ adenosine receptor ($A_1AR$) generally responds to ADO analogues in the potency order of **R**-phenylisopropyl adenosine (**R**-PIA) > 2-chloro-adenosine (2-Cl-ADO) ≥ 5'-N-ethylcarboxamine adenosine (NECA) (Ukena *et al.*, 1987; Williams, 1987); whereas $A_2AR$ responds in the order of NECA > 2-Cl-ADO > **R**-PIA (Stiles, 1991). $A_1AR$ has been shown to couple to a variety of effector systems, including

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adenylate cyclase, guanylate cyclase, membrane ion channels, and phospholipases (Olsson & Pearson, 1990). So far, the only established effector system for $A_2AR$ is adenylate cyclase (Stiles, 1991). In this study, we demonstrate that $P_1$ purinoceptor agonists act on $A_2AR$ to activate airway $Cl^-$ secretion via a $Ca^{2+}$-dependent signalling pathway, which appears to be preserved in CF airway epithelial cells.

## Methods

### *Cell culture*

CFPEo- cells were transformed as described (Cozens *et al.*, 1991; 1992). Cells were grown in Minimum Essential Medium supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, NY, U.S.A.), 100 u $ml^{-1}$ penicillin and 0.2 mg $ml^{-1}$ streptomycin (Biofluids, Rockville, MD, U.S.A.). The culture medium was replenished 2–3 times per week. Cells were used at passage 63–70.

### *$^{125}I$ efflux assay*

Cells were plated on 22 × 22-mm square plastic coverslips (VWR, San Francisco, CA, U.S.A.) and used at 90–100% confluency ~1 week after seeding. The efflux solution contained (in mM): 135 NaCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 2.4 $K_2PO_4$, 0.6 $kH_2PO_4$, 10 glucose, and 10 HEPES (pH 7.4). In high-$K^+$ (120 mM) efflux solution, 114.6 mM KCl substituted for equal amount of NaCl. Cells were first loaded with 20 μCi $ml^{-1}$ $^{125}I$ for ~2 h in a water bath gassed with 100% $O_2$ at 37°C. Extracellular $^{125}I$ was eliminated by rapidly rinsing the cell monolayer on coverslip three times in efflux solution for a cumulative time of 1 min. The efflux experiment was then carried out by sequentially transferring the cell monolayer/coverslip, at appropriate time intervals (usualy 1 min), through a series of cell culture dishes (Costar, Cambridge, MA, U.S.A.) containing 3 ml efflux solution plus the desired agent(s) at room temperature. The cell monolayer/coverslip was retained in the last culture dish of transfer. $^{125}I$ effluxed into each dish was counted individually in a gamma radiation counter (LKB, Gaithersburg, MD, U.S.A.). Non-efflux counts were determined by counting the cells together with the 3 ml efflux solution in the last cell culture dish.

### *Fluorescence measurement of $[Ca^{2+}]_i$*

CFPEo- cells were plated on 9 × 22-mm rectangular glass coverslips (Wheaton, Milville, NJ, U.S.A.) and studied at 90–100% confluency ~1 week following seeding. Cells were incubated in efflux solution containing 5–10 μM fura-2-AM and 0.05% (w/v) pluoronic F127 for 15–20 min at 37°C and rinsed with dye-free solution. The coverslip was then mounted vertically in an acrylic cuvette at an angle of ~60 degrees from the incident light. Agents were added to the cuvette during an experiment by means of a Hamilton syringe. Fluorescence was measured in a spectrofluorimeter (SLM-AMINCO, Urbana, IL, U.S.A.) at 30°C. Excitation wavelength was altered between 340 and 380 nm every 0.2 s and the emission fluorescence monitored at 510 ± 10 nm. $[Ca^{2+}]_i$ was determined as described by Grynkiewicz *et al.* (1985).

### *Electrophysiology*

Whole-cell patch-clamp experiments were performed in single CFPEo- cells grown on glass coverslips 1–2 days following seeding. Cells/coverslip were placed in a 1 ml acrylic chamber on the stage of a Zeiss IM inverted microscope and bathed in a solution containing (in mM) Tris-Cl 170, $MgCl_2$ 1, $CaCl_2$ 2.5, glucose 15, and HEPES 10 (pH 7.4; ~330 mosm $kg^{-1}$), at 25–30°C. The pipette solution contained (in mM): CsCl 140, $MgCl_2$ 2, EGTA 1, MgATP 2, glucose 10 and HEPES 5 (pH 7.35; ~300 mosm $kg^{-1}$). The bath solution was made ~30 mosm $kg^{-1}$ hypertonic compared with the pipette solution to prevent hyponicity-induced $Cl^-$ current (Worrell *et al.*, 1989). Micropipettes were made as described by Hamill *et al.* (1981) and had a tip resistance of 2–3 MΩ. Whole-cell currents were recorded with an Axopatch amplifier (Axon Instruments, Foster City, CA, U.S.A.). Voltage-clamp protocols were run with an aid of a Tecmar 12-bit A/D-D/A converter and an IBM-AT computer. Signals, filtered at 1 kHz, were displayed on a strip-chart recorder and stored in floppy disks. Data were analyzed by means of pClamp, version 5.5 (Axon Instruments).

### *Materials*

CPCA, DMPX and $N^6$-phenyladenosine were purchased from Research Biochemical Inc. (Nalick, MA, U.S.A.). Fura-2-acetoxymethyl ester (fura-2-AM), 1,2-*bis*(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid, tetracesium salt (BAPTA) and pluoronic F127 were from Molecular Probes (Eugene, OR, U.S.A.). Other reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Synthetic peptides CaMK [273-302] and CaMK [284-302] were kindly provided by Dr Howard Schulman, Stanford University.

## Results

### *Effect of purinoceptor agonists on $^{125}I$ efflux rate*

We initially studied and compared the effect of adenine nucleotides ATP, ADP, and 5'-AMP on $^{125}I$ efflux rate in CFPEo- cells to test whether the $P_2$ purinoceptor plays a predominant role in regulating membrane conductive $Cl^-$ transport. We concentrated on CF phenotype airway cells since $P_2$ purinoceptor agonists are in clinical trial as a therapeutic agent to improve airway function in CF, presumably by enhancing $Cl^-$ secretion. $^{125}I$ was used in the efflux assay because iodine is selectively transported through the $Cl^-$ conductive pathway by epithelial cells (Widdicombe & Welsh, 1980). As illustrated in Figure 1a, $^{125}I$ efflux rate

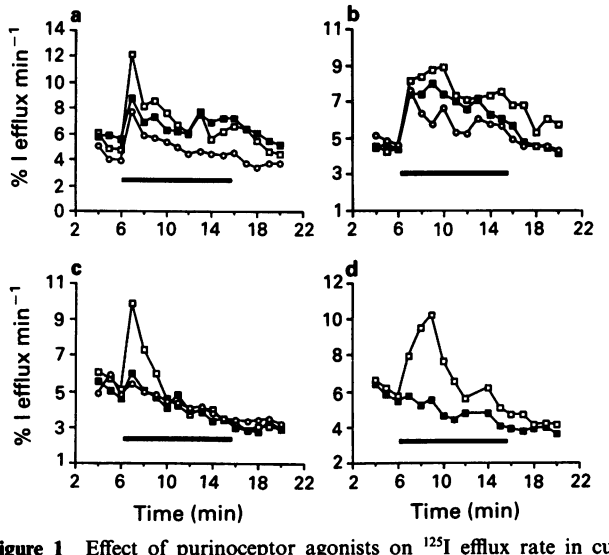


**Figure 1** Effect of purinoceptor agonists on $^{125}I$ efflux rate in cultured monolayers of CFPEo- cells. Results shown in each panel were obtained from paired experiments performed under identical conditions in the same day. Agonists were added where indicated by the bar. (a and b) Effect of ATP (○), ADP (■), and 5'-AMP (□) (1 mM) on $^{125}I$ efflux in low-$K^+$ (4.5 mM; a) and high-$K^+$ (125 mM; b) bathing medium, respectively. (c) Effect of $P_1$ purinoceptor agonists NECA (□), 2-Cl-ADO (■), and R-PIA (○) (10 μM). (d) Effect of $A_1AR$ agonist $N^6$-phenyl ADO (■; 10 μM) vs. $A_2AR$ agonist CPCA (□; 2 μM). $^{125}I$ efflux rate was normalized as % efflux $min^{-1}$ (Clancy *et al.*, 1990).

was stimulated differentially by adenine nucleotides following the rank order of potency of 5'-AMP > ADP > ATP. In order to rule out the possibility that the increased $^{125}I$ efflux rate is secondary to an activated $K^+$ conductance, which hyperpolarizes the cell membrane and increases the driving force for $Cl^-$ conductive exit, the experiment was repeated in a high-$K^+$ (120 mM) bathing medium. Under these conditions, an identical order of stimulation by the agonists was observed (Figure 1b). We have also examined and compared the effect of ADO and adenine nucleotides in a cell line transformed from CF nasal polyps, CFNPEo- (Cozens *et al.*, 1992); these agents (1 mM) stimulated $^{125}I$ efflux rate following a similar order of potency (ADO $\geqslant$ ATP > AMP > ADP; data not shown). As summarized in Figure 2, ADO and adenine nucleotides augmented $^{125}I$ efflux in CFPEo- cells in the order of potency of ADO > AMP > ADP $\simeq$ ATP, both at the dose of 1 mM and 0.1 mM. ADO showed much less effect when given at a lower dose; in a set of 'paired' experiments performed under identical conditions on the same day, additions of 0.01, 0.1 and 1 mM ADO increased $^{125}I$ efflux rate by 0.2, 1.4, and 4.1% $min^{-1}$, respectively. These results are consistent with activation of $Cl^-$ conductance via a $P_1$ purinocepter.

To decipher the subtype of $P_1$ purinoceptors involved in purine analogue-induced $Cl^-$ secretion, the effects of $P_1$ selective purinocepter agonists were subsequently examined and compared at 10 and 100 $\mu$M, respectively. As shown in Figure 1c and Figure 2, among the $P_1$ agonists tested, $^{125}I$ efflux rate was stimulated in the order of NECA > 2-Cl-ADO > R-PIA.

CPCA is a potent $A_2AR$ agonist (Bruns *et al.*, 1986; Daly, 1982). We have tested the effect of CPCA (2 $\mu$M) compared with that of a selective $A_1AR$ agonist, $N^6$-phenyl ADO (10 $\mu$M; Daly *et al.*, 1986). As shown in Figure 1d and Figure 2, $^{125}I$ efflux rate was potently stimulated by CPCA but was only slightly stimulated, if at all, by $N^6$-phenyl ADO. These results favour the involvement of the $A_2AR$ subtype.

### *Effect of $P_1$ purinoceptor agonists on $[Ca^{2+}]_i$*

CF is characterized by defective regulation of the CFTR $Cl^-$ channel by cyclic AMP-dependent protein kinase and protein kinase C (Boat *et al.*, 1989; Hwang *et al.*, 1989; Anderson *et al.*, 1992). In CF airway however, $Cl^-$ secretion remains inducible by raising $[Ca^{2+}]_i$ (Wagner *et al.*, 1991; 1992; Chan *et al.*, 1992). We have tested whether $P_1$ purinoceptor agonists stimulate $Cl^-$ secretion in CFPEo- cells by mobilizing intracellular $Ca^{2+}$. Application of ADO, at 1 and 0.1 mM, caused a marked transient increase in $[Ca^{2+}]_i$ (Figure 3a and b). The effect of ADO was much less pronounced when given

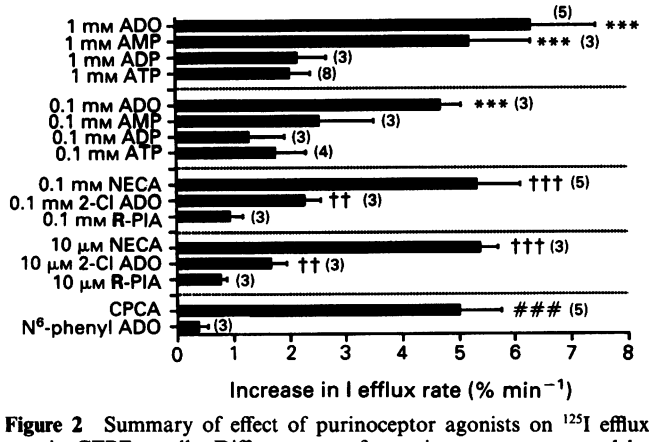


**Figure 2** Summary of effect of purinoceptor agonists on $^{125}I$ efflux rate in CFPEo- cells. Different sets of experiments are separated by dotted lines. Number of cell monolayers used in each experiment ($n$) is given in parentheses. Data are presented as mean ± s.e.mean. Statistical comparisons are made within each set of experiments by Student's unpaired $t$ test. ***$P<0.001$ compared with the same dose of ATP. ††$P<0.01$; †††$P<0.001$ compared with the same dose of R-PIA. ###$P<0.001$ compared with $N^6$-phenyl ADO.

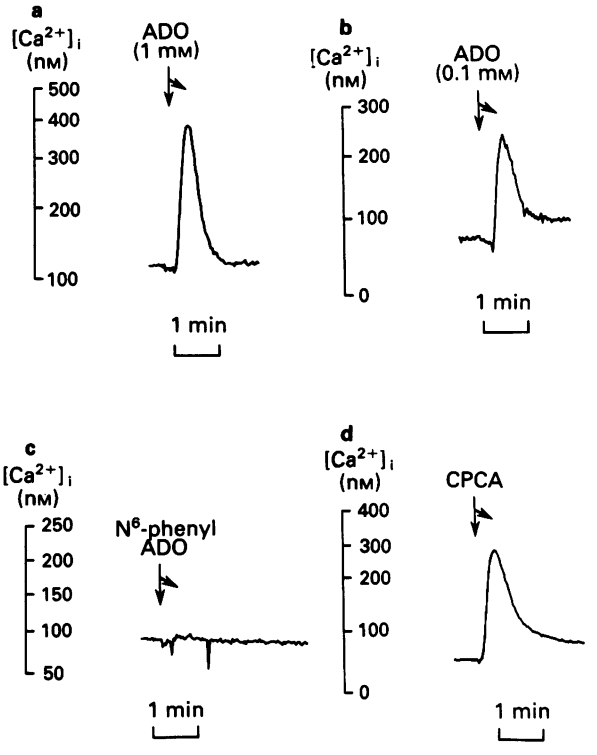


**Figure 3** Effect of $P_1$ purinoceptor agonists on $[Ca^{2+}]_i$. Agents were added where indicated by the arrows at the following concentrations: ADO, 1 mM (a) and 0.1 mM (b); $N^6$-phenyl ADO, 10 $\mu$M (c); CPCA, 2 $\mu$M (d).

at a lower dose (not shown). In concert with the $^{125}I$ efflux data (see Figure 1d), CPCA but not $N^6$-phenyl ADO induced elevation of $[Ca^{2+}]_i$ (Figure 3c and d). The effect of $P_1$ purinoceptor agonists on $[Ca^{2+}]_i$ is summarized in Table 1.

### *$A_2AR$ antagonist DMPX abolishes stimulation by CPCA*

3,7-Dimethyl-1-propargylxanthine (DMPX) is a potent and selective $A_2AR$ antagonist (Seale *et al.*, 1988). We have examined the effect of DMPX on CPCA-induced intracellular $Ca^{2+}$ mobilization and CPCA-stimulated $^{125}I$ efflux. DMPX was used at 100 $\mu$M, ~9 times the reported $K_i$ value of 11 ± 3 $\mu$M (Seale *et al.*, 1988); the dose of CPCA was increased to 10 $\mu$M. Pretreatment of cells with DMPX (6–10 min) abolished the stimulatory effect of CPCA both on $[Ca^{2+}]_i$ (Figure 4a) and on $^{125}I$ efflux (Figure 4b). These results suggest that $P_1$ purinoceptor agonists act mainly on $A_2AR$ to stimulate $Cl^-$ secretion, which is mediated through a $Ca^{2+}$-dependent pathway.

### *Activation of membrane $Cl^-$ channels by $P_1$ purinoceptor agonists: role of CaMK*

Bath application of ADO (1 mM) induced an outwardly-rectifying whole-cell current characteristic of $Ca^{2+}$-stimulated membrane $Cl^-$ current (Figure 5a). The recorded current should be predominantly due to flow of $Cl^-$ ions because $Na^+$ and $K^+$ were omitted in the bath and pipette solutions and $Cs^+$, used to replace pipette $K^+$, is known to block $K^+$ channels. The reversal potential was near 0 mV (Figure 5b), in good agreement with the predicted Nernst potential for $Cl^-$ (−5 mV).

$Ca^{2+}$/calmodulin-dependent protein kinase (CaMK) has been shown to mediate $Ca^{2+}$-stimulated $Cl^-$ currents in secretory epithelia (Worrell & Frizzell, 1991; Chan *et al.*, 1992; Wagner *et al.*, 1992) and human T lymphocytes (Nishimoto *et al.*, 1991). The $Ca^{2+}$-dependent pathway

**Table 1** Effects of addition of $P_1$ purinoceptor agonists on $[Ca^{2+}]_i$ (nM) in CFPEo- cells

| | *Baseline* | *+ Agent* | *Δ* | *n* |
|---|---|---|---|---|
| ADO (1 mM) | 67 ± 23 | 324 ± 46 | 257 ± 33* | 3 |
| ADO (0.1 mM) | 61 ± 8 | 277 ± 44 | 216 ± 43* | 5 |
| $N^6$-phenyl ADO (10 μM) | 102 ± 13 | 108 ± 12 | 7 ± 3 | 4 |
| CPCA (2 μM) | 59 ± 5 | 252 ± 31 | 193 ± 34* | 3 |
| CPCA (10 μM) in presence of DMPX (100 μM)† | 119 ± 23 | 127 ± 25 | 8 ± 2 | 3 |

Data are presented as mean ± s.e.mean. *n*, number of cell monolayers studied. *Significantly different from the baseline $[Ca^{2+}]_i$ ($P<0.05$) by Student's paired *t* test. †CPCA was added ~8 min after the addition of DMPX.

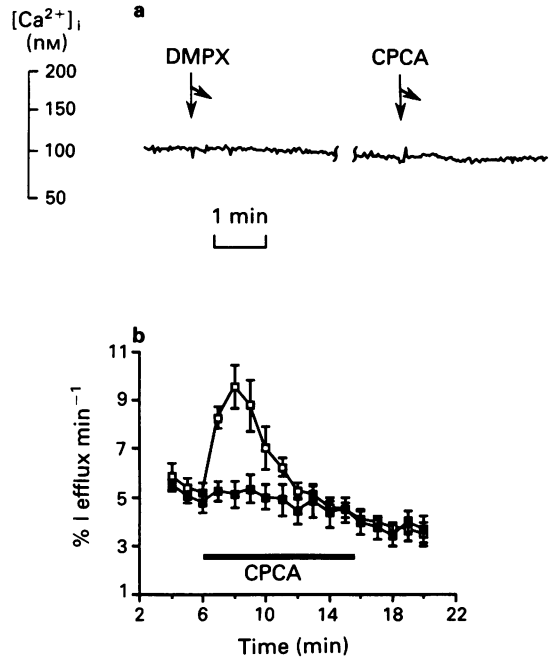


**Figure 4** Stimulation of CPCA is abolished by $A_2AR$ antagonist, DMPX. (a) Effect of CPCA (10 μM) on $[Ca^{2+}]_i$ in presence of DMPX (100 μM). The time-break shown was ~5 min. (b) Effect of CPCA on $^{125}I$ efflux in the presence (■) and absence (□) on DMPX. $n = 4$ each.

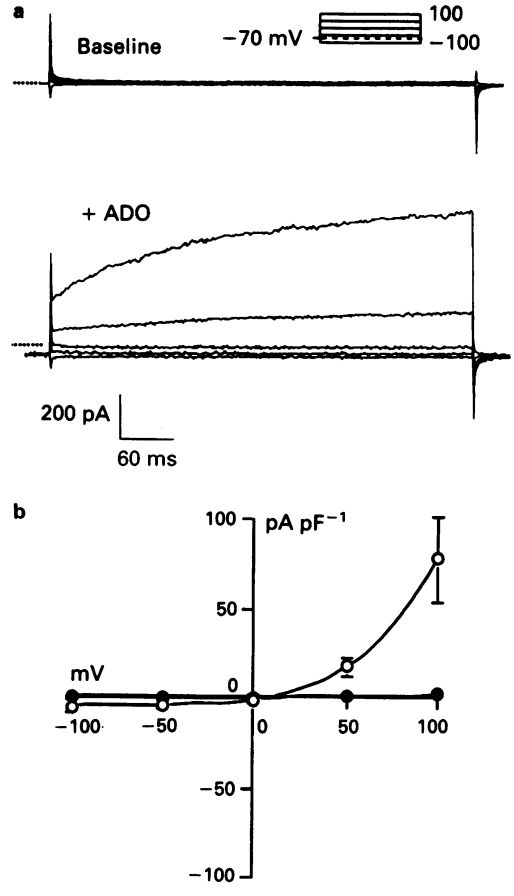


**Figure 5** Effect of ADO on membrane $Cl^-$ current. (a) Whole-cell $Cl^-$ current recorded at baseline (above) and after stimulation (below) of ADO (1 mM). Dotted line indicates zero-level $Cl^-$ current. The resting membrane potential was held at − 70 mV. To examine the current-voltage (*I-V*) relation of a recorded current, membrane potential was sequentially altered from − 100 to + 100 mV in 50 mV steps, using the voltage-clamp protocol given in the inset. Data were averaged over the final 50 ms of the current pulses. (b) *I-V* relations of $Cl^-$ currents recorded at baseline (●) and after stimulation (○) of ADO. Data were normalized by dividing by cell capacitance, an index of cell surface area (Wagner *et al.*, 1991), and presented as mean ± s.e.mean ($n = 7$ cells).

involves CaMK-mediated phosphorylation of a $Cl^-$ channel/transport protein entirely separate from CFTR, since antisense depletion of CFTR protein eliminates cyclic AMP-dependent but not $Ca^{2+}$-dependent $Cl^-$ secretion (Wagner *et al.*, 1992). As $P_1$ purinoceptor agonist-stimulated $Cl^-$ secretion correlated with intracellular $Ca^{2+}$ mobilization, we next examined whether CaMK is involved in the signal transduction pathway. A selective inhibitory peptide of CaMK (a synthetic peptide containing the autoinhibitory region of CaMK), CaMK [273-302], which inhibits brain CaMK activity at 1 μM (Malinow *et al.*, 1989), and a control peptide, CaMK [284-302], which is a truncated version of CaMK [273-302] and does not inhibit CaMK activity (Malinow *et al.*, 1989), were added separately into the cytosol of different groups of CFPEo- cells, through the patch-clamp pipette. As shown in Figure 6, ADO-stimulated $Cl^-$ currents were largely inhibited by the inhibitory peptide CaMK [273-302] ($P<0.01$) but not by the control peptide CaMK [284-302]. In concert, incorporation of BAPTA (10 mM), a $Ca^{2+}$ chelator, to the pipette solution, to prevent intracellular $Ca^{2+}$ elevation and activation of CaMK, also abolished ADO-induced whole-cell $Cl^-$ currents (baseline 2.5 ± 0.5 pA $pF^{-1}$, + ADO, 2.3 ± 0.3 pA $pF^{-1}$; $n = 3$). These results strongly suggest that the action of $P_1$ purinoceptor agonists is mediated via a $Ca^{2+}$-dependent signalling pathway involving CaMK.

## Discussion

ADO and adenine nucleotide induced $Cl^-$ ($I^-$) conductive loss in the CF airway epithelial cell lines, CFPEo- and CFNPEo-. In both cases, ADO was the most potent stimulant among the four purine analogues tested. Thus, under our experimental conditions, the $P_1$ purinoceptor agonist, ADO was more effective in stimulating $Cl^-$ secretion than the $P_2$ agonist, ATP. These results suggest that the purinoceptor agonists-stimulated $Cl^-$ secretion is mainly

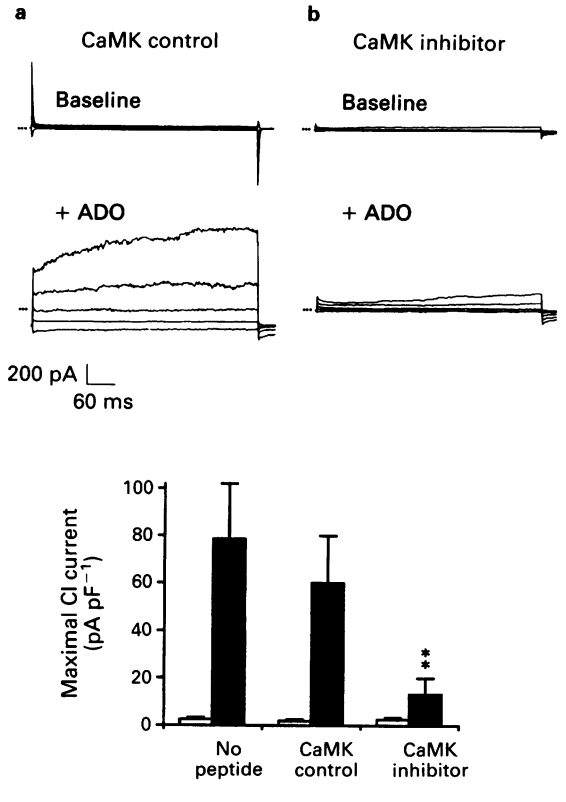


**Figure 6** ADO activates $Cl^-$ secretion via CaMK. (a) Effect of ADO (1 mM) on whole-cell $Cl^-$ current in the presence of CaMK control peptide (CaMK [284-302]; left) and in the presence of CaMK inhibitory peptide (CaMK [273-302]; right), respectively. Cell was internally perfused with the peptide (20 μM) by addition to the pipette solution. Shown are whole-cell $Cl^-$ current recorded at baseline (above) and after stimulation (below) of ADO. (b) Baseline outward $Cl^-$ current (open columns) and maximal outward $Cl^-$ current induced by ADO (solid columns) in absence of peptide (No peptide, $n = 7$), in the presence of CaMK [284-302] (CaMK control; $n = 5$), and in the presence of CaMK [273-302] (CaMK inhibitor; $n = 5$), respectively. $^{**}P < 0.01$ compared with ADO-activated $Cl^-$ current in the absence of peptide (No peptide) by Student's unpaired $t$ test.

mediated by the $P_1$ subtype. $P_1$ purinoceptor agonists differentially stimulated $Cl^-$ secretion in CFPEo- cells in the rank order of potency of NECA > 2-Cl-ADO > **R**-PIA. These results are consistent with the involvment of $A_2AR$. A higher dose of these agents and ADO was needed to induce $Cl^-$ secretion, compared with that needed in the native canine airway epithelial cells (Pratt *et al.*, 1986). ADO 0.01 mM was not sufficient to increase substantially $^{125}I$ efflux in CFPEo- (see Results). This is consistent with our previous observation (Cozens *et al.*, 1991) that transformed epithelial cells, after being passaged a number of times, tend to show reduced sensitivities to various $Cl^-$ secretagogues. In addition, the higher dose of $P_1$ agonists needed to induce $Cl^-$ secretion in CFPEo- may partly be due to an attenuated response of CF airway epithelia to $Ca^{2+}$-mediated stimulation. $Ca^{2+}$ ionophore A23187 was shown previously to enhance $Cl^-$ transport in CF airway epithelial cells to a level of ~60% that of normal cells (Frizzell *et al.*, 1986; Boucher *et al.*, 1989).

What is the signal transduction mechanism involved in $A_2AR$-mediated $Cl^-$ secretion in CFPEo-? We have shown recently that cyclic AMP does not affect membrane $Cl^-$ channel activities in transformed CF airway epithelial cells (Wagner *et al.*, 1991). The effect of ADO and $A_2AR$ agonist CPCA on $[Ca^{2+}]_i$, and the abolition of CPCA-induced increase in $[Ca^{2+}]_i$ by the $A_2AR$ antagonist DMPX, implicate the involvement of a $Ca^{2+}$-dependent pathway. The transient increase in $[Ca^{2+}]_i$ brought about by ADO and CPCA may result from the stimulation of phospholipase C and phosphatidylinositol turnover. Ali *et al.* (1990) showed recently in a rat tumour mast cell that NECA induced transient elevation of cytosolic inositol phosphates as well as $[Ca^{2+}]_i$, accompanied by a secretory response to $P_1$ purinoceptor agonists in a rank order of potency implying a role of the $A_2AR$. Our patch-clamp experiments show that CaMK also participates in ADO-stimulated $Cl^-$ secretion, as previously shown for the calcium ionophore-induced $Cl^-$ secretion (Wagner *et al.*, 1991; Worrell & Frizzell, 1991). The increase in $[Ca^{2+}]_i$ elicited by ADO or CPCA may cause autophosphorylation of CaMK, rendering it active even after $[Ca^{2+}]_i$ returns towards the baseline level (MacNicol *et al.*, 1990). These results demonstrate that the effect of ADO-induced elevation of $[Ca^{2+}]_i$ is further transmitted by CaMK. This implies that $A_2AR$ may couple to effector systems other than adenylate cyclase, which is the only reported one thus far (Stiles, 1991).

How do these findings compare to previously published reports? The exact nature of the purinoceptor that is linked to ion transport in secretory epithelia is unclear from the cumulative published literature. Several previous published studies, most notably of canine tracheal epithelium (Pratt *et al.*, 1986) and human $T_{84}$ colonic carcinoma cells (Barrett *et al.*, 1990), demonstrated ADO-induced $Cl^-$ secretion mediated through an $A_2AR$, as indicated by the rank order of potency of ADO analogues. In each of these studies, $A_2AR$ appeared to be coupled to adenylate cyclase and generation of cyclic AMP, though in the latter study (Barrett *et al.*, 1990), ion transport was stimulated at concentrations below that required to increase cyclic AMP. Barrett *et al.* (1990) further showed that ADO did not affect $[Ca^{2+}]_i$ in $T_{84}$ cells and initiated a phosphorylation profile consistent with cyclic AMP- rather than $Ca^{2+}$-dependent kinase. If ADO-induced $Cl^-$ secretion is cyclic AMP-mediated, it presumably occurs via CFTR and therefore should be defective in CF-derived cells. Our studies indicate, however, that $A_2AR$ is coupled to $Ca^{2+}$ and is preserved in CF epithelial cells, as expected for $Ca^{2+}$-dependent $Cl^-$ secretion (Wagner *et al.*, 1991).

In contrast to our findings and those cited above, Boucher and his colleagues demonstrated that extracellular nucleotides regulated ion transport across human airway epithelium, both *in vivo* (Knowles *et al.*, 1991) and in freshly excised intact tissue (Mason *et al.*, 1991), by means of a $P_2$ receptor. The potency order of nucleotide analogues and responsiveness to UTP suggested the presence of one or more $P_2$ receptor subtypes distinct from the previously characterized $P_{2x}$ and $P_{2y}$ subtypes (Burnstock & Kennedy, 1985). ADO was relatively ineffective in mediating ion transport. In this case, nucleotide-induced ion transport changes were linked to changes in $[Ca^{2+}]_i$ and were preserved in CF airway epithelium. However, changes in short-circuit current were attributed to increments in the rate of $Na^+$ absorption (Barrett *et al.*, 1990) rather than primarily through changes in $Cl^-$ secretion. Primary changes in $Na^+$ absorption could not explain, however, our whole-cell patch-clamp studies of CaMK-mediated outwardly rectifying $Cl^-$ currents. Differences in the findings with respect to the purinoceptor subtype and ionic mechanism may be partially attributable to studies of relatively intact human epithelium (Barrett *et al.*, 1990) vs. studies of the transformed airway epithelial cells. In the relatively more intact tissue preparation, metabolism of ADO may mask ADO-induced changes in $Cl^-$ secretion. In support of this, it has been shown that ADO is relatively impotent in causing $Cl^-$ secretion in rabbit ileal mucosa because of uptake and metabolism (Dobbins *et al.*, 1984).

We have shown that $P_1$ purinoceptor agonists can stimulate $Cl^-$ secretion in CF airway epithelial cells via an alternate $Ca^{2+}$-dependent signalling pathway, thus suggesting a potential means of circumventing the CF defect. In isolated cells, the $P_1$ purinoceptor agonists appeared to be con-

siderably more potent in inducing $Cl^-$ secretion than the $P_2$ agonists like ATP. Thus, nonmetabolizable $P_1$ purinoceptor agonists might be useful therapeutically in treatment of CF. It should be noted, however, that while ADO does not induce bronchoconstriction in normal subjects, it enhances bronchoconstriction in asthmatic patients (Cushley *et al.*, 1984). This side-effect of ADO can be attenuated by nedocromil sodium and sodium cromoglycate (Crimi *et al.*, 1986).

In conclusion, our results suggest that $P_1$ purinoceptor agonists act via $A_2AR$ to stimulate $Cl^-$ secretion in airway epithelial cells. The stimulatory effect of $P_1$ agonists is mediated via a $Ca^{2+}$-dependent signalling pathway, which appears to be, at least partially, preserved in CF cells.

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# Differences in the effects of $NK_1$-receptor antagonists, (±)-CP 96,345 and CP 99,994, on agonist-induced responses in guinea-pig trachea

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**1** The effects of the $NK_1$-receptor antagonists, (±)-CP 96,345 and CP 99,994, on $NK_1$-agonist evoked contractions were compared in isolated rings of guinea-pig tracheal smooth muscle.

**2** (±)-CP 96,345 and CP 99,994 were similarly effective in antagonizing responses evoked by septide, whereas CP 99,994 was more effective than (±)-CP 96,345 in inhibiting responses evoked by [$Sar^9Met^{11}(O_2)$] substance P.

**3** These results suggest that responses to septide and [$Sar^9Met^{11}(O_2)$] substance P may be operated via different populations of $NK_1$-receptors.

**Keywords:** $NK_1$-receptor subtypes; guinea-pig trachea; (±)-CP 96,345; CP 99,994

## Introduction

The development of non-peptide selective $NK_1$-receptor antagonists has shown that species differences in $NK_1$-receptors exist (Snider *et al.*, 1990; Beresford *et al.*, 1991; Garret *et al.*, 1991). It has recently been suggested that subtypes of $NK_1$-receptors may exist in different tissues from the same species, based on the differential effects of $NK_1$-receptor antagonists (Carruette *et al.*, 1992; Beresford *et al.*, 1992). Thus CP 96345 was less effective in inhibiting $NK_1$-receptor evoked responses in guinea-pig trachea than in guinea-pig ileum (Carruette *et al.*, 1992; Beresford *et al.*, 1992). However, identification of receptor subtypes by comparison of antagonist $pA_2$ values between tissues can be misleading, since values for the same antagonist may vary considerably between tissues (Kenakin, 1990).

The use of different $NK_1$-receptor agonists may aid the identification of $NK_1$-receptor subtypes. It has been suggested that a new neurokinin receptor subtype (possibly belonging to the $NK_1$-receptor family) may exist in guinea-pig ileum, since responses evoked by septide and [$APA^{9-10}$] substance P (SP) are more susceptible to inhibition by the peptide $NK_1$-receptor antagonists GR 71251 and [D-$Pro^9$, $Pro^{10}$,$Trp^{11}$]SP than are responses evoked by [$Pro^9$]SP (Petitet *et al.*, 1992; Chassaing *et al.*, 1992).

In the present experiments we compared the effects of two $NK_1$-receptor antagonists ((±)-CP 96,345 and CP 99,994) on contractile responses evoked by two supposedly selective agonists (septide and [$Sar^9Met^{11}(O_2)$]SP) in guinea-pig tracheal smooth muscle. RP 67580, an $NK_1$-receptor antagonist which is known to have a different pharmacological profile (i.e. higher affinity for $NK_1$-receptors in rat tissues than for $NK_1$-receptors in guinea-pig tissues), was used as a comparison (Garrett *et al.*, 1991).

## Methods

Tracheal rings (approximately 5 mm) were obtained from male Dunkin Hartley guinea-pigs (Interfauna, 300–400 g). The tissues were mounted for tension recording in siliconized organ baths containing Krebs physiological salt solution (PSS (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, $KH_2PO_4$ 1.2 and glucose 11.1) maintained at 37°C, pH 7.4, gassed with 95% $O_2$, 5% $CO_2$. The PSS also contained atropine sulphate (1 μM), indomethacin (1 μM), mepyramine maleate (1 μM) and methysergide (1 μM) to eliminate any indirect/non $NK_1$-receptor-mediated responses. The tissues were placed under a 1 g resting load. Following a 60 min equilibration period, SR 48968 (30 nM) an $NK_2$-receptor antagonist was added to the tissue bath to eliminate any effects of the agonists at $NK_2$-receptors; 15 min later, enzyme inhibitors (bestatin 1 μM, phosphoramidon 1 μM, bacitracin 0.04 $g\,l^{-1}$) were added: higher concentrations of these inhibitors attenuated responses to the agonists, an effect which may result from an interaction with $NK_1$-receptors (Fong, unpublished observation). A further 15 min later a cumulative concentration-effect curve to septide or to [$Sar^9Met^{11}(O_2)$]SP was obtained. The tissues were then washed. Following equilibration (60 min) with either (±)-CP 96,345, CP 99,994 or RP 67580, the concentration-effect curve to the appropriate agonist was repeated again in the presence of SR 48968 and the enzyme inhibitors (see above). Each tissue was exposed to either [$Sar^9Met^1(O_2)$]SP or septide and to a single concentration of the antagonist under test. Appropriate vehicle- and time-matched control tissues were used.

### *Analysis of data*

Responses were expressed as a percentage of the maxima of the initial concentration-effect curve. For each individual tissue, dose-ratios (DR) were calculated and were used to construct full Schild plots according to the method of Arunlakshana & Schild (1959) or to estimate the $pK_B$ value (i.e. log dissociation constant) using the following equation where:

$$\text{estimated } pK_B = \frac{\log([\text{concentration of antagonist}])}{(DR - 1)}$$

In order to allow direct comparisons of antagonist affinity, the slope of each Schild plot was constrained to unity and the $pK_B$ values were then calculated (Jenkinson, 1991). Concentration-effect curves were fitted to the data by least squares analysis of variance to the equation $Y = Y_{max}/(1 + (EC_{50}/\text{agonist concentration})^{nH})$, 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).

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*Drugs and solutions*

The following substances were used: septide ([pGlu$^6$,Pro$^9$]SP (6–11)), bestatin, phosphoramidon, bacitracin, atropine sulphate, indomethacin, mepyramine maleate (all from Sigma); [Sar$^9$,Met$^{11}$(O$_2$)]SP (CRB); methysergide maleate (RBI). SR 48968 ((S)-N-methyl-N[4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide)), (±)-CP 96,345 (racemic(2S,3S/2R,3R) (cis-2-)diphenylmethyl)-N-((2-methoxyphenyl) methyl)-1-azabicyclo(2.2.2)octan-3-amine), CP 99,994 ([(+)-(2S,3S)-3-(2-methoxybenzylamino)-2-phenylpiperidine]) and RP 67580 ((3aR,7aR)-7,7-diphenyl-2(1-imino-2-(2-methoxyphenyl/ethyl)perhydroisoindole)) were synthesized by Merck Sharp & Dohme Research Laboratories. The neurokinin agonists were dissolved in water (stock solutions of 10 mM) and neurokinin antagonists were dissolved in dimethylsulphoxide (DMSO; Fluka, final bath concentration not greater than 0.1%).

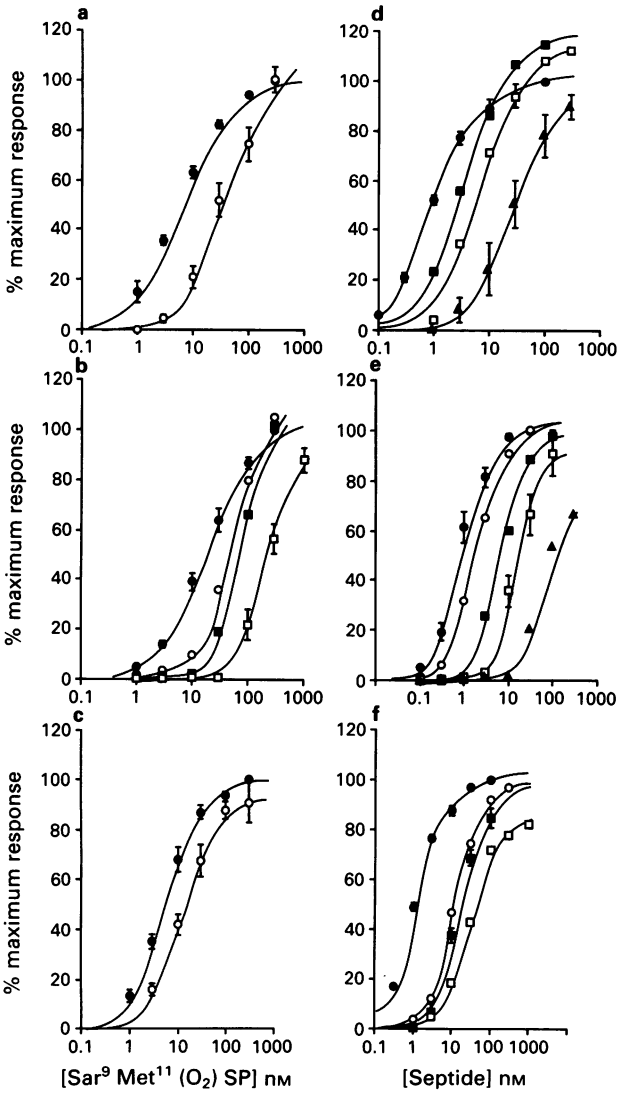


**Figure 1** Left hand panel: effects of (a) (±)-CP 96,345 (● control, ○ 300 nM), (b) CP 99,994 (● control, ○ 30 nM, ■ 100 nM, □ 300 nM) and (c) RP67580 (● control, ○ 1 μM) on [Sar$^9$Met$^{11}$ (O$_2$)]SP-induced contractile responses. Right hand panel: effects of (d) (±)-CP 96,345 (● control, ■ 10 nM, □ 30 nM, ▲ 100 nM), (e) CP 99,994 (● control, ○ 10 nM, ■ 30 nM, □ 100 nM, ▲ 300 nM) and (f) RP 67580 (● control, ○ 30 nM, ■ 1 μM, □3 μM) on septide-induced contractions. Each point, expressed as a percentage of the maximum response obtained in the initial control curve, is the mean value with s.e.mean ($n$ = 4–8). For reasons of clarity not all s.e. bars are shown. Curves were fitted by least squares analysis of variance (see text for details).

**Table 1** p$K_B$ values (with 95% confidence limits) for (±)-CP 96,345, CP 99,994 and RP 67580

| *Agonist* | *Antagonist* | *p$K_B$* | *95% CL* |
|---|---|---|---|
| [Sar$^9$Met$^{11}$(O$_2$)] substance P | (±)-CP 96345 | 6.9* | 6.7–7.1 |
| | CP 99994 | 7.7 | 7.6–7.8 |
| | RP 67580 | 6.4* | 6.1–6.7 |
| Septide | (±)-CP 96345 | 8.4 | 8.3–8.6 |
| | CP 99994 | 8.5 | 8.2–8.7 |
| | RP 67580 | 7.0 | 6.8–7.2 |

Values were determined from Schild analysis where the slope has been constrained to unity except * where p$K_B$ values are estimates obtained from a single antagonist concentration (CP 96,345 = 300 nM; RP 67580 = 1 μM; $n$ = 8).

## Results

Figure 1 shows the effects of (±)-CP 96,345, CP 99,994 and RP 67580 on the concentration-effect curves to septide and [Sar$^9$Met$^{11}$ (O$_2$)]SP. In vehicle- and time-matched control tissues the dose-ratios were less than two. (±)-CP 96,345, CP 99,994 and RP 67580 produced parallel shifts in the position of the concentration-effect curves with little or no effect on the maximum of the curve, suggesting competitive antagonism. The slopes of the Schild plots were not significantly different from unity supporting the assumption of the competitive nature of the antagonism. The p$K_B$ values (calculated from constrained Schild plots) for (±)-CP 96,345, CP 99,994 and RP 67580 are shown in Table 1. It should be noted that due to the weak antagonist effects of (±)-CP 96,345 and RP 67580 on [Sar$^9$Met$^{11}$(O$_2$)] SP-evoked responses, the p$K_B$ values are estimates obtained with a single antagonist concentration.

## Discussion

In the present study we compared the effects of (±)-CP 96,345 and CP 99,994 on NK$_1$-receptor-mediated responses in guinea-pig trachea evoked by two NK$_1$-receptor agonists. (±)-CP 96,345 and CP 99,994 are selective NK$_1$-receptor antagonists which have been shown to exert similar effects in both binding and functional studies. Thus both (±)-CP 96,345 and CP 99,994 inhibit $^{125}$I-labelled BH substance P in hIM-9 cells with $K_i$ values of 0.6 and 0.3 nM respectively, they inhibit substance P-induced increases in firing rate in guinea-pig locus coeruleus neurones ($IC_{50}$ values of 90 and 50 nM respectively) and reverse [Sar$^9$Met$^{11}$(O$_2$)]SP-induced locomotor activity in guinea-pigs ($ED_{50}$ values 1.9 and 0.6 mg kg$^{-1}$, s.c. Desai *et al.*, 1992; McLean *et al.*, 1992; Snider *et al.*, 1991).

In the present study, (±)-CP 96,345 and CP 99,994 were similarly effective in inhibiting responses evoked by septide (p$K_B$ values were 8.4 and 8.5 respectively) in guinea-pig trachea. The p$K_B$ value for RP 67580 was approximately 1.5 log units less. The effects of the antagonists on septide-evoked responses are consistent with their previously reported actions on NK$_1$-receptor-mediated responses in guinea-pig isolated tissue (Carruette *et al.*, 1992).

When [Sar$^9$Met$^{11}$(O$_2$)]SP was used as the agonist, differences in the effects of the antagonists were observed. Firstly, (±)-CP 96,345, CP 99,994 and RP 67580 were less effective in antagonizing responses evoked by [Sar$^9$Met$^{11}$(O$_2$)]SP than in antagonizing responses evoked by septide. Secondly, when using different agonists there was a difference in the rank order of effectiveness of the antagonists. In the case of septide, the rank order was (±)-CP 96,345 = CP 99,994 > RP 67580. However, when [Sar$^9$Met$^{11}$(O$_2$)]SP was used as the agonist, the rank order was CP 99,994 > (±)-CP 96,345 > RP 67580. Guinea-pig trachea is known to contain a mixed

population of $NK_1$- and $NK_2$-receptors (Ireland *et al.*, 1991). However, since septide and [$Sar^9Met^{11}(O_2)$]SP are selective $NK_1$-receptor agonists and experiments were carried out in the presence of SR 48968 (a $NK_2$-receptor antagonist), it is unlikely that differences in the effects of the $NK_1$-receptor antagonists result from activation of $NK_2$-receptors.

It has been shown recently that only two amino acid residues in the binding site of the rat and human $NK_1$-receptors are responsible for the large differences in the affinity of these receptors for RP 67580 and (±)-CP 96,345 respectively, leading to the suggestion that rat and human $NK_1$-receptors are species variants of the same receptor (Fong *et al.*, 1992a). In the present report, the rank order for (±)-CP 96,345 and RP 67580 were preserved, regardless of the agonist used. These observations cannot be explained on the basis of a 'rat-type $NK_1$-receptor' being present in guinea-pig trachea.

It is possible that the differences in the effects of (±)-CP 96,345 and CP 99,994 may reflect that in guinea-pig trachea, two subtypes of $NK_1$-receptors exist, one of which is activated by septide and shows equal sensitivity to the effects of (±)-CP 96,345 and CP 99,994, and a second subtype which is activated by [$Sar^9Met^{11}(O_2)$]SP and shows differential sensitivity to these antagonists. Alternatively, it has been reported that the human $NK_1$ receptor can exist in alternative spliced variants (Fong *et al.*, 1992b) where each isoform is coupled to a separate second messenger system. It is possible that in guinea-pig trachea different agonists activate different isoforms of $NK_1$-receptors and differences in second messenger coupling can influence measured antagonist affinity constants (Kenakin, 1990).

In conclusion, in guinea-pig trachea there are differences in the effects of (±)-CP 96,345 and CP 99,994 which may reflect the existence of two populations of $NK_1$-receptors.

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# Effects of muscarinic $M_2$ and $M_3$ receptor stimulation and antagonism on responses to isoprenaline of guinea-pig trachea *in vitro*

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**1** In guinea-pig and canine airway smooth muscle, there is reduced β-adrenoceptor agonist sensitivity in tissues pre-contracted with muscarinic agonists when compared to tissues pre-contracted with other spasmogens, such as histamine or leukotriene $D_4$. This reduced sensitivity may be the result of an interaction between muscarinic receptors and β-adrenoceptors. In this study the effects of $M_2$ receptor antagonism and stimulation have been investigated on the relaxant potency of isoprenaline in guinea-pig isolated tracheal smooth muscle.

**2** (+)-*cis*-Dioxolane contracted isolated tracheal strips in a concentration-dependent manner ($EC_{50}$ = 11.5 ± 0.9 nM). The rank order of antagonist apparent affinities (with $pA_2$ values in parentheses) was atropine (9.4 ± 0.1) > zamifenacin (8.2 ± 0.1) > para-fluoro-hexahydro-siladiphenidol (p-F-HHSiD, 7.2 ± 0.1) > pirenzepine (6.5 ± 0.1) > methoctramine (5.5 ± 0.1). Schild slopes were not significantly different from unity. This was consistent with a role of muscarinic $M_3$ receptors in mediating contraction.

**3** In tissues pre-contracted to 3 g isometric tension using (+)-*cis*-dioxolane (0.2 μM, approximately $EC_{80}$), the relaxant potency of isoprenaline was significantly ($P<0.05$) increased by 0.3 μM methoctramine (control $EC_{50}$ = 32.2 ± 4.3 nM, plus methoctramine $EC_{50}$ = 19.1 ± 4.5 nM). This concentration of methoctramine had no effect on contractile responses to (+)-*cis*-dioxolane (control, $EC_{50}$ = 17.6 ± 3.2 nM, plus methoctramine, $EC_{50}$ = 21.0 ± 4.4 nM).

**4** When acetylcholine (non-selective), (+)-*cis*-dioxolane (non-selective), L-660,863 ((±)-3-(3-amino-1,2,4-oxadiazole-5-yl)-quinuclidine, $M_2$-selective) or SDZ ENS 163 (thiopilocarpine, mixed $M_2$ antagonist, partial $M_3$ agonist) were used to achieve isometric tensions of 3 g, the relaxant potency of isoprenaline ranged from 3.7 ± 0.3 nM (SDZ ENS 163) to 49.4 ± 3.2 nM ((+)-*cis*-dioxolane). Reducing the concentration of these agonists (and therefore the level of developed tension to 2 g), significantly ($P<0.05$) increased the relaxant potency of isoprenaline. In contrast, when histamine was used to pre-contract tissues to either 2 or 3 g ($EC_{50}$ = 4.2 ± 0.6 and 3.8 ± 1.1 nM, respectively), there was no significant effect on the relaxant potency of isoprenaline.

**5** There was a slight but significant ($P<0.05$) reduction in the relaxant potency of isoprenaline, in tissues pre-contracted to 3 g using histamine in combination with (+)-*cis*-dioxolane (30 nM). This effect was reversed by $M_2$ receptor antagonism, using methoctramine (1 μM).

**6** These data suggest that in guinea-pig isolated trachea, the relaxant potency of isoprenaline may depend not only on the level of developed tension but also, on the level of muscarinic $M_2$ receptor stimulation/blockade of the spasmogen inducing the tension. However, the lack of selective $M_2$ agonist and the low $M_2/M_3$ selectivity of antagonists in this tissue do not permit definitive conclusions to be made about the role of these receptors in modulating isoprenaline potency.

**Keywords:** Muscarinic cholinoceptors; β-adrenoceptors; tracheal smooth muscle; $M_2/M_3$ receptor agonists and antagonists

## Introduction

In guinea-pig and canine airway smooth muscle, the relaxant potency of β-adrenoceptor agonists depends both upon the nature of the agonist used to elevate the resting tension and the magnitude of the contracture from which relaxations are elicited (Torphy, 1984; Russell, 1984; Torphy *et al.*, 1985). Thus, from equivalent levels of developed isometric tension, isoprenaline is less potent in relaxing tissues pre-contracted with muscarinic agonists than tissues pre-contracted with either histamine or leukotriene $D_4$, (Torphy, 1984; Russell, 1984; Koenig *et al.*, 1989). One explanation for the difference in isoprenaline relaxant potency, is an inhibitory action of muscarinic receptor stimulation on β-adrenoceptor function (Torphy, 1984; Gunst *et al.*, 1989).

Activation of muscarinic receptors in smooth muscle results in inhibition of adenylyl cyclase activity and stimulation of phosphoinositide specific phospholipase C activity (Jones *et al.*, 1987; Sankary *et al.*, 1988; Yang *et al.*, 1991; Pyne *et al.*, 1992). This is due to activation of two muscarinic receptor subtypes, $M_2$ (80–88%) and $M_3$ (12–20%) respectively (Fryer & El Fakahany, 1990; Mahesh *et al.*, 1992). Muscarinic $M_3$ receptors mediate smooth muscle contraction (Roffel *et al.*, 1990; Ten Berge *et al.*, 1993), but the role, if any, of the majority $M_2$ receptor population is unclear.

In smooth muscle, adenylyl cylcase activity is enhanced by β-adrenoceptor activation, resulting in relaxation. Activation of muscarinic $M_2$ receptors inhibits this stimulation, by coupling to a pertussis toxin-sensitive guanine nucleotide binding protein, $G_i$ (Sankary *et al.*, 1988; Yang *et al.*, 1991; Griffin & Ehlert, 1992; Pyne *et al.*, 1992). In canine isolated trachea pre-contracted with muscarinic agonists, selective antagonism of $M_2$ receptors enhances the relaxant potency of isoprenaline (Fernandes *et al.*, 1992). Pertussis toxin, which ADP-ribosylates and thereby inactivates the alpha subunit of $G_i$, has a similar effect (Mitchell *et al.*, 1993). Thus, activation of $M_2$ receptors may attenuate β-adrenoceptor-mediated relaxation, thereby facilitating $M_3$ receptor-mediated contraction (Torphy *et al.*, 1985; Sankary *et al.*, 1988).

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Alternatively, it is also possible that attenuation of relaxation to β-adrenoceptor activation involves $M_3$ receptors, directly. Stimulation of $M_3$ receptors activates a phosphoinositide specific phospholipase C, via a pertussis toxin-insensitive G protein, $G_q$. This results in the formation of inositol (1,4,5) trisphosphate and 1,2 diacylglycerol (see Chilvers & Nahorski, 1990, for review), which causes intracellular release of calcium and activation of a protein kinase C, respectively. These two processes may lead to phosphorylation of β-adrenoceptors, guanine nucleotide binding proteins ($G_s$) or adenylyl cyclase (Van Amsterdam *et al.*, 1989; 1990). Functional antagonism by $M_3$ receptor activation may, therefore, offset relaxations to β-adrenoceptor agonists, without involving $M_2$ receptors. In support of this hypothesis, Meurs *et al.* (1993) have failed to demonstrate an effect of selective $M_2$ receptor antagonism on the relaxant potency of isoprenaline in bovine trachea. However, a correlation was demonstrated by these workers, between the inhibitory effects on the relaxant potency of isoprenaline and the potency at $M_3$ receptors mediating enhanced inositol phospholipid metabolism (Meurs *et al.*, 1993).

The aim of the present studies was to explore further the role of muscarinic receptor subtypes in modulating relaxations of guinea-pig, isolated trachea to isoprenaline. The lack of potent and selective $M_2$ and $M_3$ receptor agonists (see Caulfield, 1993, for review) mandated that indirect approaches be employed. These were, firstly, the use of a non-selective muscarinic agonist, (+)-*cis*-dioxolane, in the presence of selective $M_2$ receptor antagonism using methoctramine. Secondly, the use of tissues pre-contracted with muscarinic agonists possessing varying intrinsic efficacies at muscarinic receptors. Thirdly, by studying the effects of selective $M_2$ receptor stimulation using (+)-*cis*-dioxolane in the presence of $M_3$ receptor antagonism by *p*-F-HHSiD (*para*-fluoro-hexahydrosiladiphenidol). A preliminary account of this work was presented to the British Pharmacological Society (Watson & Eglen, 1993).

## Methods

### *General*

Male, Dunkin-Hartley guinea-pigs (250–350 g) were killed by exposure to $CO_2$. Tracheae were isolated and placed in aerated, modified Krebs solution (composition mM: KCl 4.6, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, NaCl 118.2, glucose 10.0, $NaHCO_3$ 24.3 and $CaCl_2$ 2.5) and cleaned of extraneous tissue. Tracheae were opened along their ventral surface and strip preparations were cut transversely, with each strip containing 3–4 cartilaginous rings. Silk (4–0) sutures were attached to the cartilaginous portions on either side of the smooth muscle bands and the preparations were suspended, at a resting tension of 1 g, in 10 ml organ baths containing aerated, modified Krebs solution (pH 7.4, 37°C). This 1 g applied tension was considered the baseline, from which all further tension charges were recorded. Indomethacin (1 μM) was present in the Krebs solution throughout, to inhibit prostaglandin synthesis. Tetrodotoxin (0.1 μM) was also present throughout, to eliminate pre-junctional effects of muscarinic agonists. Corticosterone (30 μM) was present in all studies with β-adrenoceptor agonists, to inhibit extraneuronal monoamine uptake. All preparations were allowed 60 min to equilibrate, prior to construction of concentration-effect curves. These were established in a cumulative manner, using incremental concentrations at 0.5 $\log_{10}$ intervals. Each successive concentration was added once a sustained contracture to the previous concentration was attained.

### *Receptor characterization*

Concentration-effect curves to (+)-*cis*-dioxolane, were constructed and tissues were washed and re-equilibrated, for 60 min, in the presence of a single concentration of one of the following antagonists: atropine (10, 30 or 100 nM), pirenzepine (1, 3 or 10 μM), methoctramine (1, 3 or 10 μM), p-F-HHSiD (0.03, 0.3, 1 or 3 μM) and zamifenacin (10–100 nM). A second concentration-effect curve to (+)-*cis*-dioxolane was then established in the presence of antagonist. Parallel studies were undertaken in the absence of antagonist to correct for temporal changes in sensitivity.

### *The effect of muscarinic $M_2$ receptor antagonism, on the relaxant potency of isoprenaline in tissues pre-contracted with (+)-*cis*-dioxolane*

A concentration-effect curves was initially obtained to (+)-*cis*-dioxolane (1 nM–1 μM) in all tissues, to establish both the maximal contractile response and the concentration required to give approximately a 3 g increase in isometric tension. During this initial exposure to (+)-*cis*-dioxolane, methoctramine (0.3 μM) was present to inhibit $M_2$ receptor desensitization occurring at high (+)-*cis*-dioxolane concentrations. Tissues were then washed at 15 min intervals over the following 120 min period and during the final 60 min, separate tissues were equilibrated in the absence or presence of a single concentration of methoctramine (0.3 μM). Tissues were then pre-contracted to 3 g using (+)-*cis*-dioxolane (0.2 μM) and concentration-effect curves to isoprenaline (01. nM–1 μM) were established.

### *The effect of muscarinic agonists and histamine on the relaxant potency of isoprenaline*

Concentration-effect curves to L-660,863 (1 nM–0.3 μM), SDZ ENS 163 (0.1–30 μM), acetylcholine (ACh, 1 nM–10 μM), (+)-*cis*-dioxolane (1 nM–1 μM) or histamine (0.1–0.3 mM) were obtained to establish both the maximal response to these agonists and the concentration required to increase isometric tension by approximately 3 g. Tissues were then washed at 15 min intervals over a 60 min period and allowed to re-attain baseline isometric tension. The tension was then increased to 3 g by addition of either muscarinic agonist or histamine. Each tissue was exposed to only one agonist. Once a stable contracture was attained, relaxant concentration-effect curves to isoprenaline (0.1 nM–1 μM) were established. Similar experiments were also undertaken, in separate tissues pre-contracted with a lower concentration of the above agonists, to an isometric tension of 2 g. Physostigmine (0.3 μM) was present in the studies with acetylcholine, to inhibit acetylcholinesterase activity.

### *The effect of $M_2$ receptor stimulation and antagonism on the relaxant potency of isoprenaline in tissues pre-contracted with histamine*

In this series of experiments, the effect of muscarinic $M_2$ receptor activation, using (+)-*cis*-dioxolane, was investigated on the relaxant potency of isoprenaline. In short, this was achieved by raising the level of isometric tension to 3 g with histamine and, in the presence of $M_3$ receptor antagonism (p-F-HHSiD, 0.3 μM), activating $M_2$ receptors with (+)-*cis*-dioxolane (0.1 μM).

Concentration-effect curves to histamine (0.1 μM–0.3 mM) were obtained in all tissues, to establish both the maximal contractile response and the concentration required to give approximately a 3 g isometric tension change. Tissues were then washed and re-equilibrated for 60 min in the absence or presence of a single concentration of methoctramine (1.0 μM). (+)-*cis*-Dioxolane (0.1 μM) was added to all but control tissues and the tone was raised to a final isometric tension of 3 g using histamine. A concentration-effect curve to isoprenaline was then constructed.

The apparent affinity ($pA_2$) of p-F-HHSiD at $M_2$ receptors is 6.0 (Lambrecht *et al.*, 1988; Eglen *et al.*, 1990). Therefore, at the concentration of p-F-HHSiD (0.3 μM) used to inhibit

$M_3$ receptor-mediated contractions in this experiment, 23% of $M_2$ receptor would be occupied. This level of $M_2$ receptor occupancy may compromise putative inhibitory effects of (+)-*cis*-dioxolane on the isoprenaline relaxant potency. Therefore, these experiments were repeated in the absence of p-F-HHSiD. To retain $M_2$ receptor activity but reduce $M_3$ receptor-mediated contractions, the concentration of (+)-*cis*-dioxolane was reduced from 0.1 μM to 30 nM. The final 3 g increase in isometric tension was therefore achieved by a combination of histamine and (+)-*cis*-dioxolane (test tissues) or histamine alone (control tissues).

### *Measurement and analysis of responses*

All responses were recorded as changes in isometric tension (g). Contractile responses were normalized to the maximal contractile responses in each tissue during the first exposure to agonist. Relaxant responses were expressed as a percentage of the isometric tension induced by the agonist, before application of isoprenaline. Data were analysed by the relationship of Parker & Waud (1971), using a non-linear iterative curve fitting procedure (Kaleidagraph, Synergy software, Reading, PA 19606, U.S.A., Leung *et al.*, 1992). The potency (defined as the $EC_{50}$) and maximal responses determined by this procedure were corrected for changes in sensitivity with time, where necessary. Apparent antagonist affinities ($pA_2$) were determined, where appropriate, by Schild regression analysis (Arunlakshana & Schild, 1959). Values quoted are those obtained when the slope, not being significantly different from unity, was constrained to unity.

Statistical analysis of the data was performed using paired and unpaired Student's *t* tests where appropriate, with $P < 0.05$ being considered significant. All values quoted are the mean ± s.e.mean from five animals, unless otherwise stated.

### *Compounds used*

(+)-*cis*-Dioxolane (L-(+)-*cis*-2-methyl-4-trimethylammonium methyl-1,3-dioxolane iodide, a 60:40 mixture of *cis*:*trans*), atropine, pirenzepine, methoctramine, p-F-HHSid (*para*-fluoro-hexa-hydro-sila-diphenidol) and histamine were obtained from Research Biochemicals Inc. (Natick, MA, USA). L-660,863 ((±)-3-(3-amino-1,2,4-oxadiazole-5-yl)-quinuclidine) was synthesized in the Institute of Organic Chemistry (Syntex Discovery Research, Palo Alto, CA, U.S.A.). Tetrodotoxin, physostigmine, indomethacin, corticosterone, isoprenaline, acetylcholine and ascorbic acid were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). SDZ ENS 163 (thiopilocarpine) was a gift from Sandoz Pharma Ltd (Basel, Switzerland) and zamifenacin was generously provided by Pfizer Ltd. (Sandwich, U.K.).

Indomethacin was made up as a 1 mg $ml^{-1}$ solution in propylene glycol and solubilized by a brief period (2–3 min) of sonication. Corticosterone was made up as a 0.1 M solution in dimethyl sulphoxide. Tetrodotoxin was made up as a 1.0 mM stock solution in 0.01 M acetic acid, which was then divided into 200 μl volumes and frozen until use. Ascorbic acid (22 μM) was added to solutions of histamine and isoprenaline as an anti-oxidant and these solutions were kept on ice for the duration of the experiments.

## Results

### *Receptor characterization*

(+)-*cis*-Dioxolane caused concentration-dependent contractions of tracheal strips with a maximal response of 4.2 ± 0.1 g and potency ($EC_{50}$) of 11.5 ± 0.9 nM. No significant time-dependent shift in the concentration-effect curves to (+)-*cis*-dioxolane was observed. All muscarinic antagonists caused parallel concentration-dependent dextral shifts in the concentration-effect curves to (+)-*cis*-dioxolane. The apparent antagonist affinities ($pA_2$), were atropine 9.4 ± 0.1, pirenzepine 6.5 ± 0.1, methoctramine 5.5 ± 0.1, p-F-HHSiD 7.2 ± 0.1 and zamifenacin 8.2 ± 0.1 The Schild slopes were not significantly different from unity.

### *The effect of muscarinic $M_2$ receptor antagonism, on the relaxant potency of isoprenaline in tissues pre-contracted with (+)-cis-dioxolane*

Isoprenaline caused concentration-dependent relaxations in tissues pre-contracted with 0.2 μM (+)-*cis*-dioxolane to approximately 3 g (Figure 1). Isoprenaline completely reversed contractions induced by (+)-*cis*-dioxolane, with a potency ($EC_{50}$) of 32.2 ± 4.3 nM. In the presence of methoctramine (0.3 μM), the concentration-effect curve to isoprenaline was shifted to the left in a parallel fashion (Figure 1) with a potency ($EC_{50}$ value) of 19.1 ± 4.5 nM ($P < 0.05$). There was no significant difference in the magnitude of the developed tension in either of these two groups, prior to performing concentration-effect curves to isoprenaline (2.9 ± 0.2 g controls and 3.2 ± 0.2 g plus methoctramine). There was no significant effect of this concentration of methoctramine (0.3 μM) on the potency of (+)-*cis*-dioxolane (control, $EC_{50}$ = 17.6 ± 3.2 nM, plus methoctramine, $EC_{50}$ = 21.0 ± 4.4 nM) nor the magnitude of the maximum response to agonist ($n = 6$).

### *The effect of muscarinic agonists and histamine on the relaxant potency of isoprenaline*

All muscarinic agonists produced concentration-dependent contractions of tracheal strips (Table 1) and were full agonists with respect to (+)-*cis*-dioxolane, with the exception of SDZ ENS 163 (Figure 2, Table 1). The rank order of potency was L-660,863 ≥ (+)-*cis*-dioxolane > ACh > SDZ ENS 163 (Table 1). Physostigmine (0.3 μM), added 15–20 min prior to the addition of acetylcholine, increased isometric tension by 1.4 ± 0.2 g (see Figure 3).

Concentration-dependent relaxations to isoprenaline were seen in all tissues pre-contracted to either 3 or 2 g (Figure 4, Table 2). In tissues pre-contracted with the higher concentration of muscarinic agonist, to approximately 3 g, isoprenaline was most potent when SDZ ENS 163 was used to induce the

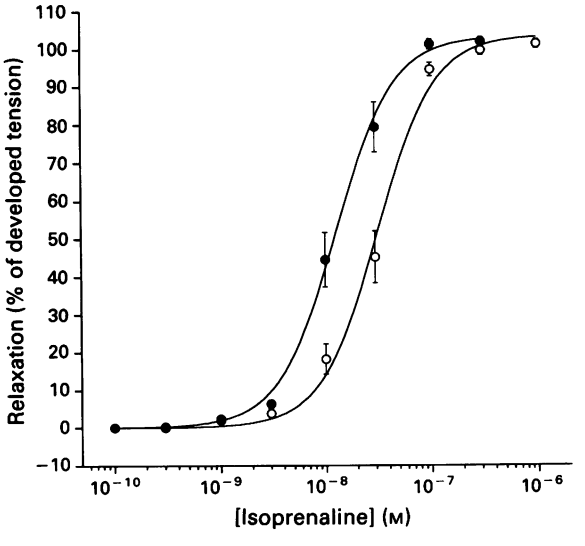


**Figure 1** Isoprenaline-induced relaxation in tissues pre-contracted with (+)-*cis*-dioxolane in the absence (○) and presence of 0.3 μM (●) methoctramine. Relaxations are expressed as a percentage of the developed tension induced by (+)-*cis*-dioxolane, which were not significantly different between groups. Values are the mean ± s.e. mean, $n = 6$.

**Table 1** The contractile potency ($EC_{50}$) and maximal tension changes associated with a range of muscarinic agonists in guinea-pig tracheal smooth muscle

| | *SDZ ENS 163* | *L-660,863* | *Acetylcholine* | *(+)-cis-dioxolane* |
|---|---|---|---|---|
| Maximal response (g) | 2.4 ± 0.3* | 3.8 ± 0.2 | 3.7 ± 0.2 | 3.8 ± 0.2 |
| Potency ($EC_{50}$ nM) | 1000 ± 100 | 5.7 ± 0.8 | 159 ± 19 | 8.1 ± 1.6 |
| *n* | (15) | (15) | (15) | (18) |

Values are the mean ± s.e.mean, where *n* = number of experiments. *$P<0.05$ Student's unpaired *t* test ((+)-*cis*-dioxolane maxima v SDZ ENS 163 maxima).

**Table 2** The relaxant potency ($EC_{50}$) of isoprenaline in tissues pre-contracted using a range of muscarinic agonists

**A** *Pre-contracted to approximately 3 g*

| | *SDZ ENS 163* | *L-660,863* | *Acetylcholine* | *(+)-cis-dioxolane* |
|---|---|---|---|---|
| Developed tension (g) | 2.8 ± 0.3 | 2.8 ± 0.2 | 3.0 ± 0.1 | 3.0 ± 0.2 |
| Potency ($EC_{50}$ nM) | 3.7 ± 0.3† | 7.0 ± 0.8† | 20.8 ± 4.3† | 49.4 ± 3.2† |
| *n* | (3) | (5) | (6) | (12) |

**B** *Pre-contracted to approximately 2 g*

| | *SDZ ENS 163* | *L-660,863* | *Acetylcholine* | *(+)-cis-dioxolane* |
|---|---|---|---|---|
| Developed tension (g) | 2.1 ± 0.1 | 2.0 ± 0.1 | 2.0 ± 0.1 | 2.0 ± 0.1 |
| Potency ($EC_{50}$ nM) | 1.1 ± 0.3 | 3.6 ± 0.7 | 4.5 ± 1.6 | 6.2 ± 1.1 |
| *n* | (5) | (5) | (6) | (5) |

The concentrations of agonist required to achieve these developed tensions were: (A) SDZ ENS 163 = 30 μM, L-660,863 = 30 nM, ACh = 10 μM, (+)-*cis*-dioxolane = 0.1 μM and (B) SDZ ENS 163 = 12.6 ± 1.2 μM, L-660,863 = 9.6 ± 1.4 nM, ACh = 0.4 ± 0.2 μM, (+)-*cis*-dioxolane = 21.2 ± 5.4 nM. Values are the mean ± s.e.mean, where *n* = the number of experiments. *$P<0.05$. Student's paired *t* test, †$P<0.02$, Student's unpaired *t* test.

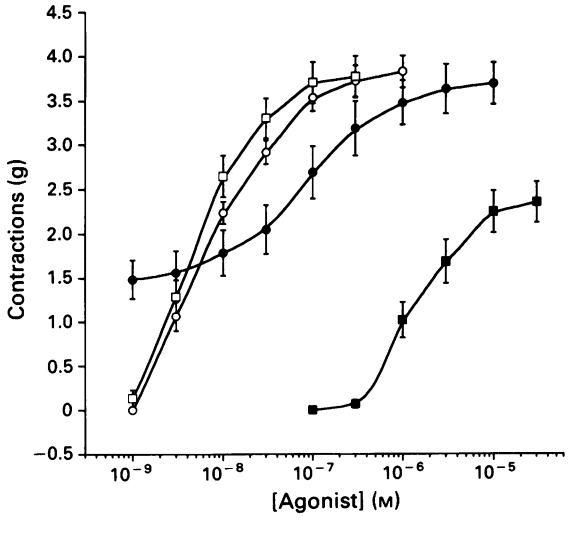


**Figure 2** Contractions of the trachea induced by a range of muscarinic agonists; (+)-*cis*-dioxolane (○), acetylcholine (●), L-660,863 (□) and SDZ ENS 163 (■), given as absolute changes in isometric tension. The potencies of these agonists are shown in Table 2. Values are the mean ± s.e.mean, *n* = 15–18 experiments.

contracture and least potent when (+)-*cis*-dioxolane was used (Table 2). The relaxant potency of isoprenaline was significantly different in all cases, although the initial level of isometric tension, induced by the muscarinic agonists, were not significantly different. Isoprenaline completely reversed contractions induced by all the muscarinic agonists, with the exception of ACh in which case the contracture was reversed by 71 ± 5% (Figures 3 and 4).

When tissues were pre-contracted with the lower concentration of agonist, to approximately 2 g, isoprenaline was again most potent against contractures induced by SDZ ENS 163 and least potent against (+)-*cis*-dioxolane-induced contracture (Table 2). Under these conditions, isoprenaline completely reversed the contracture induced by all agonists (Figure 4) and the relaxant potency of isoprenaline was significantly increased when compared to that obtained in tissues pre-contracted with the higher concentration of agonist (to approximately 3 g).

Histamine produced concentration-dependent contractions of tracheal strips with a potency ($EC_{50}$) of 5.1 ± 0.9 μM and maximal tension change of 3.5 ± 0.1 g. Isoprenaline caused concentration-dependent relaxations of tissues pre-contracted with high and low concentrations of histamine, to approximately 3 g (2.6 ± 0.2 g) or 2 g (2.1 ± 0.1 g). These two levels of initial contracture were significantly different from each other, but were not significantly different from those elicited by the high and low concentrations of muscarinic agonists described above. Isoprenaline completely reversed histamine-induced contractions at either 3 or 2 g isometric tension, with potencies ($EC_{50}$) of 4.2 ± 0.6 nM and 3.8 ± 1.1 nM, respectively. These values were not significantly different from one another, despite the fact that the initial levels of isometric tension were significantly different from each other (2.6 ± 0.2 g vs 2.1 ± 0.1 g).

In tissues pre-contracted to 3 g, isoprenaline was significantly more potent at relaxing tissues contracted by histamine than L-660,863, (+)-*cis*-dioxolane or acetylcholine. However, at this level of isometric tension, there was no significant difference between the relaxant potency of isoprenaline in tissues pre-contracted with either histamine or SDZ ENS 163. In tissues pre-contracted to 2 g, there was no significant difference between the potency of isoprenaline in tissues pre-contracted with muscarinic agonists or with histamine.

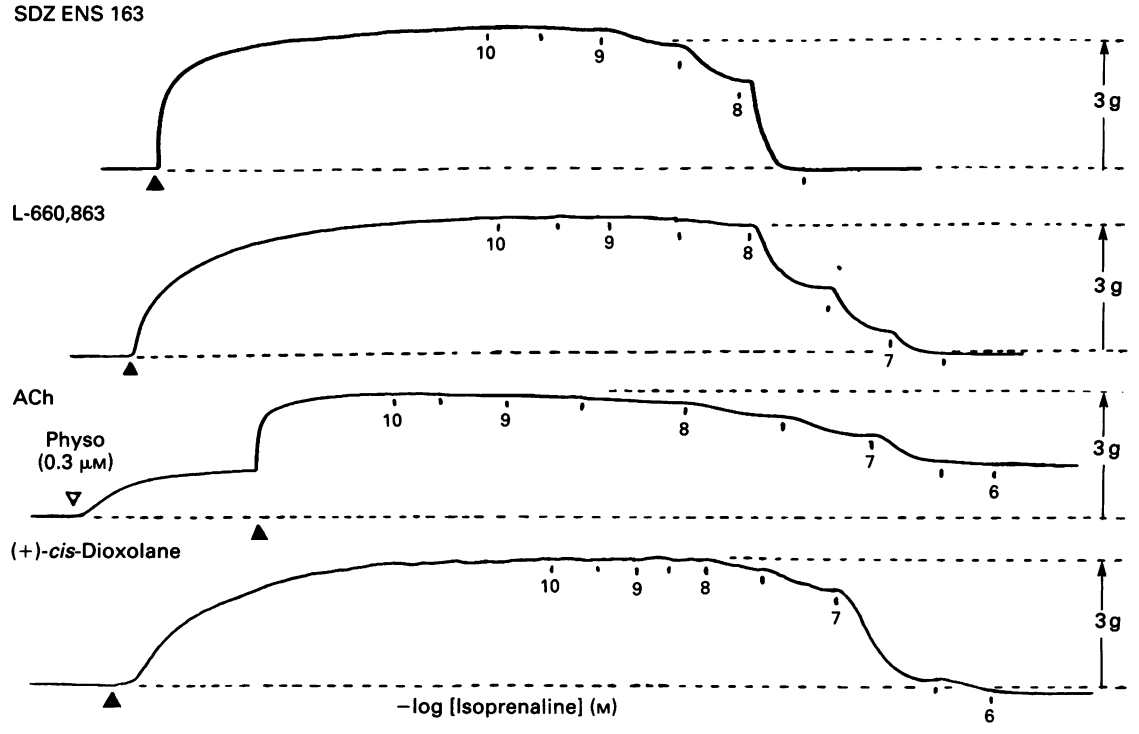


**Figure 3** Representative results showing relaxations to isoprenaline in tracheal strips pre-contracted to 3 g by a range of muscarinic agonists. The broken lines indicate 3 g isometric tension and the concentrations of isoprenaline in 0.5 $\log_{10}$ increments are indicated.

### *The effect of $M_2$ receptor stimulation and antagonism, on the relaxant potency of isoprenaline in tissues pre-contracted with histamine*

In the presence of p-F-HHSiD (0.3 μM), the relaxant potency ($EC_{50}$) of isoprenaline against histamine pre-contracture, was not significantly altered by agonism of $M_2$ receptors using (+)-*cis*-dioxolane (0.1 μM). Similarly, $M_2$ receptor antagonism using methoctramine (1 μM), did not significantly alter the relaxant potency of isoprenaline in tissues pre-contracted with histamine in the presence of (+)-*cis*-dioxolane (Figure 5; Table 3). Despite the presence of 0.3 μM p-F-HHSiD, (+)-*cis*-dioxolane (0.1 μM) caused a significant increase in isometric tension (0.4 ± 0.1 g). However, the concentration of histamine used to induce tone was adjusted, such that the magnitude of the contracture prior to performing concentration-effect curves to isoprenaline, was not significantly different between the three groups (Figure 5; Table 3).

In the absence of p-F-HHSiD, the relaxant potency of isoprenaline was significantly reduced in tissues pre-contracted to 3 g with a combination of histamine and (+)-*cis*-dioxolane (30 nM), when compared to tissues pre-contracted to equivalent isometric tension with histamine alone. This effect was reversed by 1.0 μM methoctramine (Figure 5; Table 3). In the absence of p-F-HHSiD, the increase in isometric tension induced by (+)-*cis*-dioxolane (30 nM) was 3.1 ± 1.0 g. However, the concentration of histamine used to induce tone was adjusted, such that the magnitude of the contracture prior to performing concentration-effect curves to isoprenaline, was not significantly different between the three groups (Figure 5; Table 3).

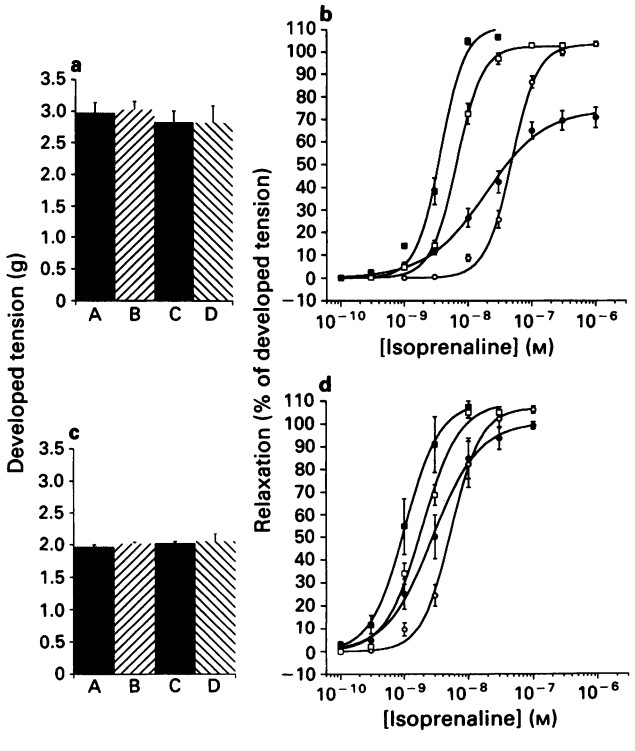


**Figure 4** Isoprenaline-induced relaxation of tissues pre-contracted to 3 g (b) and 2 g (d) isometric tension by a range of muscarinic agonist; (+)-*cis*-dioxolane (○ and A), acetylcholine (● and B), L-660,863 (□ and C) and SDZ ENS 163 (■ and D). Relaxations are expressed as a % of the developed tension induced by these agonists and are shown in absolute values in the histograms (a) and (c) (see also Table 3). Values are the mean ± s.e.mean. The number of experiments for each group are shown in Table 3, along with the $EC_{50}$ values for isoprenaline-induced relaxation under these conditions.

## Discussion

In airway smooth muscle, muscarinic receptors mediate contraction and appear to modulate the relaxant potency of β-adrenoceptor agonists. In all species studied to date, $M_2$

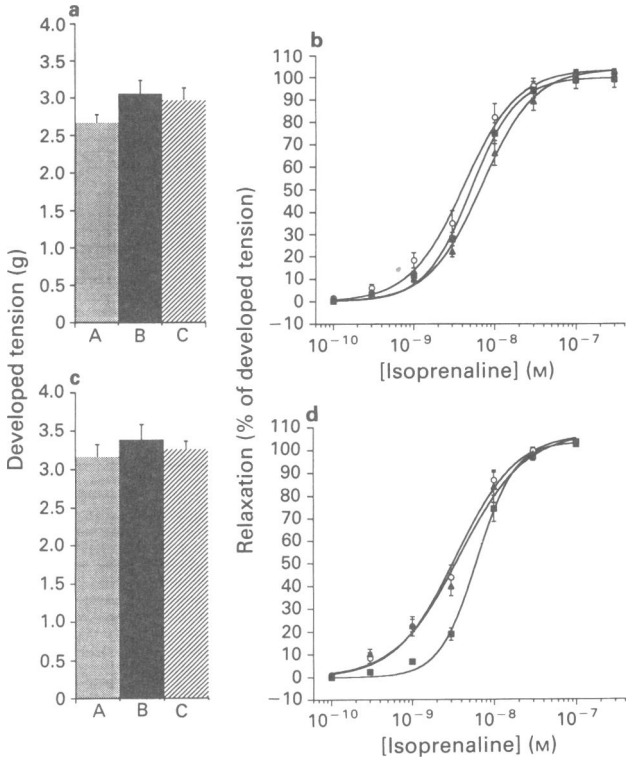


**Figure 5** Isoprenaline-induced relaxation in tissues pre-contracted with histamine under the following conditions; in the absence of (+)-*cis*-dioxolane and methoctramine (○ and A), in the presence of 0.1 μM (b) or 30 nM (d) (+)-*cis*-dioxolane (■ and B) and in the presence of (+)-*cis*-dioxolane (as above) and 1 μM methoctramine (▲ and C). (a) and (b) show responses when 0.3 μM p-F-HHSiD was present to antagonize $M_3$ receptors and (c) and (d) show responses in the absence of p-F-HHSiD. Relaxations are expressed as a percentage of the developed tension induced either by histamine alone or in combination with (+)-*cis*-dioxolane. The developed tensions for each group are shown in absolute values in the histogram (a) and (c) (see also Table 1). Values are the mean ± s.e.mean, $n = 6$ for each of the six treatment groups.

**Table 3** The effect of (+)-*cis*-dioxolane (0.1 μM or 30 nM), alone or in the presence of methoctramine (1 μM) on the relaxant potency of isoprenaline in tissues pre-contracted with histamine in the presence and absence of p-F-HHSiD (0.3 μM)

| | *Control* | *Test 1* | *Test 2* |
|---|---|---|---|
| **A** *In the presence of p-F-HHSiD* | | | |
| Developed tension (g) | 2.7 ± 0.1 | 3.1 ± 0.2 | 3.0 ± 0.2 |
| Potency ($EC_{50}$ nM) | 4.8 ± 0.7 | 5.5 ± 0.6 | 6.0 ± 1.1 |
| **B** *In the absence of p-F-HHSiD* | | | |
| | *Control* | *Test 1* | *Test 2* |
| Developed tension (g) | 3.2 ± 0.2 | 3.4 ± 0.2 | 3.3 ± 0.2 |
| | | ** | |
| Potency ($EC_{50}$ nM) | 4.4 ± 0.6 | 6.7 ± 0.8 | 3.6 ± 0.9 |
| | | NS | |

Control tissues received neither (+)-*cis*-dioxolane nor methoctramine and the developed tension was achieved with histamine at a concentration of (A) 49 ± 17 μM and (B) 27 ± 2 μM. Test 1 tissues received (+)-*cis*-dioxolane (A) 0.1 μM and (B) 30 nM and the developed tension was achieved by the addition of histamine (A) 21 ± 4 μM and (B) 2.8 ± 0.7 μM. Test 2 tissues received (+)-*cis*-dioxolane in the concentrations already noted, along with methoctramine (1 μM). In test 2 tissues the developed tension was achieved by the addition of histamine (A) 20 ± 5 μM and (B) 2.8 ± 0.8 μM. Values are the mean ± s.e.mean, $n = 6$ per treatment group. $^{**}P < 0.02$ and NS: not significant, by Student's paired $t$ test.

and $M_3$ receptors are present in airway smooth muscle in the ratio approximately 4:1 (Fryer & El Fakahany, 1990; Mahesh *et al.*, 1992). Both these muscarinic receptor subtypes have been implicated in the muscarinic receptor-mediated inhibition of β-adrenoceptor agonist-mediated relaxation (Van Amsterdam *et al.*, 1989; Fernandes *et al.*, 1992). The aim of the present study was to characterize further the role of muscarinic $M_2$ receptors in modulating relaxations of guinea-pig, isolated trachea to isoprenaline. In order to achieve this, three separate approaches were used, after first establishing an affinity profile for a range of muscarinic antagonists at the post-junctional receptor mediating contraction.

### *Receptor characterization*

The antagonist affinity profile was consistent with stimulation of $M_3$ receptors causing contraction with no involvement of $M_2$ receptors (Ten Berge *et al.*, 1993). The $pA_2$ for the novel $M_3$ receptor antagonist, zamifenacin, was similar to that previously reported by Wallis *et al.* (1993). The $pA_2$ value for p-F-HHSiD (7.2) was lower than reported at $M_3$ receptors in other smooth muscles, including guinea-pig ileum (7.8–8.0, Lambrecht *et al.*, 1988) but consistent with previous findings in our laboratory (Eglen *et al.*, 1990). The $M_3$ receptor in guinea-pig trachea may be atypical, since p-F-HHSiD, RDS 129 (Saihin & Ilhan, 1986) and zamifenacin (Wallis *et al.*, 1993; this study) discriminate between $M_3$ receptors in guinea-pig trachea and ileum. However, in the absence of evidence for structural heterogeneity of $M_3$ receptors, the reasons for these differences in $pA_2$ values remain unknown (Caulfield, 1993). A consequence of the atypical nature of the tracheal $M_3$ receptor is that studies attempting to characterize $M_2$ or $M_3$ receptor function alone in this tissue are compromised by the low tracheal $M_2$:$M_3$ selectivity of p-F-HHSiD and zamifenacin.

### *The effect of muscarinic $M_2$ receptor antagonism on the relaxant potency of isoprenaline in tissues pre-contracted with (+)-cis-dioxolane*

The first approach to study the role of $M_2$ receptors in guinea-pig trachea reproduced the findings of Fernandes and colleagues (1992) in canine trachea. Methoctramine (0.3 μM) increased the relaxant potency of isoprenaline, in (+)-*cis*-dioxolane pre-contracted tissues. The small but statistically significant increase in isoprenaline relaxant potency (1.7 fold shift) in the presence of methoctramine may be an underestimate, since comparison with the potency of isoprenaline in tissues not previously exposed to methoctramine indicates a 2.6 fold shift induced by methoctramine (see below). As the apparent affinities of methoctramine at $M_2$ and $M_3$ receptors are 7.8 and 5.8, respectively (Melchiorre *et al.*, 1987; Eglen *et al.*, 1988), it is unlikely that $M_3$ receptor antagonist properties of methoctramine account for the effect observed. To support this conclusion, 0.3 μM methoctramine had no significant effect on $M_3$-mediated contractile responses (this study). Furthermore, higher concentrations of methoctramine (1 and 3 μM), which increase $M_3$ receptor occupancy, did not further augment the potency of isoprenaline (data not shown).

### *The effect of muscarinic agonists and histamine on the relaxant potency of isoprenaline*

The second approach was to stimulate the $M_2$ receptors with agonists possessing different intrinsic activities at muscarinic receptors. Acetylcholine and (+)-*cis*-dioxolane are agonists of high intrinsic efficacy, although non-selective between $M_2$ and $M_3$ receptors (Ford *et al.*, 1991). L-660,863 is a relatively selective muscarinic $M_2$ receptor agonist (Eglen *et al.*, 1992). However, its efficacy at this receptor is low and therefore, in poorly coupled $M_2$ receptor systems it acts as an antagonist (Freeman *et al.*, 1990; Eglen *et al.*, 1992). SDZ ENS 163, is a

full agonist at $M_1$ receptors, a partial $M_3$ receptor agonist and an $M_2$ receptor antagonist (Enz *et al.*, 1992). Under the present experimental conditions, agonist effects of SDZ ENS 163 at $M_1$ receptors in the parasympathetic ganglia (Bloom *et al.*, 1987), were inhibited by the inclusion of tetrodotoxin, while post-junctional $M_1$ receptors have not been detected in airway smooth muscle (Mak & Barnes, 1990). Therefore, the use of SDZ ENS 163 permitted the relaxant potency of isoprenaline to be assessed under conditions of partial $M_3$ receptor stimulation and $M_2$ receptor antagonism. This agent, unlike L660,863 shows no selectivity between receptor subtypes on the basis of affinity, although it is 'functional selective' (atria $M_2$ $-\log K_B = 5.9 \pm 0.2$; tracheal $-\log K_A = 5.8 \pm 0.1$; Watson & Eglen, 1993).

All compounds were full agonists at $M_3$ receptors mediating contraction of tracheal strips, with the exception of SDZ ENS 163 which acted as a partial agonist. This finding is consistent with previous reports (Eglen *et al.*, 1990; Ford *et al.*, 1991; Enz *et al.*, 1992). The isoprenaline relaxant potencies were not greatly different between preparations contracted with the muscarinic agonists to 2 g isometric tension. Moreover, the isoprenaline relaxant potencies were not significantly different from potency estimates in tissues pre-contracted with histamine. This suggests that at low concentrations of muscarinic agonists, there is little effect of muscarinic receptor activation on the attenuation of relaxant responses to isoprenaline. Therefore, despite activation of $M_3$ receptors, to elicit a 2 g increase in tension, no inhibition of β-adrenoceptor-mediated relaxant responses could be detected.

However, increasing the level of both $M_2$ and $M_3$ receptor activation, significantly reduced the relaxant potency of isoprenaline. Under this condition, significant differences were seen in the relaxant potency of isoprenaline between tissues pre-contracted with full muscarinic agonists ((+)-*cis*-dioxolane, acetylcholine and L-660,863) and those pre-contracted with the partial $M_3$ agonist/$M_2$ antagonist, SDZ ENS 163. These differences may suggest that differential agonist activity at $M_2$ or $M_3$ receptors, rather than the developed tension *per se*, may influence the isoprenaline relaxant potency. In contrast, differences in isoprenaline relaxant potency were not seen in tissues pre-contracted with histamine to 2 or 3 g. Since histamine does not interact at muscarinic receptors, these data implicate a specific role for muscarinic receptors in the attenuation of relaxant responses to isoprenaline, which is in agreement with the work of others (Torphy, 1984; Jones *et al.*, 1987; Fernandes *et al.*, 1992).

### *The effect of $M_2$ receptor agonism and antagonism, on the relaxant potency of isoprenaline in tissues pre-contracted with histamine*

The final approach in the elucidation of the role of $M_2$ receptors was to stimulate the $M_2$ receptors in the absence of $M_3$ receptor activation. Ideally, an agonist with high potency and selectivity at $M_2$ receptors, which lacks intrinsic efficacy at $M_3$ receptors, is required. However, such a compound is unavailable. Thus, the alternative was to use (+)-*cis*-dioxolane, to activate $M_2$ receptors, in the presence of $M_3$ receptor antagonism by p-F-HHSiD. In this manner, the effect of $M_2$ receptor activation on relaxant potency of isoprenaline was examined in tissues pre-contracted to 3 g using histamine in the presence of 0.3 μM p-F-HHSiD. Under these conditions, there was no effect of (+)-*cis*-dioxolane on the relaxant potency of isoprenaline. This finding may imply that activation of $M_3$ receptors is required to mediate the reduction in isoprenaline relaxant potency, supporting conclusions reached by Meurs *et al.* (1993).

Alternatively, an involvement of $M_2$ receptors cannot be excluded, since in guinea-pig trachea, p-F-HHSiD shows relatively low selectivity between $M_3$ and $M_2$ receptors (16 fold Eglen *et al.*, 1990). Assuming a $pA_2$ of 6.0 for p-F-HHSiD (Lambrecht *et al.*, 1988; Eglen *et al.*, 1990) 23% of $M_2$ receptors would be occupied at the concentration required to antagonize $M_3$ receptor-mediated contractions (0.3 μM). This may be sufficient, in a poorly coupled system, to antagonize the effect of $M_2$ receptor-mediated inhibition of isoprenaline-induced relaxations. In an attempt to clarify the problem, experiments were repeated in the absence of p-F-HHSiD, and with a lower concentration of (+)-*cis*-dioxolane (30 nM), to reduce effects at $M_3$ receptors, while maintaining detectable effects on isoprenaline relaxant potency. In these studies, (+)-*cis*-dioxolane reduced the relaxant potency of isoprenaline, an effect which was reversed by methoctramine. The modest effect of (+)-*cis*-dioxolane (30 nM) was unsurprising given our previous observations that low concentrations of muscarinic agonists have small effects on isoprenaline relaxant potency. Taken together these data may suggest that the level of isometric tension achieved, is less important in determining the relaxant potency of isoprenaline compared to the level of muscarinic receptor activation.

Some data obtained in these studies require further explanation; (1) In the first study, an equilibration step with methoctramine, was used during construction of concentration-effect curves to (+)-*cis*-dioxolane. As discussed above, in tissues treated in this way, the relaxant potency of isoprenaline was 1.5 fold greater than in tissues not exposed to methoctramine. This suggests that residual $M_2$ receptor antagonism by methoctramine may have occurred. (2). There was no significant difference in the relaxant potency of isoprenaline in tissues pre-contracted to 2 g with either muscarinic agonists or histamine. This indicates a lack of muscarinic inhibition of isoprenaline relaxant responses. (3) In preparations pre-contracted to 3 g with L-660,863, the relaxant potency of isoprenaline was 1.7 fold less than in tissues pre-contracted with histamine (lacking $M_2$ activity) and 7 fold greater than in tissues pre-contracted with (+)-*cis*-dioxolane. (4) In the presence of $M_3$ receptor antagonism by p-F-HHSiD (0.3 μM) approximately 23% of the $M_2$ receptors would be occupied and no effect of $M_2$ receptor stimulation by (+)-*cis*-dioxolane (0.1 μM) could be demonstrated.

One explanation for all four observations is that muscarinic inhibition of β-adrenoceptors is mediated by $M_2$ receptors that are poorly coupled. This would be predicted to have the following consequences. Firstly, residual $M_2$ receptor antagonism (as a result of prior exposure to methoctramine) would reverse inhibitory effects of (+)-*cis*-dioxolane and thus increase the potency of isoprenaline. Secondly, high concentrations of agonists would be required to achieve adequate $M_2$ receptor occupancy in order to detect a functional inhibition. Thirdly, agonists with low intrinsic efficacy at $M_2$ receptors, such as L-660,863 or SDZ ENS 163, would behave as antagonists in such a system. Finally, antagonist occupation of a small porportion of $M_2$ receptors would have a large effect on the functional response. However, without direct measurement of the receptor reserve associated with $M_2$ inhibitory effects, this can only be speculated upon.

These studies have therefore provided some evidence for the involvement of $M_2$ receptors in the inhibitory effects of (+)-*cis*-dioxolane on the relaxant potency of isoprenaline, although a role for $M_3$ receptor cannot be excluded. In this respect these data concur with reports that AF-DX 116 or gallamine enhance the potency of isoprenaline in canine and rabbit trachea (Fernandes *et al.*, 1992; Arjona *et al.*, 1993). In the former tissue, Mitchell *et al.* (1993) has also demonstrated a similar effect of pertussis toxin, which functionally uncouples $M_2$ receptors from inhibition of adenylyl cyclase. In contrast, in bovine and guinea-pig trachea, gallamine has been reported not to augment the relaxant potency of isoprenaline (Meurs *et al.*, 1993; Roffel *et al.*, 1993). The reason for these discrepancies is unclear, although differences in methodology may be important. Thus, differences in the level of basal tension, the presence or absence of epithelium or of indomethacin, and the recording of isometric or isotonic tensions may account for the differences. However, it is important to note that in guinea-pig ileum, when $M_3$ receptors have been inactivated by alkylation, stimulation of $M_2$

receptors causes contraction by inhibiting relaxations elicited by isoprenaline, in tissues pre-contracted to histamine (Thomas *et al.*, 1993). However, similar studies have yet to be reported in guinea-pig trachea.

In conclusion, these data suggest that it is the degree of muscarinic receptor activation that is important in determining the relaxant potency of isoprenaline and this is related to the efficacy of the muscarinic agonist at $M_2$ and $M_3$ receptors. Since muscarinic $M_2$ receptor antagonism augments the relaxant potency of isoprenaline, these data provide some evidence for an inhibitory role of $M_2$ receptors on relaxant responses to isoprenaline. However, the involvement of $M_3$ receptors cannot be definitively excluded due to (a) the lack of selective $M_2$ and $M_3$ receptor agonists and (b) the low $M_2/M_3$ selectivity of antagonists in guinea-pig trachea.

We would like to thank Jenson Wong for his contribution to the work determining antagonist apparent affinities and to Dr Helen Reddy for her helpful discussion. We would also like to thank Sandoz Pharma Ltd. (Basel, Switzerland), for providing SDZ ENS 163 and Pfizer Ltd. (Sandwich, U.K.), for providing the zamifenacin. Our thanks also to Seth Michelson for performing the statistical analysis.

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# Attenuation by chlormethiazole administration of the rise in extracellular amino acids following focal ischaemia in the cerebral cortex of the rat

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**1** *In vivo* microdialysis has been used to investigate the concentration of various amino acids and lactate in the extracellular fluid of the rat cortex following focal ischaemia, the probe being placed in the core of the infarct area.

**2** An ischaemic infarct was produced in the cortex by use of a photochemical dye (Rose Bengal) and light irradiation. There was a marked increase in lactate concentration (300%) over the next 4 h. Substantial increases were also seen in the concentration of the excitatory (glutamate and aspartate), inhibitory (GABA and taurine) and other amino acids (serine, alanine, asparagine).

**3** Administration of chlormethiazole (200 mg $kg^{-1}$, i.p.) 5 min after the onset of ischaemia reduced the ischaemia-induced neurodegeneration by approximately 30%, measured histologically 24 h later.

**4** Chlormethiazole (200 mg $kg^{-1}$, i.p.) administration also reduced the rise in the concentration of lactate and all the amino acids by between 30–60% during the first 4 h after the onset of ischaemia.

**5** Analysis of the time course of the amino acid changes suggested that chlormethiazole is not neuroprotective because of the inhibition of excitatory amino acid release but rather that the attenuated rise in the concentration of all the amino acids is reflective of neuroprotection and therefore decreased cell death.

**6** This conclusion was supported by the observation that the enhanced efflux of glutamate from slices of cerebral cortex which had been induced by incubation of the slices in an hypoxic medium was unaltered by the presence of a high concentration of chlormethiazole (1 mM) in the medium.

**7** Overall the data strengthen the evidence for the neuroprotective effect of chlormethiazole in this model of focal ischaemia.

**Keywords:** Focal ischaemia; chlormethiazole; excitatory amino acids; amino acids; neuroprotection; glutamate; GABA; lactate; *in vivo* microdialysis

## Introduction

Watson and colleagues (1985) have developed a relatively non-invasive method of producing an ischaemic infarct in the cortex by means of a photochemically induced thrombosis of cerebral arteries. An intravenous injection of the photosensitive dye, Rose Bengal, is given and a green light used to penetrate the skull of anaesthetized rats and illuminate subdural blood vessels. A photochemical reaction occurs with subsequent damage to the endothelial lining of blood vessels, platelet aggregation and thrombosis (Dietrich *et al.*, 1987a,b; 1988; Grome *et al.*, 1988; De Ryck *et al.*, 1989; Laursen *et al.*, 1991). In the irradiated area of the cortex there is vascular stasis and ischaemic cell death (Watson *et al.*, 1985; Snape *et al.*, 1993; Baldwin *et al.*, 1993b,c).

Recently we reported that chlormethiazole, a drug already shown to be an effective neuroprotective agent in the gerbil model of transient global ischaemia (Cross *et al.*, 1991; Baldwin *et al.*, 1993a) reduced the size of the infarction in the photochemical model of focal ischaemia (Snape *et al.*, 1993). Dizocilpine, the N-methyl-D-aspartate antagonist, despite being neuroprotective in the gerbil model (Gill *et al.*, 1987; 1988; Cross *et al.*, 1991) was without protective effect in the photochemical model as was the AMPA antagonist NBQX [2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo (F) quinoxaline] (Baldwin *et al.*, 1993c). As both dizocilpine and NBQX are protective in other models of focal ischaemia (Gill *et al.*, 1988; 1992; Park *et al.*, 1988; Sheardown *et al.*, 1990) this raises questions as to the involvement of glutamate in the mechanisms involved in cell death in the photochemical model.

The current study was undertaken therefore to investigate by use of *in vivo* microdialysis, the extracellular concentrations (and therefore presumably efflux) of various transmitter and non-transmitter amino acids and also lactate following a photochemically induced ischaemic episode and the effect of chlormethiazole on these concentrations. In this way it was hoped that knowledge would be gained both of the changes that follow photochemically induced ischaemia and also the mechanisms involved in the neuroprotective action of chlormethiazole.

Since a previous study (Baldwin *et al.*, 1993c) found that the extracellular concentrations of amino acids and lactate did not change in non-ischaemic animals in the course of the experiment, it was considered reasonable to perform the study comparing only drug and non-drug treated ischaemic rats.

## Methods

### *Animals*

Male Lister hooded rats (Olac, U.K.) weighing 280–360 g at the time of surgery were used. Rats were housed in groups of 5 in a room with a 12 h light:12 h dark cycle (lights on at 07 h 00 min) and given food and water *ad libitum*.

### *Implantation of microdialysis probes*

Rats were anaesthetized with halothane (1.5–5.0%) in a $O_2/N_2O$ mixture (1:2) and secured in a Kopf stereotaxic frame with the tooth bar at −3.3 mm below interaural zero. A 3 mm concentric dialysis probe (240 μm diameter; CMA

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Microdialysis AB, Sweden) was implanted horizontally via a guide cannule (CMA11, CMA Microdialysis AB) into the right cortex: −2.3 mm posterior and −2.0 mm ventral to the skull surface at bregma and the tip of the probe was located at ±0.0 mm from the midline. A 6 mm diameter plastic ring was placed directly over the right cortex, behind bregma and tangential to the bregmoidal and midline skull sutures. The probe and plastic ring were secured to the skull with 2 screws and dental acrylic, leaving the skull inside the ring, free of acrylic. Rats were given 2 ml of 4% glucose in saline (s.c.) as a nutrient and placed in individual perspex cages (30 × 30 × 25 cm) to recover for 20 h.

### *Induction of ischaemia*

Rats were anaesthetized with halothane (1.5–5.0%) in an oxygen/nitrous oxide mixture (1:2). Rose bengal (7.5 mg $ml^{-1}$) in saline was then injected slowly into the right jugular vein at a dose of 20 mg $kg^{-1}$ (Ca. 0.5–0.8 ml injected over 20 s). Immediately after the injection a fibre optic was positioned directly over the centre of the plastic ring above the skull (distance from fibre to skull <2.0 mm). This area of skull was irradiated for 7 min with light from a 150 W halogen source that had been passed through a green filter (Olympus Highlight 3000, Olympus, U.K.).

Five minutes after induction of ischaemia rats received an injection of either chlormethiazole (200 mg $kg^{-1}$, i.p.) or NaCl, 0.9% w/v (saline). Since the chlormethiazole-treated animals were sedated, they were placed on a heating mat under a blanket for the experiment. Saline-treated animals were replaced in the perspex boxes. The rectal temperature of both groups of animals was monitored and maintained at 37°C throughout the remainder of the experiment.

### *Collection of dialysate samples*

Dialysis probes were perfused with artificial cerebrospinal fluid (composition, mM: NaCl 125, KCl 2.5, $MgCl_2$ 1.18 and $CaCl_2$ 1.26) at a rate of 1 μl $min^{-1}$. The first 60 min sample was discarded and the next three 30 min baseline samples collected. Rats were then anaesthetized for the induction of ischaemia, replaced in the perspex boxes and samples collected every 30 min for the next 4 h.

After collection, animals were replaced in their cages overnight before perfusion and histological assessment.

Each dialysis sample was divided into 2 aliquots and frozen at −30°C until assay; 5 μl was taken for lactate determination and 25 μl for amino acid determination.

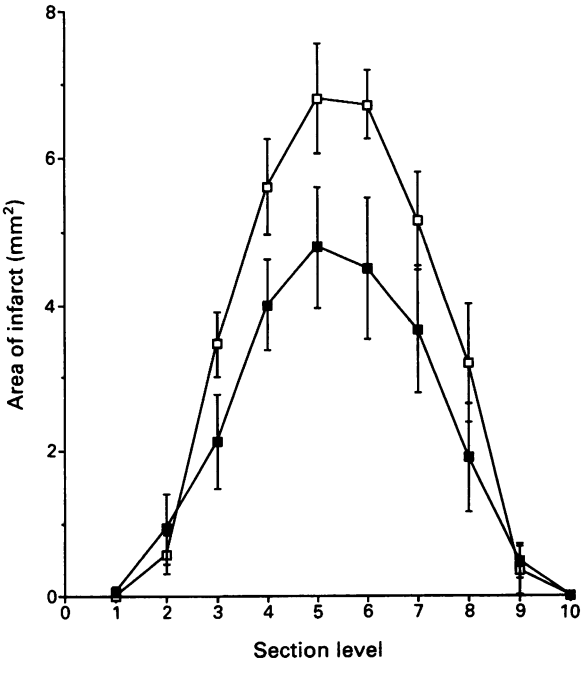


**Figure 1** The effect of chlormethiazole (200 mg $kg^{-1}$, i.p., $n = 9$; ■) or saline (i.p., $n = 10$; □) given 5 min post-ischaemia, on the size of the cortical infarct shown by tetrazolium chloride staining measured 24 h post-ischaemia. Values are mean (± s.e.mean) area of infarct ($mm^2$) at each section level (i.e. 1 mm sections taken from anterior to posterior of brain). There was a significant effect of 'level' (ANOVA $F(8,136) = 40.59$, $P<0.001$) and a significant 'drug' × 'level' interaction (ANOVA $F(8,136) = 2.02$, $P<0.05$).

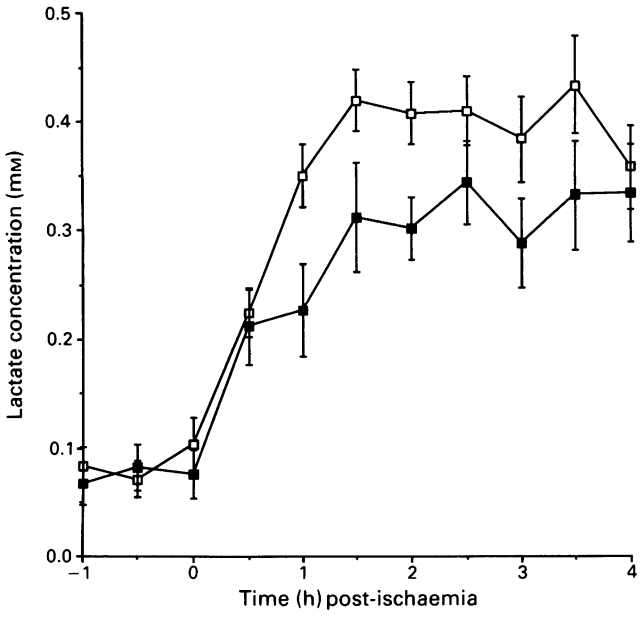


**Figure 2** Measurement of lactate concentration in dialysate from ischaemic rats. Mean (± s.e.mean) lactate concentration (mM) from rats receiving either chlormethiazole (200 mg $kg^{-1}$, i.p., $n = 10$; ■) or saline (i.p., $n = 10$; □) 5 min after induction of ischaemia. There was a significant effect of 'time' (ANOVA $F(10,180) = 55.45$, $P<0.001$) and a significant 'drug' × 'time' interaction (ANOVA $F(10,180) = 1.97$, $P<0.05$).

### *Measurement of lactate and amino acids concentrations in dialysate*

Lactate concentrations were determined by the enzymatic assay method of Lowry & Passonneau (1972) as described by Baldwin *et al.* (1993c). Amino acid concentrations were measured by high performance liquid chromatography (h.p.l.c.) with fluorometric detection following precolumn derivatisation with *o*-ptthalaldehyde as described by Lindroth *et al.* (1985) and briefly detailed by Baldwin *et al.* (1993c).

### *Histological assessment of damage*

Twenty four hours after induction of ischaemia rats were perfused with 2,3,5-triphenyltetrazolium chloride (4% w/v in saline) and the brain subsequently prepared for fixing and slicing into 1 mm thick sections. The stained slices were photographed under a dissecting microscope and measures of the infarct size made with a digitizing tablet and computer by an observer unaware of treatment condition. Full experimental details are given in the paper of Snape *et al.* (1993) which also shows photographs of typical infarct damage.

### *Measurement of glutamate release from cortical slices* in vitro

Rats were killed by cervical dislocation, the brains removed and cortical slices prepared with a McIlwain tissue chopper

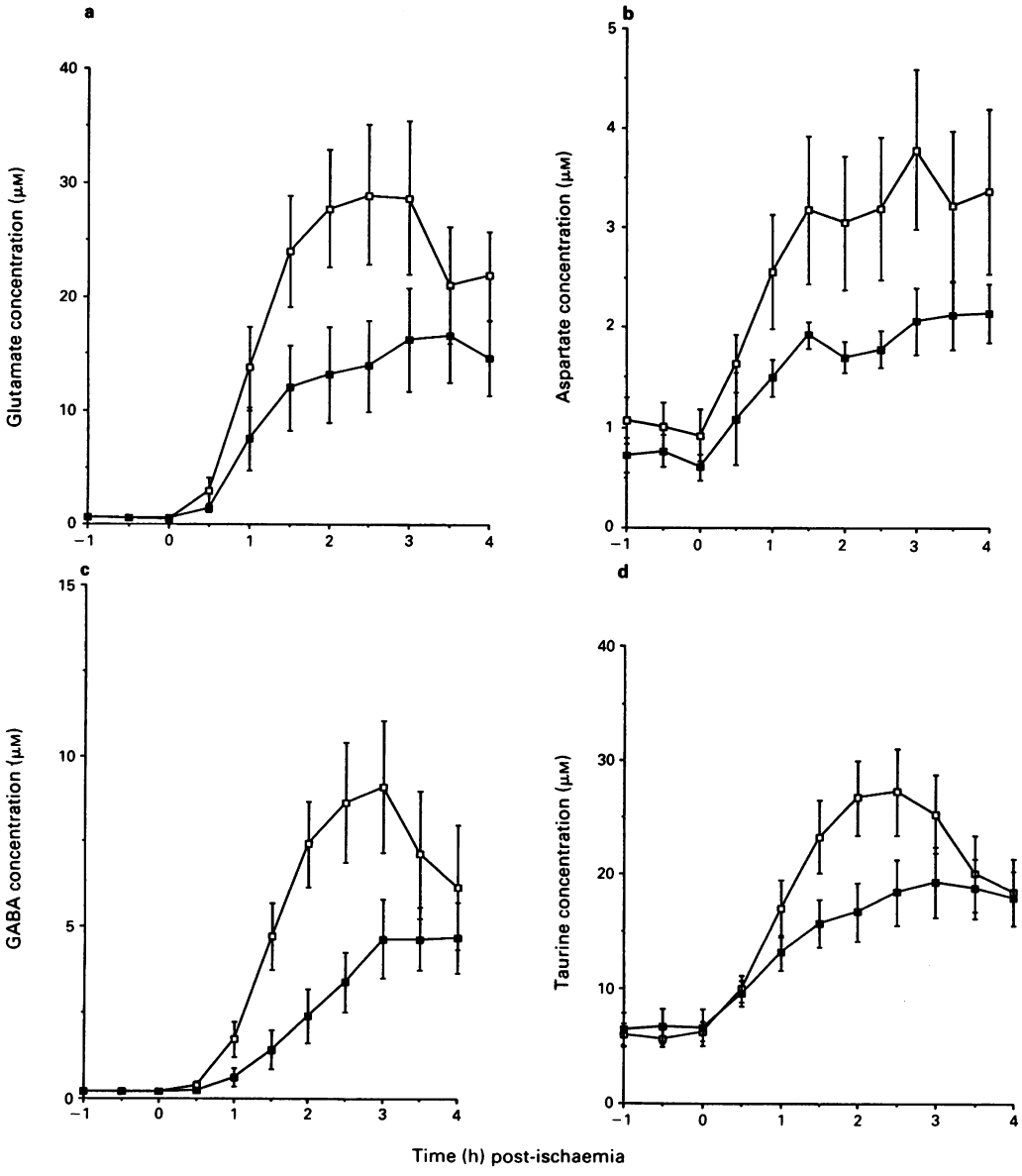


**Figure 3** Measurement of glutamate, aspartate, GABA and taurine concentration in dialysate from ischaemic rats. Rats received either chlormethiazole (200 mg $kg^{-1}$, i.p., $n = 10$; ■) or saline (i.p., $n = 10$; □) 5 min after induction of ischaemia. (a) Mean (± s.e.mean) glutamate concentration (μM) in dialysate. There was a significant effect of 'time' (ANOVA $F(10,180) = 24.04$, $P < 0.001$) and a significant 'drug' × 'time' interaction (ANOVA $F(10,180) = 2.50$, $P < 0.05$). (b) Mean (± s.e.mean) aspartate concentration (μM) in dialysate. There was a significant effect of 'time' (ANOVA $F(10,180) = 16.34$, $P < 0.001$) but no significant 'drug' × 'time' interaction. (c) Mean (± s.e.mean) GABA concentration (μM) in dialysate. There was a significant effect of 'drug' (ANOVA $F(1,18) = 4.56$, $P < 0.05$) and 'time' (ANOVA $F(10,180) = 26.96$, $P < 0.001$) and a significant 'drug' and 'time' interaction (ANOVA $F(10,180) = 3.90$, $P < 0.001$). (d) Mean (± s.e.mean) taurine concentration (μM) in dialysate. There was a significant effect of 'time' (ANOVA $F(10,180) = 34.24$, $P < 0.001$) and a significant 'drug' × 'time' interaction (ANOVA $F(10,180) = 3.00$, $P < 0.005$).

(500 μm, cut in two perpendicular directions). Slices were incubated in oxygenated Krebs buffer (pH 7.4, 37°C) for 15 min. The buffer was removed and the procedure repeated two further times. The final wash buffer was removed and 100 μl of packed slices was transferred to mesh baskets. The baskets were placed in vials containing 2 ml of oxygenated Krebs buffer and incubated for 3 min. Baskets were then transferred to vials containing 2 ml of fresh solution at 3 min intervals. In some cases the buffer had been bubbled with $N_2$ instead of $O_2$ and chlormethiazole (1 mM) was added to some of the buffer solutions. After 18 min, baskets were placed in HCl (1 M) for 60 min to release all remaining amino acids. Aliquots of the release medium and tissue extract were frozen until assay for amino acids. Amino acid release was expressed as a percentage of the total content of the tissue slices plus medium.

### *Drugs and reagents*

The following drugs and reagents were used (source in parentheses): dichlormethiazole edisylate (Astra Arcus, Södertälje, Sweden); halothane (ICI, U.K.); sodium pentobarbitone ('Sagatal', RMD Animal Health Ltd., U.K.); rose bengal [acid red 94; tetraiodotetrachlorofluorescein sodium salt], 2,3,5-triphenyltetrazolium chloride, *o*-phthalaldehyde, lactic acid glutamic acid, γ-aminobutyric acid (GABA), aspartic acid, taurine, serine, alanine and asparagine (Sigma Chemical Co., Poole, U.K.). All other reagents were obtained from Merck Ltd., Poole, U.K.

### *Statistics*

Separate analyses were performed for each amino acid and

for lactate. Dialysate amino acid and lactate concentrations were analysed by two-way Analysis of Variance (ANOVA) with 'drug' (i.e. saline or chlormethiazole) as the between groups factor and 'time' as the repeated measure. The histological data from the dialysis study were analysed by 2-way ANOVA with 'drug' as the between groups factor and 'section level' as the repeated measure. Data from the *in vitro* release experiments were analysed by 2-way ANOVA with 'condition' (i.e. $O_2$, hypoxia or hypoxia with chlormethiazole) as the between groups factor and 'time' as the repeated measure.

## Results

### *Effect of chlormethiazole on the infarct size following the ischaemic episode*

The size of the cortical damage 24 h after the induction of ischaemia was measured in a series of sections taken through the irradiated area of cortex. The area of damage was reduced by approximately 30% in animals given chlormethiazole (200 mg $kg^{-1}$, i.p.) 5 min after the ischaemic episode (Figure 1).

### *Effect of ischaemia on dialysate lactate concentrations*

The concentration of lactate in the dialysate rose rapidly following the light exposure and onset of the ischaemic episode with a final concentration 4 h after the onset of ischaemia being around 300% higher than baseline (Figure 2). Administration of chlormethiazole (200 mg $kg^{-1}$, i.p.) 5 min after the onset of ischaemia attenuated this change although there was no difference in the first 30 min sample following the start of the ischaemic episode (Figure 2).

### *Effect of chlormethiazole on glutamate, aspartate, GABA and taurine concentrations in the dialysate*

The glutamate concentration in the dialysate rose nearly 30 fold within 2 h of the start of ischaemia and this increase was decreased by approximately 40% in the chlormethiazole-treated rats (Figure 3a). The ischaemia-induced rise in aspartate concentration was much smaller, but was also decreased by nearly 40% in chlormethiazole-injected rats (Figure 3b).

The concentration of GABA and taurine in the dialysate also rose significantly following the start of the ischaemic episode and these increases were attenuated by administration of chlormethiazole (Figures 3c and d). The peak concentration of all 4 compounds was observed to occur approximately 2 h after the onset of ischaemia (Figure 3).

### *Effect of chlormethiazole on serine, alanine and asparagine concentrations in the dialysate*

The concentrations of serine, alanine and asparagine amino acids increased in the dialysate following the onset of ischaemia with the concentration appearing to increase throughout the 4 h collection period (Figure 4). Chlormethiazole-treated animals had a significantly smaller increase in the concentration of these amino acids in the dialysate in each case (Figure 4).

### *Effect of hypoxia and chlormethiazole on glutamate release from cortical slices*

Incubation of cortical slices in an oxygenated buffer resulted in a steady and modest release of glutamate into the medium (Figure 5). When the slices were transferred to an hypoxic medium there was a marked increase in glutamate release, which was not affected by the presence of a high concentration (1 mM) of chlormethiazole in the buffer (Figure 5).

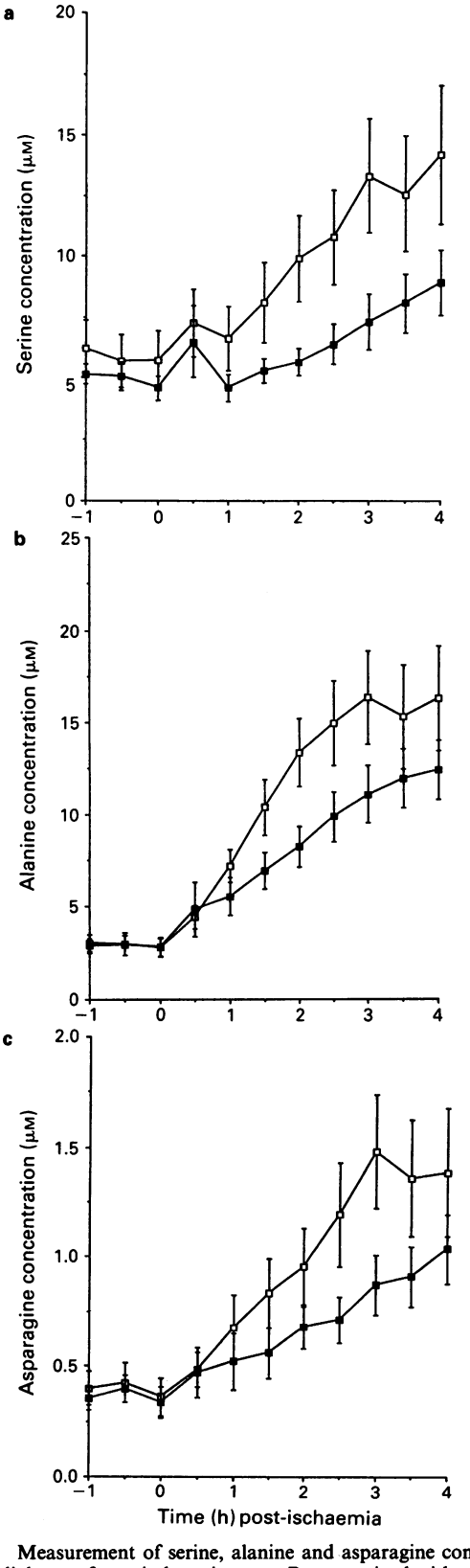


**Figure 4** Measurement of serine, alanine and asparagine concentration in dialysate from ischaemic rats. Rats received either chlormethiazole (200 mg $kg^{-1}$, i.p., $n = 10$; ■) or saline (i.p., $n = 10$; □) 5 min after induction of ischaemia. (a) Mean (± s.e.mean) serine concentration (μM) in dialysate. There was a significant effect of 'time' (ANOVA $F(10,180) = 15.64$, $P < 0.001$) and a significant 'drug' × 'time' interaction (ANOVA $F(10,180) = 2.93$, $P < 0.005$). (b) Mean (± s.e.mean) alanine concentration (μM) in dialysate. There was a significant effect of 'time' (ANOVA $F(10,180) = 41.80$, $P < 0.001$) and a significant 'drug' × 'time' interaction (ANOVA $F(10,180) = 2.44$, $P < 0.01$). (c) Mean (± s.e.mean) asparagine concentration (μM) in dialysate. There was a significant effect of 'time' (ANOVA $F(10,180) = 23.17$, $P < 0.001$) and a significant 'drug' × 'time' interaction (ANOVA $F(10,180) = 2.49$, $P < 0.01$).

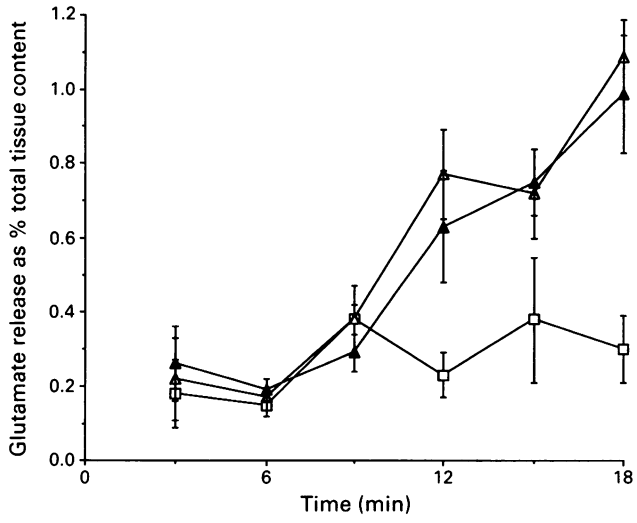


**Figure 5** The effect of hypoxia on glutamate release from cortical slices. Slices of cortex were incubated in oxygenated buffer. After 6 min one group was continued in oxygenated buffer (□) while two other groups were incubated with either buffer bubbled with $N_2$ (△) or $N_2$ buffer containing 1 mM chlormethiazole (▲). Results shown as glutamate release as % of total glutamate content ± s.e.mean.

## Discussion

The finding of Snape *et al.* (1993) that chlormethiazole is neuroprotective in the photochemical model of ischaemic cell death was confirmed in the current investigation (Figure 1), thereby allowing confidence in the data obtained when the drug was used to investigate changes in the extracellular neurotransmitter amino acid concentrations which occur following ischaemia.

Previous data from this laboratory (Snape *et al.*,1993) have indicated that neuronal damage occurs very rapidly after the onset of ischaemia in the photochemical model of stroke, the damage observed at 4 h being approximately 75% of the total damage present at 24 h, when damage is near maximal. This degree of damage is almost identical to that reported by Grome *et al.* (1988). It therefore seems reasonable to suppose that the major biochemical changes which lead to the observed pathology are also occurring in the first 4 h.

We previously demonstrated by use of *in vivo* microdialysis that there was a marked increase in the extracellular concentration of lactate following the induction of focal ischaemia using the photochemical model (Baldwin *et al.*, 1993c). This increase presumably indicated an increase in anaerobic metabolism and was consistent with findings in other ischaemia models (Kuhr & Korf, 1988). We also found a clear increase in the efflux of the excitatory amino acid, glutamate (Baldwin *et al.*, 1993c), in agreement with the findings of others using different models of focal and global ischaemia (Benveniste *et al.*, 1984; Globus *et al.*, 1988; Butcher *et al.*, 1990). The current study has confirmed these observations and also found a marked increase in the excitatory amino acid, aspartate. All these data could therefore be used to suggest a key role for the excitatory amino acids in the development of the neurodegenerative changes which follow an ischaemic episode in the model, as has been proposed in others (Rothman & Olney, 1986; Choi *et al.*, 1988; Meldrum, 1990).

The efflux of the inhibitory amino acid, GABA, was also found to increase at the same time (Baldwin *et al.*, 1993c; this paper) as did the concentration of taurine, in agreement with studies in other ischaemic models (Benveniste *et al.*, 1984; Butcher *et al.*, 1990; Lekieffre *et al.*, 1992). It remains uncertain as to whether taurine is an inhibitory neurotransmitter. However, there is certainly evidence for an inhibitory neuromodulatory role in the brain, possibly through an action at GABA receptors (see Oja & Kontro, 1990) and taurine has also been shown to assist in the recovery of neuronal function following hypoxia (Schurr *et al.*, 1987). There is also evidence that taurine is an important factor in cerebral osmoregulation (Kimelberg *et al.*, 1990; Lehmann, 1990; Puka *et al.*, 1991). It could be postulated therefore that the release of taurine may be indirectly linked to mechanisms involved in limiting the development of the infarct, particularly as the infarct in this model is associated with a significant degree of oedema (Dietrich *et al.*,1987a; Laursen *et al.*, 1991; Snape *et al.*, 1993; Green & Cross, unpublished observations).

Chlormethiazole administration decreased the efflux of the excitatory amino acids in the infarct region, which would provide an attractive explanation for its neuroprotective action. However, it was also observed to decrease the efflux of the inhibitory amino acids, GABA and taurine. Furthermore the efflux of the amino acids not thought to have a neurotransmitter role in the brain (serine, alanine and asparagine) was decreased by a similar amount.

Since the dialysis probe was placed in the centre of the infarct region, directly under the illumination source, the changes in dialysate concentration reflect biochemical changes in the core of the infarct. This is important as chlormethiazole decreases the spread of damage (Snape *et al.*, 1993; this paper). We therefore have confidence that the neurochemical changes discussed above reflect neurochemical differences in the infarct region, not the fact that less tissue has been damaged in the region of the probe.

Following occlusion of the middle cerebral artery in rats, the rise in extracellular glutamate and aspartate peaked within the first hour post-ischaemia (Butcher *et al.*,1990). After transient forebrain ischaemia, the extracellular concentrations of these amino acids rose even more rapidly (Benveniste *et al.*, 1984). In both models, extracellular levels of 'metabolic' amino acids (such as alanine and serine) increased relatively slowly and reached peak concentrations which were considerably lower than those of the neuroactive amino acids. In the present study the extracellular concentrations of neuroactive amino acids also rose to a greater extent than the metabolic amino acids; however, peak concentrations were not reached until at least 2 h after the onset of ischaemia.

What seems probable therefore is that the rise in the extracellular concentrations of all amino acids reflects the breakdown of damaged cells in the ischaemic area. The attenuation of this rise in the chlormethiazole-treated rats therefore relates to the neuroprotective effect of the drug, and is a reflection of fewer cells being damaged, rather than an inhibition of the ischaemia-induced release of glutamate and aspartate. Consistent with this contention was the observation that chlormethiazole, even at a high (1 mM) concentration, did not inhibit the hypoxia-induced release of glutamate *in vitro*.

Further support for these proposals comes from the fact that chlormethiazole administration afforded around 30% protection measured histologically and also decreased the efflux of amino acids into the extracellular space in the infarct area by approximately the same amount.

It should also be noted that the increase in extracellular lactate concentrations was diminished in the chlormethiazole-treated rats. However, there was no difference between control and chlormethiazole-treated rats in the lactate concentration in the dialysate collected over the first 30 min following the ischaemic episode. This suggests that chlormethiazole did not affect this immediate effect of ischaemia. In this model, damage occurs rapidly (Grome *et al.*, 1988) with Snape *et al.* (1993) observing that approximately 50% of the area of oedema and extravasation measured at 24 h was apparent 30 min after the infarct, a time when chlormethiazole had no effect on lactate concentrations.

Again it is likely that the difference in extracellular lactate after this time relates to neuroprotection. However, what cannot be ruled out at present is that a difference was not observed in lactate concentration between control and experimental groups in the first 30 min because chlormethiazole was either altering blood flow or routes of energy metabolism.

As stated earlier glutamate has often been claimed to play a key role in the pathological processes associated with neurodegeneration following an ischaemic episode. We have previously questioned its involvement in the photochemical model, based on our finding that dizocilpine and NBQX were not neuroprotective (Baldwin *et al.*, 1993a). The current study does not suggest that this opinion is unreasonable. Chlormethiazole did decrease the rise in extracellular glutamate which followed the ischaemic insult. However, it failed to have any effect on the release of this neurotransmitter from brain slices exposed to an hypoxic insult. We are therefore forced to conclude that the chlormethiazole-induced effect on glutamate *in vivo* is a reflection of its neuroprotective action which prevents cell death and concomitant glutamate release rather than an inhibition of glutamate release thereby affording neuroprotection. In this model of ischaemia therefore increased extracellular glutamate concentrations appear to be a consequence of neurodegeneration rather than the cause.

In conclusion, the *in vivo* microdialysis data described in this study have provided neurochemical data which support the histological evidence for the neuroprotective action of chlormethiazole in the photochemical model (Snape *et al.*, 1993; this paper). Although the results do not give any plausible explanation for the mechanism(s) by which chlormethiazole produces its protective effect, they do indicate that a specific action on excitatory amino acid release is unlikely.

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# Prevention by specific chemical classes of $\alpha_1$-adrenoceptor antagonists of veratrine-contractures in rat left atria independently of $\alpha_1$-adrenoceptor blockade

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**1** The putative direct protective effects of a series of chemically diverse $\alpha_1$-adrenoceptor antagonists against veratrine alkaloid-induced tetanic contractures in rat isolated left atria have been investigated.

**2** Atria were mounted in organ baths containing normal, oxygenated physiological salt solution (20 ml, pH 7.4), for isometric tension recording. Atria were electrically driven at 4 Hz and were maintained at 34°C. Veratrine (100 $\mu$g ml$^{-1}$) was applied to the atria to elicit tetanic (diastolic) contracture.

**3** Concentration-dependent protective effects against veratrine-contractures, in the absence of negative inotropic responses, were observed with the quinazoline congeners, prazosin and doxazosin and with the benzodioxane-related compounds, WB 4101 and its thio analogue, benoxathian. $IC_{50}$ concentrations and apparent Hill coefficients of all four drugs ranged from 0.27 to 0.93 $\mu$M, and from 0.86 to 1.09, respectively, and are consistent with interaction at a single site.

**4** In contrast, no protective activity versus veratrine-contractures was observed with corynanthine, 5-methyl-urapidil, phenoxybenzamine, phentolamine or chloroethylclonidine (10 $\mu$M).

**5** Contractures were prevented by prazosin at concentrations 2–3 log units higher than those which antagonized methoxamine-evoked inotropic responses. In addition, concommitant $\alpha_1$-adrenoceptor occupancy by high concentrations of methoxamine (100 $\mu$M), phentolamine (10 $\mu$M, inactive *per se* in preventing contracture), or both drugs together, failed, in each case, to modify significantly the protective effects of prazosin or WB 4101 against veratrine-contractures.

**6** Our findings demonstrate that $\alpha_1$-adrenoceptor antagonists which prevent veratrine-contractures belong to specific chemical classes of the quinazoline- and benzodioxane-type. The mechanism by which these drugs afford protection is apparently independent of an interaction with defined $\alpha_1$-adrenoceptors.

**Keywords:** $\alpha_1$-Adrenoceptor antagonists; veratrine; tetanic contracture; $\alpha_1$-adrenoceptors; sodium channel

## Introduction

Impaired $Na^+$ channel inactivation is a potentially important mechanism which can lead to myocardial contractile failure under conditions of energy deprivation, such as ischaemia (Kohlhardt *et al.*, 1989; Undrovinas *et al.*, 1992; Ver Donck *et al.*, 1993). Veratrine-alkaloid intoxication of isolated cardiac tissues is a useful model of contractile failure, since veratridine, the active veratrine constituent, impairs $Na^+$ channel inactivation, which ultimately leads to $Ca^{2+}$ loading and tetanic contracture (Pang & Sperelakis, 1982; Honerjäger, 1983) by well-described mechanisms (Leibowitz *et al.*, 1986; Ver Donck & Borgers, 1991).

In a recent study, in which veratrine was employed to evoke tetanic contractures of rat isolated left atria, we discovered that the $\alpha_1$-adrenoceptor antagonists, prazosin and WB 4101, effectively prevented contracture development (Le Grand *et al.*, 1993). However, our findings left undetermined whether the protective effects of prazosin and WB 4101 were mediated by interaction with atrial $\alpha_1$-adrenoceptors and whether other $\alpha_1$-adrenoceptor antagonists share this previously undescribed property.

The objectives of the present investigation were therefore to determine (i) whether $\alpha_1$-adrenoceptor antagonists in general afford protection against veratrine-contractures, and (ii) whether or not this activity is mediated by $\alpha_1$-adrenoceptor blockade.

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## Methods

### *Isolation of atria*

In accordance with French Law and the local ethical committee guidelines for animal research, male Wistar rats (360–420 g, OFA, Iffa Credo, France) were anaesthetized with sodium pentobarbitone (50 mg kg$^{-1}$, i.p.) and treated with heparin (500 iu, i.v.). The chest was opened, the left atrium rapidly excised and carefully mounted vertically in an organ bath containing 20 ml of Krebs solution of the following composition (mM): NaCl 119.0, KCl 5.6, $MgSO_4$ 1.17, $CaCl_2$ 2.1, $NaH_2PO_4$ 1.0, $NaHCO_3$ 25.0, glucose 10.0, pH 7.4. The bath was continuously maintained at 34°C and the solution gassed with a mixture of 95% $O_2$ and 5% $CO_2$. The atria were stimulated electrically via two electrodes (Grass stimulator, S48) at a frequency of 4 Hz (impulse duration 1 ms, 2 × threshold current). The force of contraction was measured with a transducer (Statham, UC2) and registered on a pen recorder (Gould Instruments, RS 3400); 1 g pretension was applied to each atrium.

### *Positive inotropic responses*

After a 30 min equilibration period in the presence of atenolol (10 $\mu$M), a single concentration of $\alpha_1$-adrenoceptor antagonist or vehicle was injected into the organ bath; 15 min later, cumulative concentrations of the $\alpha_1$-adrenoceptor agonist, methoxamine, were sequentially injected at intervals of 15 min and the peak developed systolic tension recorded for each methoxamine concentration.

The agonist $pD_2$ value was calculated by taking the negative logarithm of the methoxamine concentration re-

quired to induce 50% of the maximum response ($EC_{50}$); 95% confidence intervals are given in parentheses.

### *Veratrine-induced contractures*

After a 30 min equilibration period, a single concentration of drug or solvent was injected into the organ bath and 15 min later, veratrine (100 μg ml$^{-1}$) was added. Systolic isometric tension development was measured before drug or solvent injection and just before the addition of veratrine in order to detect any positive or negative inotropic drug or solvent effects. The maximum developed diastolic tension of veratrine-induced contracture was measured irrespective of time. One contracture was elicited per atrium.

### *Drugs*

Veratrine HCl, methoxamine HCl, atenolol, prazosin HCl, and phentolamine HCl were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.); 5-methyl-urapidil, chloroethylclonidine 2HCl, corynanthine HCl, phenoxybenzamine HCl, benoxathian HCl and WB 4101 (2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane) HCl from Research Biochemicals Inc. (Natick, MA, U.S.A.) and were dissolved in distilled water. Ultrasonication was required to dissolve prazosin. Doxazosin mesylate was a gift from the Pfizer Corporation (Brussels, Belgium).

### *Data analysis*

Values are expressed as mean ± s.e.mean. The drug concentrations necessary to inhibit by 50% active (systolic) tension development ($ATD_{50}$) and veratrine-induced contracture ($VIC_{50}$) were calculated graphically. Curve fitting was performed using the Marquardt algorithm (Marquardt, 1963) which gave $IC_{50}$ values with 95% confidence intervals and the corresponding apparent Hill coefficients (nH). Data homogeneity was verified by variance analysis (ANOVA) which included a Dunnett's test. Any $P$ value lower than 0.05 was considered significant.

## Results

### *Effects of a range of $\alpha_1$-adrenoceptor antagonists on veratrine-induced contracture*

After the atria underwent a 30 min equilibration period, veratrine (100 μg ml$^{-1}$) induced a transient initial positive inotropic response followed by sustained increases in diastolic tension (tetanic contracture; Figure 1a). A series of chemically diverse $\alpha_1$-adrenoceptor antagonists was then examined for potential protective activity against veratrine-contractures. 5-Methyl-urapidil (Figure 1b), corynanthine, phenoxybenzamine, phentolamine and chloroethylclonidine, were totally ineffective in preventing veratrine-contracture, even at high concentrations (10 μM, Table 1). On the other hand, the quinazoline congeners, prazosin and doxazosin (Figure 1c), and the benzodioxane-related $\alpha_1$-adrenoceptor antagonists, WB 4101 and benoxathian, potently and concentration-dependently suppressed the development of tetanic contracture. The range of $IC_{50}$ and nH values obtained for the latter four drugs versus veratrine was 0.27–0.93 μM, and 0.86–1.09, respectively (Table 1).

In order to examine whether the protective effect of prazosin against veratrine is species-specific, similar experiments were conducted in both guinea-pig and rabbit isolated, electrically stimulated (4 Hz) left atria.

Prazosin (0.1–10 μM) produced concentration-dependent protection against veratrine contractures in both guinea-pig ($IC_{50}$ 2.80 μM [1.23–5.29]; nH 1.11; $n = 22$) and rabbit atria ($IC_{50}$ 1.96 μM [1.19–7.60]; nH 0.55; $n = 21$), which was less potent than that observed in rat atria. Veratrine (100 μg

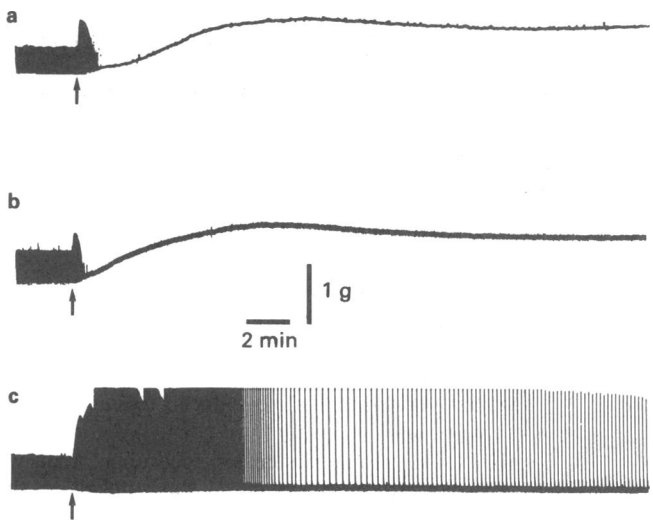


**Figure 1** Typical isometric tension recordings of veratrine-contractures in rat isolated left atria. (a) Control contracture; (b) same as (a) but with 5-methyl-urapidil (10 μM) added 15 min before veratrine; (c) same as (a) but with doxazosin (10 μM) added 15 min before veratrine (peak systolic tension cut off at full scale). Arrows indicate addition of veratrine (100 μg ml$^{-1}$).

**Table 1** Drug concentrations required to reduce by 50% active tension development ($ATD_{50}$) and veratrine-induced contracture ($VIC_{50}$)

| *Compound* | *$ATD_{50}$* (μM) | *$VIC_{50}$* (μM) | *nH* | n |
|---|---|---|---|---|
| Prazosin | >10 | 0.62 (0.08–1.0) | 0.91 | 36 |
| Prazosin + Methoxamine (100 μM) | >10 | 0.68 (0.05–1.41) | 0.90 | 20 |
| Doxazosin | >10 | 0.27 (0.06–0.8) | 1.09 | 24 |
| WB 4101 | >10 | 0.93 (0.17–2.2) | 0.93 | 24 |
| Benoxathian | >10 | 0.84 (0.01–4.2) | 0.86 | 28 |
| 5-methylurapidil | >10 | >10 | – | 6 |
| Corynanthine | >10 | >10 | – | 7 |
| Phenoxybenzamine | >10 | >10 | – | 6 |
| Phentolamine | >10 | >10 | – | 13 |
| Chloroethylclonidine | >10 | >10 | – | 6 |

95% confidence intervals are indicated in parentheses.
Hill coefficients (nH) correspond to concentration-inhibition curves generated versus VIC.
*n* indicates the number of atria studied.

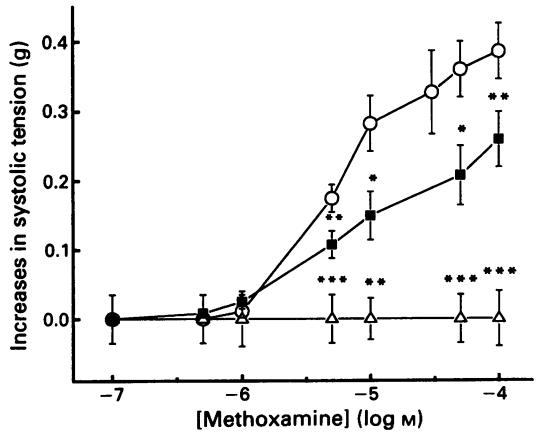


**Figure 2** Concentration-response relationships for methoxamine-induced increases in systolic tension and inhibition by prazosin. Effect of methoxamine alone (○, $n = 6$); methoxamine + prazosin 100 pM (■); methoxamine + prazosin 10 nM (△). All experiments were conducted in the presence of atenolol (10 μM) to prevent $\beta_1$-adrenoceptor stimulation. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. Data are expressed as mean ± s.e.mean.

$ml^{-1}$)-induced contractures were less marked in rabbit atria than those observed in rat and guinea-pig.

### *Functional evidence for the presence of $\alpha_1$-adrenoceptors in rat left atria*

The relatively selective $\alpha_1$-adrenoceptor agonist, methoxamine (0.1–100 μM), induced concentration-dependent positive inotropic responses, increasing systolic tension development by 71 ± 2% ($P<0.001$, $n=6$) at the highest concentration studied ($pD_2$ 5.26 [4.45–5.54]; Figure 2). All experiments were conducted in the presence of atenolol (10 μM) to prevent $\beta_1$-adrenoceptor activation. Concentrations of methoxamine higher than 100 μM were found to intoxicate the atria (arrhythmias and diastolic contracture occurred frequently), and were therefore not employed.

Methoxamine-induced positive inotropic responses were potently and concentration-dependently antagonized by relatively low concentrations of prazosin (100 pM–10 nM; Figure 2) although in a non-competitive manner. It is of note that these concentrations of prazosin are considerably lower than those which were required to prevent veratrine-contractures ($IC_{50}$ 0.62 μM, Table 1).

### *Involvement of $\alpha_1$-adrenoceptors in veratrine-contractures and the protective effect of prazosin and WB 4101*

The effects of $\alpha_1$-adrenoceptor stimulation on the development of veratrine-contractures are shown in Table 2. Methoxamine (100 μM) in the presence or absence of $\beta_1$-adrenoceptor blockade with atenolol (10 μM), had no significant effect on the time-course or the peak developed tension of tetanic contracture. Atenolol (10 μM) *per se* had no effect upon veratrine-induced contractures (Table 2).

Phentolamine (1 μM) fully antagonized methoxamine-mediated positive inotropic responses ($P<0.001$, $n=6$, not shown), but was ineffective *per se* at concentrations as high as 10 μM against veratrine-induced contracture (Table 2, Figure 3).

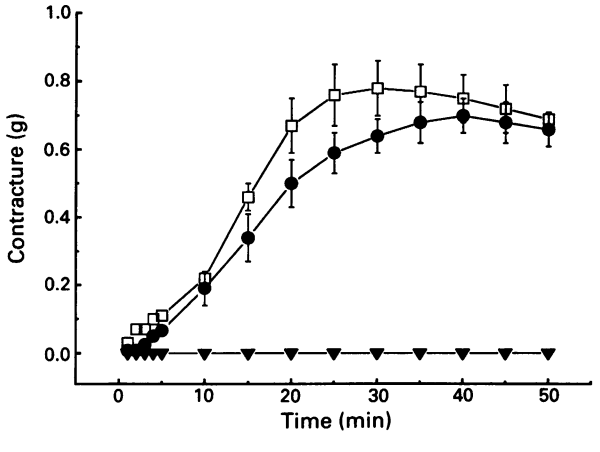


**Figure 3** Effects of $\alpha_1$-adrenoceptor occupancy on the time course of veratrine-(100 μg $ml^{-1}$)-induced tetanic contracture in rat isolated atria and protection against contracture by prazosin. Effects of methoxamine (100 μM; □, $n=6$); methoxamine (100 μM) + phentolamine (10 μM; ●, $n=6$); methoxamine (100 μM) + phentolamine (10 μM) + prazosin (10 μM; ▲, $n=6$). Values for prazosin (10 μM) + phentolamine (10 μM; $n=6$) were superimposed upon those for prazosin + methoxamine + phentolamine (not shown for clarity). Control curve or control + phentolamine (10 μM; not shown for clarity), were not statistically significantly different at any time point from that of methoxamine (100 μM) alone, or methoxamine (100 μM) and phentolamine (10 μM) together. Contracture from 10 min onwards for methoxamine + phentolamine + prazosin are statistically significantly different ($P<0.001$) from corresponding control, methoxamine, phentolamine or methoxamine + phentolamine. Data are expressed as mean ± s.e.mean.

**Table 2** Maximum generated tension of tetanic contractures induced by veratrine

| *Compound* | *Contracture max* (g) | n | P |
|---|---|---|---|
| Control | 0.87 ± 0.02 | 20 | |
| Prazosin (10 μM) | 0.0 | 6 | <0.001 |
| Atenolol (10 μM) | 0.83 ± 0.09 | 8 | NS[a] |
| Methoxamine (100 μM) | 0.85 ± 0.02 | 6 | NS[a] |
| Methoxamine (100 μM) + atenolol (10 μM) | 0.75 ± 0.07 | 8 | NS |

NS indicates not significantly different from control or from methoxamine + atenolol[a]. *n* indicates the number of atria studied.

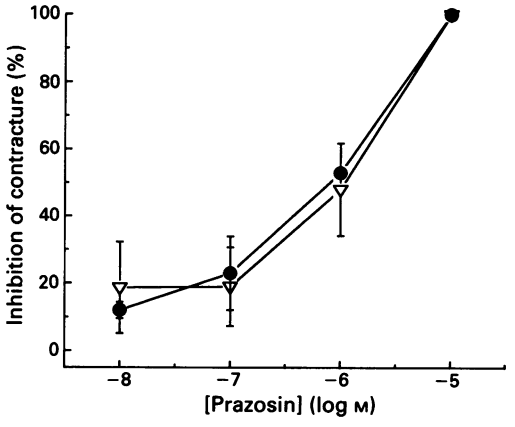


**Figure 4** Concentration-response relationships for the protective effects of prazosin versus veratrine-induced tetanic contracture in the presence or absence of $\alpha_1$-adrenoceptor stimulation with methoxamine. Prazosin (●); prazosin + methoxamine (100 μM; Δ). No statistically significant differences were found between any of the corresponding values. Data are expressed as mean ± s.e.mean.

Prazosin (10 μM) fully prevented tetanic contracture, even in the presence of phentolamine (10 μM), or phentolamine (10 μM) and methoxamine (100 μM; Figure 3). Furthermore, the prazosin inhibition curve against veratrine-contracture, was not significantly modified by methoxamine (100 μM; Table 1; Figure 4). Similar results were obtained with WB 4101 (data not shown).

## Discussion

The aims of the present study were to determine (i) whether $\alpha_1$-adrenoceptor antagonists in general afford protection against veratrine-contractures, and (ii) whether or not this activity is mediated by blockade of $\alpha_1$-adrenoceptors. Veratrine intoxicates isolated cardiac tissues by impairing $Na^+$ channel inactivation, leading to $Ca^{2+}$ loading and contractile failure (Ver Donck *et al.*, 1986; Patmore *et al.*, 1989; Wermelskirchen *et al.*, 1991). Although veratrine is an alkaloid mixture, the mechanism of action of the active constituent, veratridine, is well characterized. Veratridine binds to the open state of the $Na^+$ channel, thereby markedly enhancing the open probability (Honerjäger, 1983; Leibowitz *et al.*, 1986), and evokes sustained, reversible increases in diastolic tension (tetanic contracture) of isolated atria (Le Grand *et al.*, 1993).

### *Effects of a series of chemically diverse $\alpha_1$-adrenoceptor antagonists*

We first investigated whether $\alpha_1$-adrenoceptor antagonists in general afford protection versus veratrine-contractures. Con-

centration-dependent protective effects in the absence of negative inotropic responses were found with the quinazoline congeners, prazosin and doxazosin, and with the benzodioxane-related compounds, WB 4101 and its thio-analogue, benoxathian (concentration range 10 nM–10 μM for all 4 drugs). nH values for the 4 drugs were in the range 0.86–1.09, and are therefore compatible with a single site of action. Conversely, no protective activity at all was noted with representative $\alpha_1$-adrenoceptor antagonists of other chemical classes, including corynanthine, 5-methyl-urapidil, phenoxybenzamine, phentolamine and chloroethylclonidine, even at high concentrations (10 μM). This finding clearly illustrates that, although at relatively high concentrations, only $\alpha_1$-adrenoceptor blocking drugs of the quinazoline or benzodioxane-type afford protection in the present experimental model. That selective blockade of one of the $\alpha_{1A}$ or $\alpha_{1B}$ subtypes could mediate protection against veratrine-contractures can be definitively excluded, since the $\alpha_{1A}$-subtype selective antagonists, WB 4101 and 5-methyl-urapidil (Hanft & Gross, 1989) were respectively protective and inactive; and the $\alpha_{1B}$-subtype selective antagonist, chloroethylclonidine (Minneman *et al.*, 1988) was ineffective. A serious doubt is thus raised as to whether the protective effect of the active compounds investigated is mediated by interaction with $\alpha_1$-adrenoceptors.

### *Involvement of atrial $\alpha_1$-adrenoceptors*

We then sought to determine whether the protective effects of $\alpha_1$-adrenoceptor antagonists are mediated by interaction with atrial $\alpha_1$-adrenoceptors. Functional $\alpha_1$-adrenoceptors were demonstrated in rat isolated left atria by use of the relatively selective $\alpha_1$-adrenoceptor agonist, methoxamine, which elicited concentration-dependent inotropic responses, in agreement with previous reports (Nakashima & Hagino, 1972; Nawrath, 1989). Methoxamine-evoked positive inotropic responses were concentration-dependently inhibited by prazosin and were abolished by concentrations as low as 10 nM. This concords with the high affinity values reported for prazosin for rat atrial $\alpha_1$-adrenoceptors ($K_D$ 0.24 nM, Chess-Williams & Broadley, 1987).

Veratrine-contractures, however, remained unaffected by high methoxamine concentrations. In addition, methoxamine failed to modify either the $IC_{50}$ or nH values obtained for prazosin against veratrine-contractures. Moreover, prazosin fully prevented tetanic contracture development even in the presence of high concentrations of phentolamine (which were inactive *per se*) or methoxamine, or both.

These results indicate that the concentrations of prazosin required to inhibit veratrine-contractures are 2–3 log units higher than those that effectively antagonize positive inotropic responses elicited by $\alpha_1$-adrenoceptor stimulation. In addition, prazosin prevented veratrine-contractures in a similar manner in rat, rabbit and guinea-pig isolated left atria, thereby excluding a species-specific effect.

Collectively, our findings strongly suggest that prazosin, doxazosin, WB 4101 and benoxathian elicit protective activity against veratrine-contractures by a mechanism which is independent of blockade of defined $\alpha_1$-adrenoceptors.

### *Possible mechanisms involved in the protective effects of prazosin, doxazosin, WB 4101 and benoxathian*

Impaired $Na^+$ channel inactivation, and $Ca^{2+}$ entry via voltage-operated $Ca^{2+}$ channels and/or $Na^+$-$Ca^{2+}$ exchange appear to be the main mechanisms by which veratrine elicits tetanic contracture (Pang & Sperelakis, 1982; Honerjäger, 1983), and therefore represent the main possible targets for the actions of prazosin, doxazosin, WB 4101 and benoxathian. Blockade of $Ca^{2+}$ entry via voltage-operated L-type $Ca^{2+}$ channels can be excluded, since it has previously been shown that various $Ca^{2+}$ channel blockers afford little or no protection against veratrine (Wermelskirchen *et al.*, 1991; Le Grand *et al.*, 1993). Voltage-operated T-type channels do not appear to be implicated either, since NiCl in concentrations as high as 100 μM failed to afford protection against veratrine (B. Le Grand *et al.*, unpublished results). However, blockade of $Ca^{2+}$ entry occurring via $Na^+$-$Ca^{2+}$ exchange by these $\alpha_1$-adrenoceptor antagonists cannot be ruled out at present.

Several studies (Northover, 1983; Dukes & Vaughan Williams, 1984; Rosen *et al.*, 1984) have shown that certain $\alpha_1$-adrenoceptor antagonists, including phenoxybenzamine, phentolamine, prazosin and WB 4101, reduce the maximum upstroke velocity ($V_{max}$) of the action potential, possibly suggestive of inhibitory activity upon $Na^+$ channel function. However, since phenoxybenzamine and phentolamine were ineffective against veratrine-contractures, interference with $V_{max}$ appears to be an unlikely explanation, particularly since compounds which strongly reduce $V_{max}$, such as class 1 anti-arrhythmic agents, fail to afford protection against veratrine intoxication (Le Grand *et al.*, 1993). Since tetrodotoxin (TTX) but not the class 1 anti-arrhythmics, quinidine and lignocaine, effectively prevented veratrine-contractures (Le Grand *et al.*, 1993), our previous proposal that prazosin interacts with a TTX-sensitive, quinidine-insensitive site on the $Na^+$ channel merits further investigation. It is noteworthy that both TTX and the novel cytoprotective agent, R 56865 (Ver Donck & Borgers, 1991), normalize impaired $Na^+$ channel inactivation kinetics (Carmeliet & Tytgat, 1991).

In conclusion, our observations show that veratrine-contractures of rat isolated left atria are prevented by $\alpha_1$-adrenoceptor antagonists of the quinazoline and benzodioxane-type, but not by $\alpha_1$-adrenoceptor antagonists from other chemical classes. The protective mechanism of action of prazosin, doxazosin, WB 4101 and benoxathian is apparently unrelated to an interaction with defined $\alpha_1$-adrenoceptors.

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# Attenuated renal response to moxonidine and rilmenidine in one kidney-one clip hypertensive rats

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**1** $I_1$ non-adrenoceptor, imidazoline receptor agonists, such as moxonidine, increase urine flow rate and sodium excretion following infusion into the renal artery. The functions of these agonists in genetic and acquired models of hypertension have not been determined.

**2** We therefore studied the renal effects of two known non-adrenoceptor, imidazoline receptor agonists, rilmenidine and moxonidine, in 1K-1C hypertensive and 1K-sham normotensive rats. Rilmenidine (0, 3, 10, 30 nmol $kg^{-1}$ $min^{-1}$) or moxonidine (0, 1, 3, 10 nmol $kg^{-1}$ $min^{-1}$) was infused directly into the renal artery (30 gauge needle) of 1K-sham normotensive and 1K-1C hypertensive rats.

**3** In 1K-sham normotensive rats, rilmenidine and moxonidine produced dose related increases in urine flow rate, sodium excretion and osmolar clearance. Both rilmenidine and moxonidine failed to increase urine flow rate, sodium excretion and osmolar clearance in 1K-1C hypertensive rats to the same extent as in 1K-sham animals. At comparable doses, rilmenidine had no effect, while moxonidine (3 and 10 nmol $kg^{-1}$ $min^{-1}$) did result in a small increase in urine volume and osmolar clearance which was less than that observed in the 1K sham control animals.

**4** These studies indicate that the renal effects of non-adrenoceptor, imidazoline receptor stimulation are diminished in 1K-1C hypertensive rats compared with 1K-sham normotensive rats. Whether this decrease in activity of the natriuretic non-adrenoceptor, imidazoline receptors contributes to the increase in blood pressure in the 1K-1C acquired model of hypertension remains to be determined.

**Keywords:** Rilmenidine; moxonidine; one kidney-one clip hypertension; non-adrenoceptor, imidazoline receptors

## Introduction

In acquired forms of hypertension, renal α-adrenoceptor density has been found to be unchanged or decreased (Yamada *et al.*, 1980; Fukuda *et al.*, 1983; Saiz *et al.*, 1987; Michel *et al.*, 1989b; Wilson, 1991), whereas in spontaneously hypertensive rats, the density of renal $\alpha_2$-adrenoceptors has been found to be elevated as compared to Wistar-Kyoto rats (Pettinger *et al.*, 1982; Saiz *et al.*, 1987; Sanchez *et al.*, 1989; Michel *et al.*, 1990a). Furthermore, previous studies in our laboratory indicated that the renal response to $\alpha_2$-adrenoceptor agonists such as clonidine was decreased in spontaneously hypertensive rats (Li *et al.*, 1992) but not in one kidney, one clip (1K-1C) hypertensive rats (Li & Smyth, 1994). The diuretic and natriuretic effects of $\alpha_2$-adrenoceptor stimulation have been shown to be mediated by an inhibition of the renal action of vasopressin (Blandford & Smyth, 1990; Gellai, 1990). Thus, the decreased renal response to a V2-vasopressin receptor antagonist in spontaneously hypertensive rats but not 1K-1C hypertensive rats (Li & Smyth, 1993) was consistent with the decreased response to $\alpha_2$-adrenoceptor stimulation in this model of genetic hypertension.

Recent studies in our laboratory have proposed that the effects of two purported $\alpha_2$-adrenoceptor agonists, clonidine and 2,6-dimethyl clonidine (2,6-DMC), may in fact be mediated by two $\alpha_2$-adrenoceptor subtypes or two distinct receptors, $\alpha_2$-adrenoceptors and non-adrenoceptor, imidazoline receptors (Smyth *et al.*, 1992a). Whereas 2,6-DMC caused an increase in urine flow rate which was secondary to an increase in osmolar clearance, clonidine resulted in an increase in urine flow rate which was secondary to an increase in free water clearance (Smyth *et al.*, 1992a). Furthermore, we have recently found that the effects of 2,6-DMC but not clonidine were significantly attenuated in rats with 1K-1C hypertension (Li & Smyth, 1994) and there was a decrease in [$^3$H]-idazoxan binding sites in kidneys from 1K-1C hypertensive rats. The renal actions of 2,6-DMC can be antagonized by idazoxan (Smyth & Li, 1991) which would be consistent with those effects being mediated by the non-adrenoceptor, imidazoline receptor (Feldman *et al.*, 1990; Gomez *et al.*, 1991; Allan *et al.*, 1993). Nevertheless, it has not been determined as to whether the decreased response to 2,6-DMC was due to a decreased activity at the $\alpha_2$-adrenoceptor or the non-adrenoceptor, imidazoline receptor.

In the present study, we determined whether or not, in 1K-1C hypertensive rats, a decreased response was found with non-adrenoceptor, imidazoline receptor agonists in general. Two agonists (rilmenidine and moxonidine) which have been shown to display a much greater selectivity and affinity for the $I_1$ non-adrenoceptor, imidazoline receptor over the $\alpha_2$-adrenoceptor (Ernsberger *et al.*, 1992), were used to investigate further the non-adrenoceptor, imidazoline receptor in 1K-1C hypertensive and 1K-sham normotensive rats.

## Methods

### *Experimental animals*

The standard procedures have been described previously (Blandford & Smyth, 1988; 1990). Male Sprague-Dawley rats (100–125 g) were obtained from the University of Manitoba (Charles River Breeding Stock) and cared for according to regional animal care standards protocol. Animals were housed at 22°C with an environmental humidity maintained at 50% with a light/dark cycle from 07 h 00 min to 19 h 00 min. Rats were fed Purina rat chow and received tap water for drinking.

The animals were separated into two groups. In the first group (1K-1C), rats were anaesthetized with ether and the kidneys exposed by an abdominal incision. A silver clip

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(0.254 mm) was placed around the left renal artery and the right kidney was removed. In the second group (1K-sham), animals underwent the identical procedure except, when the left renal artery was separated from the renal vein, no silver clip was placed around the artery and the right kidney was removed. The animals were kept for 12 to 16 days after surgery and subsequently underwent the protocol described below.

### *Renal effects of rilmenidine or moxonidine*

On the day of the experiment, the rats were anaesthetized with pentobarbitone (BDH Chemicals, Poole, England; 50 mg $kg^{-1}$, i.p.). Additional anaesthetic was administered in a bolus dose of 3 mg $kg^{-1}$ i.v. as needed. The rats were placed on a thermostatically controlled heating blanket. The rectal thermometer was connected to a Harvard Animal Blanket Control Unit which was used to maintain body temperature at 37.5°C. The trachea was cannulated with polethylene tubing (PE-240) and the animal was allowed to breathe spontaneously. The left carotid artery was cannulated with polyethylene tubing (PE-50) and blood pressure was measured with a Statham pressure transducer (model P23Dc) connected to a Grass Polygraph Model V. The left jugular vein was cannulated (PE-160) and connected to an infusion pump (Syringe pump model 355) for the infusion of saline. The left kidney was exposed by a flank incision and the left ureter was cannulated (PE-50) for the collection of urine. A 30 guage stainless steel needle was inserted into the aorta and advanced into the origin of the left renal artery. A single drop of glue (Superglue, LePage's Limited) was used to secure the base of the needle to surrounding tissue. This was connected by Tygon Tubing (size 0.25 mm) to an infusion pump (Harvard infusion/withdrawal pump model 600–900) for infusion of either saline or study drug at 3.4 μl $min^{-1}$.

Following the surgical procedure, saline was administered by intravenous infusion (97 μl $min^{-1}$). The animals were allowed to stabilize for a 45 min period. After the stabilization period, a 15 min control urine collection was obtained. This was followed by infusion of saline (vehicle), rilmenidine (3, 10 or 30 nmol $kg^{-1}$ $min^{-1}$) or moxonidine (1, 3 or 10 nmol $kg^{-1}$ $min^{-1}$) directly into the renal artery for the duration of the experiment at 3.4 μl $min^{-1}$. During this infusion, two additional urine collections of 15 min and 45 min were obtained into preweighed tubes.

The sodium concentration in plasma and urine were determined with a Beckman KLiNa Flame Photometer. Creatinine concentration was determined with a Beckman Creatinine Analyzer Model 2. The urine and plasma osmolality was analysed with a MicroOsmette (Precision Systems). Urine volume was determined gravimetrically.

### *Data analysis*

All data are presented as the mean ± the standard error of the mean (s.e.mean). Statistical analysis was performed with a repeated-measures ANOVA using SAS System Version 6.07. Significant interactions were further analyzed with Least Squares Means Difference Test. Baseline values (first urine collection) prior to the administration of the saline vehicle or study drugs (moxonidine and rilmenidine) were compared and presented in table format. The effects of the saline vehicle and study drugs were expressed graphically as the absolute change from the first to final urine collection period. This allowed the determination of the magnitude of the changes for each variable within the different groups. Each group comprised 5 to 8 animals. In the figures, *denotes $P<0.05$ between groups (1K vs 1K-1C) receiving the same drug infusion rate and #denotes $P<0.05$ as compared with the control within the same group receiving the vehicle infusion.

### *Drugs*

Moxonidine (supplied by Beiersdorf, AG, Hamburg, Germany) and rilmenidine (supplied by I.R.I. Servier, France) were used.

## Results

### *Effect of intrarenal infusion of rilmenidine*

Baseline values for experimental groups prior to the administration of the experimental treatments (vehicle, rilmenidine) are shown in Table 1. The first collection period was used as an indication of the effects of the surgical preparation in the different groups. The baseline values were similar for all the different doses within either the 1K sham or 1K-1C hypertensive groups. The 1K-1C hypertensive rats consistently had higher blood pressures ($P<0.05$) than the 1K sham animals. The incidence of hypertension in the animals which received the renal clip was 100%.

The mean arterial blood pressure was not altered by rilmenidine in either the 1K-sham normotensive rats or the 1K-1C hypertensive rats (Figure 1). Heart rate was decreased

**Table 1** Absolute baseline values for experimental groups prior to the administration of vehicle (0 nmol $kg^{-1}$ $min^{-1}$) or rilmenidine (3, 10 and 30 nmol $kg^{-1}$ $min^{-1}$)

| | *Rilmenidine* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *1K-Sham* | | | | *1K-1C* | | | |
| | 0 | 3 | 10 | 30 | 0 | 3 | 10 | 30 |
| BP (mmHg) | 121 ± 2 | 126 ± 1 | 130 ± 4 | 127 ± 3 | 175 ± 7[a] | 150 ± 10[a] | 165 ± 4[a] | 168 ± 7[a] |
| HR (b.p.m.) | 433 ± 10 | 423 ± 14 | 427 ± 6 | 420 ± 19 | 447 ± 8 | 417 ± 15 | 437 ± 13 | 463 ± 6 |
| $C_{cr}$ (ml $min^{-1}$) | 1.6 ± 0.3 | 1.8 ± 0.2 | 1.5 ± 0.1 | 1.5 ± 0.1 | 1.6 ± 0.2 | 2.0 ± 0.4 | 1.5 ± 0.2 | 1.5 ± 0.2 |
| UV (μl $min^{-1}$) | 14 ± 2 | 19 ± 4 | 15 ± 2 | 17 ± 3 | 13 ± 1 | 14 ± 2 | 16 ± 2 | 20 ± 3 |
| UNaV (μEq $min^{-1}$) | 2.0 ± 0.4 | 3.3 ± 0.6 | 2.9 ± 0.8 | 2.7 ± 0.6 | 1.8 ± 0.2 | 2.2 ± 0.8 | 2.0 ± 0.5 | 3.4 ± 0.7 |
| $C_{osm}$ (μl $min^{-1}$) | 53 ± 4 | 74 ± 9 | 64 ± 6 | 54 ± 6 | 46 ± 3 | 58 ± 8 | 57 ± 6 | 63 ± 8 |
| $C_{H_2O}$ (μl $min^{-1}$) | − 39 ± 4 | − 56 ± 5 | − 49 ± 5 | − 38 ± 4 | − 33 ± 3 | − 44 ± 7 | − 41 ± 6 | − 43 ± 5 |

BP, blood pressure; HR, heart rate; $C_{cr}$, creatinine clearance; UV, urine flow rate; UNaV, sodium excretion; $C_{osm}$, osmolar clearance; $C_{H_2O}$, free water clearance. Values represent mean ± standard error. [a] denotes $P<0.05$ between 1K-sham normotensives and 1K-1C hypertensive rats.

only in 1K-1C rats at the highest infusion rate of rilmenidine (30 nmol $kg^{-1}$ $min^{-1}$). Rilmenidine did not alter creatinine clearance. All doses of rilmenidine resulted in an increase in urine flow rate and sodium excretion in 1K-sham rats (Figure 2). However, in 1K-1C hypertensive rats, no change in urine flow rate or sodium excretion was observed (Figure 2). This was reflected in significantly greater sodium excretions and urine flow rates for all doses in the 1K-sham animals. Rilmenidine increased osmolar clearance in 1K-sham normotensive rats but failed to have any effect in 1K-1C hypertensive rats (Figure 3). Free water clearance was not significantly altered in 1K-sham rats or 1K-1C hypertensive rats (Figure 3).

### *Effect of intrarenal infusion of moxonidine*

Baseline values for experimental groups prior to the administration of the experimental treatments (vehicle, moxonidine) are shown in Table 2. The first collection period was used as an indication of the effects of the surgical preparation in the different groups. As would be anticipated, a significant interaction effect by group (1K Sham versus 1K-1C) was observed for blood pressure in that the animals which had received renal clips had a significantly higher blood pressure ($P<0.05$). Within the 1K sham rats the baseline values for heart rate (HR), osmolar clearance ($C_{osm}$) and free water clearance ($C_{H_2O}$) in the animals that received 3 nmol $kg^{-1}$ $min^{-1}$ of moxonidine were different from the group which received the saline vehicle (Table 2). The differences were, a lower heart rate, increased osmolar clearance and decreased free water clearance (Table 2).

The mean arterial blood pressure was not altered by moxonidine in either the 1K-sham normotensive rats or the 1K-1C hypertensive rats (Figure 4). Heart rate was decreased in both 1K-sham and 1K-1C rats at the highest infusion rate of moxonidine (10 nmol $kg^{-1}$ $min^{-1}$). Moxonidine did not alter creatinine clearance. All doses of moxonidine resulted in an increase in urine flow rate in 1K-sham rats (Figure 5), whereas, in 1K-1C hypertensive rats, the two highest infusion rates increased urine flow rate (Figure 5). The increase in urine flow rate was also greater in the 1K-sham rats as compared to the 1K-1C rats. Sodium excretion was also increased by moxonidine in 1K-sham normotensive rats at all infusion rates investigated, but only at the highest infusion rate in 1K-1C hypertensive rats (Figure 5). Moxonidine increased osmolar clearance at all infusion rates investigated in 1K-sham normotensive rats but only at the highest dose in 1K-1C hypertensive rats (Figure 6). Free water clearance was increased in the 1K-sham rats at the lowest and highest doses of moxonidine but not the 1K-1C hypertensive rats.

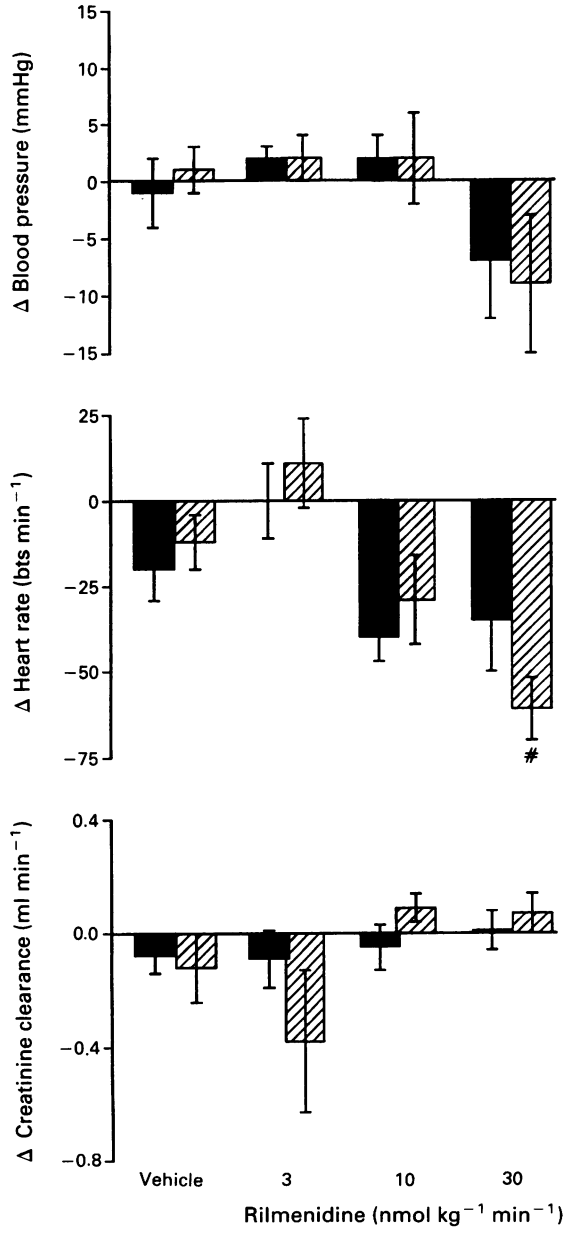


**Figure 1** The effects of rilmenidine on mean arterial blood pressure, heart rate and creatinine clearance. Data are presented as means ± standard error of the mean. The solid columns represent the 1K-sham rats and the hatched columns represent the 1K-1C hypertensive rats. #denotes $P<0.05$ as compared with the respective control group.

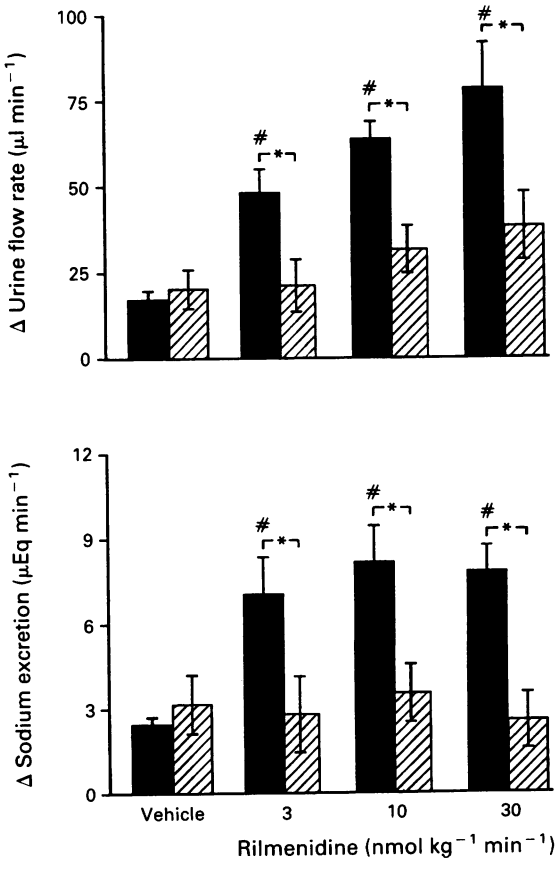


**Figure 2** The effects of rilmenidine on urine flow rate and sodium excretion. The solid columns represent the 1K-sham rats and the hatched columns represent the 1K-1C hypertensive rats. *denotes $P<0.05$ between 1K-sham normotensive and 1K-1C hypertensive rats. #$P<0.05$ as compared with the respective control group.

**Table 2** Absolute baseline values for experimental groups prior to the administration of vehicle (0 nmol $kg^{-1}$ $min^{-1}$) or moxonidine (1, 3 and 10 nmol $kg^{-1}$ $min^{-1}$)

| | *Moxonidine* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *1K-Sham* | | | | *1K-1C* | | | |
| | 0 | 1 | 3 | 10 | 0 | 1 | 3 | 10 |
| BP (mmHg) | 121 ± 2 | 121 ± 2 | 120 ± 1 | 132 ± 4 | 175 ± 7[a] | 157 ± 6[a] | 157 ± 3[a] | 161 ± 5[a] |
| HR (b.p.m.) | 433 ± 10 | 447 ± 11 | 393 ± 8* | 440 ± 11 | 447 ± 8 | 440 ± 9 | 427 ± 10 | 455 ± 11 |
| $C_{cr}$ (ml $min^{-1}$) | 1.6 ± 0.3 | 1.5 ± 0.1 | 2.1 ± 0.2 | 1.6 ± 0.1 | 1.6 ± 0.2 | 1.4 ± 0.1 | 1.7 ± 0.4 | 1.5 ± 0.1 |
| UV (μl $min^{-1}$) | 14 ± 2 | 16 ± 1 | 17 ± 2 | 15 ± 3 | 13 ± 1 | 12 ± 2 | 16 ± 3 | 13 ± 1 |
| UNaV (μEq $min^{-1}$) | 2.0 ± 0.4 | 3.5 ± 0.4 | 3.1 ± 0.6 | 2.9 ± 0.8 | 1.8 ± 0.2 | 2.1 ± 0.6 | 2.7 ± 0.3 | 1.9 ± 0.2 |
| $C_{osm}$ (μl $min^{-1}$) | 53 ± 4 | 65 ± 6 | 80 ± 6* | 60 ± 9 | 46 ± 3 | 48 ± 5 | 57 ± 3 | 50 ± 3 |
| $C_{H_2O}$ (μl $min^{-1}$) | − 39 ± 4 | − 50 ± 4 | − 63 ± 6* | − 45 ± 7 | − 33 ± 3 | − 36 ± 5 | − 41 ± 4 | − 37 ± 2 |

BP, blood pressure; HR, heart rate; $C_{cr}$, creatinine clearance; UV, urine flow rate; UNaV, sodium excretion; $C_{osm}$, osmolar clearance; $C_{H_2O}$, free water clearance. Values represent mean ± standard error. [a] denotes $P<0.05$ between 1K-sham normotensives and 1K-1C hypertensive rats and *denotes $P<0.05$ versus the vehicle control group (0) within the 1K-sham or 1K-1C groups.

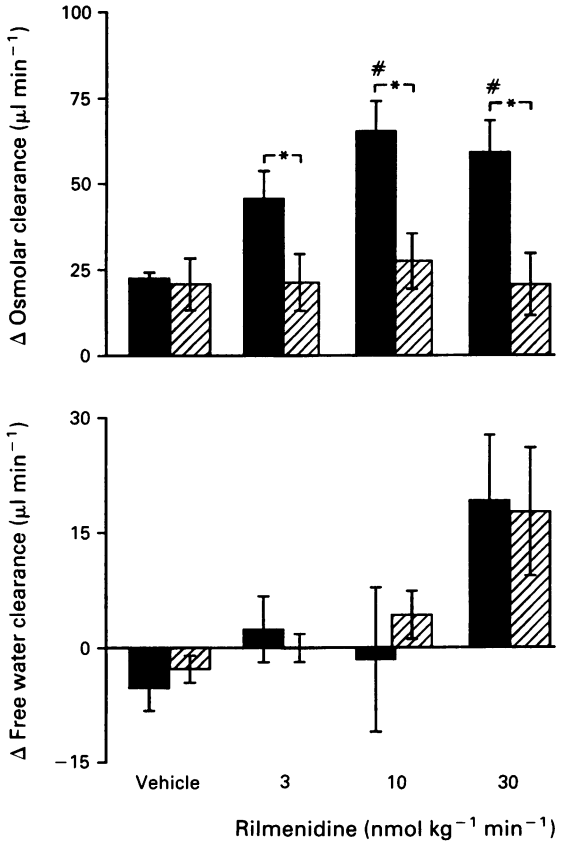


**Figure 3** The effects of rilmenidine on free water clearance and osmolar clearance. The solid columns represent the 1K-sham rats and the hatched columns represent the 1K-1C hypertensive rats. *denotes $P<0.05$ between 1K-sham normotensive and 1K-1C hypertensive rats. #denotes $P<0.05$ as compared with the respective control group.

## Discussion

Boyajian *et al.* (1987) and Coupry *et al.* (1987) reported that two purported $\alpha_2$-adrenoceptor antagonists, rauwolscine and idazoxan, labelled two distinct populations of receptors. Michel *et al.* (1989a; 1990b) also found that [$^3$H]-idazoxan labelled a non-adrenoceptor site which was distinct from the $\alpha_2$-adrenoceptor. These non-adrenoceptor sites also had a greater affinity for imidazoline based compounds than phenylethylamines (catecholamines) (Bricca *et al.*, 1989; Ernsberger *et al.*, 1990). Recently these non-adrenoceptor, imidazoline sites have been found to exist as at least two subtypes, the $I_1$ which was labelled by [$^3$H]-clonidine and the $I_2$ which was labelled by [$^3$H]-idazoxan (Ernsberger *et al.*, 1992; Reis *et al.*, 1992).

A number of lines of evidence suggested that 2,6-DMC may be a non-adrenoceptor, imidazoline receptor agonist. Previous studies in our laboratory indicated that the two purported $\alpha_2$-adrenoceptor agonists 2,6-DMC and clonidine increased urine flow rate by two distinct mechanisms (Smyth *et al.*, 1992a). 2,6-DMC increased urine flow rate secondary to an increase in osmolar clearance while clonidine increased urine flow rate by increasing free water clearance. In addition, pretreatment with a $V_2$ vasopressin antagonist had no effect on the response to 2,6-DMC but completely attenuated the effects of clonidine. These results are consistent with the effects of 2,6-DMC and clonidine being mediated by two distinct mechanisms and/or sites, $\alpha_2$-adrenoceptors and non-adrenoceptor, imidazoline receptors (Smyth *et al.*, 1992a). Effects similar to those reported with 2,6-DMC have been found by Allan *et al.* (1993) using moxonidine, an $I_1$ non-adrenoceptor, imidazoline receptor agonist with an approximately 700 times higher affinity for $I_1$ non-adrenoceptor, imidazoline receptors than that for $\alpha_2$-adrenoceptors in the kidney (Ernsberger *et al.*, 1993). They found that moxonidine produced an increased urine flow rate in Sprague-Dawley rats and this increase was secondary to an increase in osmolar clearance. This increase was blocked by pretreatment with idazoxan (non-adrenoceptor, imidazoline receptor antagonist) but not by pretreatment with rauwolscine ($\alpha_2$-adrenoceptor antagonist). The renal effects of 2,6-DMC were also blocked by idazoxan but not rauwolscine (Smyth & Li, 1991), indicating that 2,6-DMC and moxonidine may be acting at the same site and/or receptor.

Rilmenidine has at least a 3 fold greater affinity for $I_1$ non-adrenoceptor, imidazoline receptors than for $\alpha_2$-adrenoceptors (Gomez *et al.*, 1991; Ernsberger *et al.*, 1992) and the cardiovascular action of central administration of rilmenidine was blocked by idazoxan (Feldman *et al.*, 1990). The present study demonstrated that, like moxonidine (Allan *et al.*, 1993) and 2,6-DMC (Smyth *et al.*, 1992a), rilmenidine increased urine flow rate and sodium excretion in a dose-related fashion. Similarly, the increase in flow rate was associated

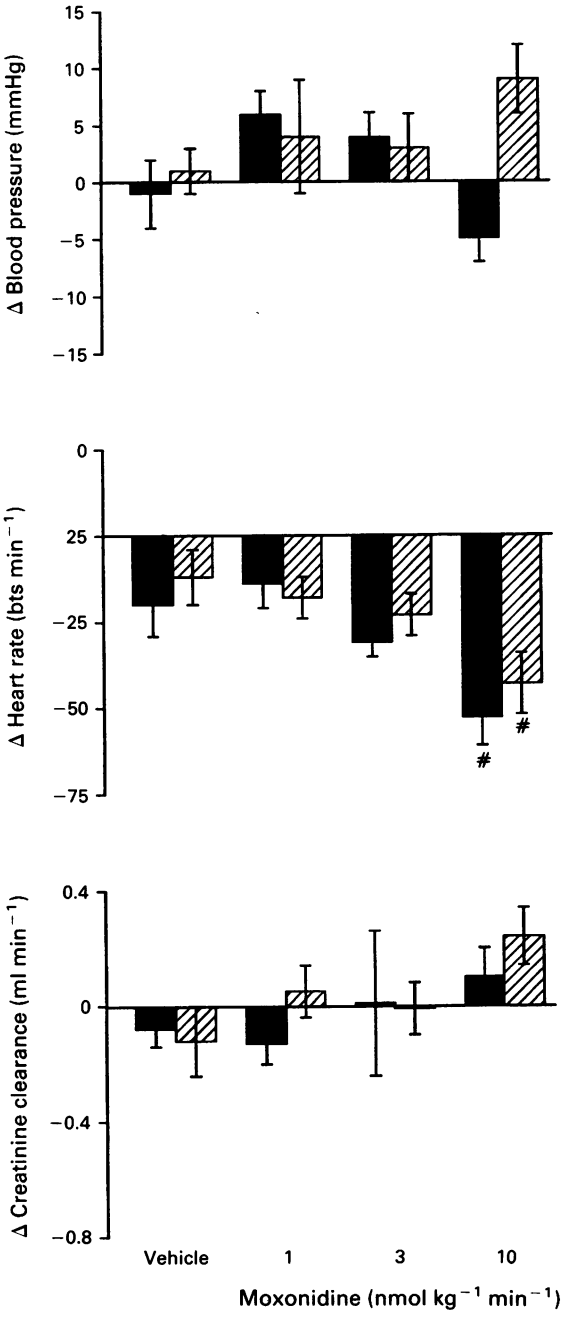


**Figure 4** The effects of moxonidine on mean arterial blood pressure, heart rate and creatinine clearance. The solid columns represent the 1K-sham rats and the hatched columns represent the 1K-1C hypertensive rats. *denotes $P<0.05$ between 1K-sham normotensive and 1K-1C hypertensive rats. #denotes $P<0.05$ as compared with the respective control group.

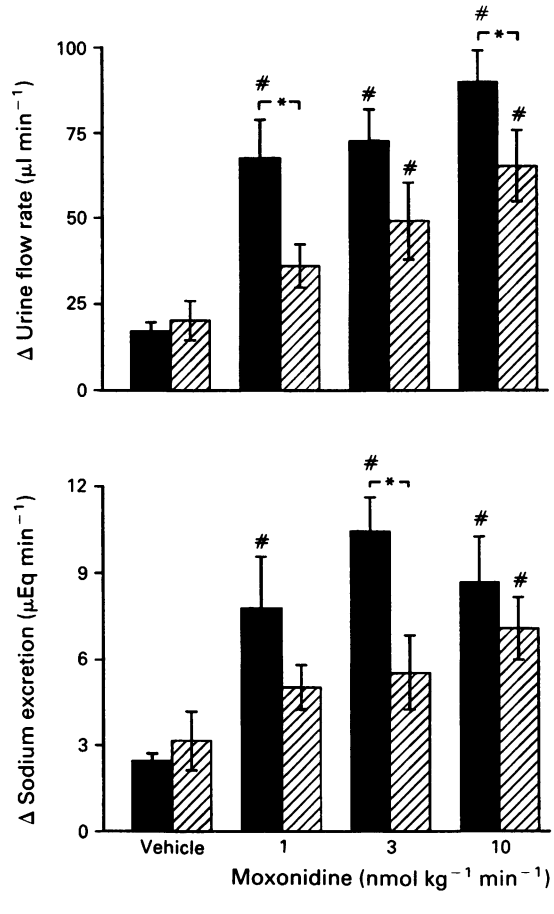


**Figure 5** The effects of moxonidine on urine flow rate and sodium excretion. The solid columns represent the 1K-sham rats and the hatched columns represent the 1K-1C hypertensive rats. *denotes $P<0.05$ between 1K-sham normotensive and 1K-1C hypertensive rats. #denotes $P<0.05$ as compared with the respective control group.

with an increase in osmolar clearance rather than an increase in free water clearance as has been documented for $\alpha_2$-adrenoceptor agonists (Blandford & Smyth, 1990; Gellai, 1990). This similarity of 2,6-DMC with moxonidine and rilmenidine would be consistent with 2,6-DMC acting at the same site, i.e. the non-adrenoceptor, imidazoline receptor. However, radioligand binding studies will be required to support this contention.

In the present study, the renal effects of rilmenidine and moxonidine were significantly attenuated in 1K-1C hypertensive rats as compared with 1K-sham normotensive rats. These results were similar to those found for 2,6-DMC in 1K-1C hypertensive rats (Li & Smyth, 1994). It is important to note that this decrease in response to non-adrenoceptor, imidazoline agonists may be specific since the natriuretic response to an $\alpha_2$-adrenoceptor agonist (Li & Smyth, 1994) and a $V_2$ vasopressin receptor antagonist (Li & Smyth, 1993) were not found to be altered in 1K-1C hypertension.

During the chronic phase of 1K-1C hypertension, blood pressure is maintained by an excess of water and sodium retention (Zandberg, 1984; Nabel *et al.*, 1985). Conceivably, the attenuated effects of renal imidazoline receptor stimulation in 1K-1C hypertensive rats could contribute to the sodium and water retention and may be caused by one of two factors. First, an increase in the endogenous levels of the non-adrenoceptor, imidazoline receptor agonist could result in a decrease in the number of these receptors available (receptor occupation) which would decrease the response to the exogenous non-adrenoceptor, imidazoline receptor agonists. At present, the endogenous agonist for non-adrenoceptor, imidazoline receptors has not been determined. However, recent studies suggest that clonidine-displacing substance may be the endogenous non-adrenoceptor, imidazoline receptor agonist (Atlas, 1991; Regunathan *et al.*, 1991). Second, this decreased response may represent a decrease in receptor activity. Insel & Motulsky (1988) found that $\alpha_2$-adrenoceptors in tissues such as those in rat kidney may be down- or up-regulated. Antagonists induced an up-regulation and agonists mediated a down-regulation of $\alpha_2$-adrenoceptors in spontaneously hypertensive rats, DOCA-salt hypertensive rats and New Zealand Genetically Hypertensive rats (Saiz *et al.*, 1987; Sanchez *et al.*, 1989; Smyth *et al.*, 1992b). The $I_1$

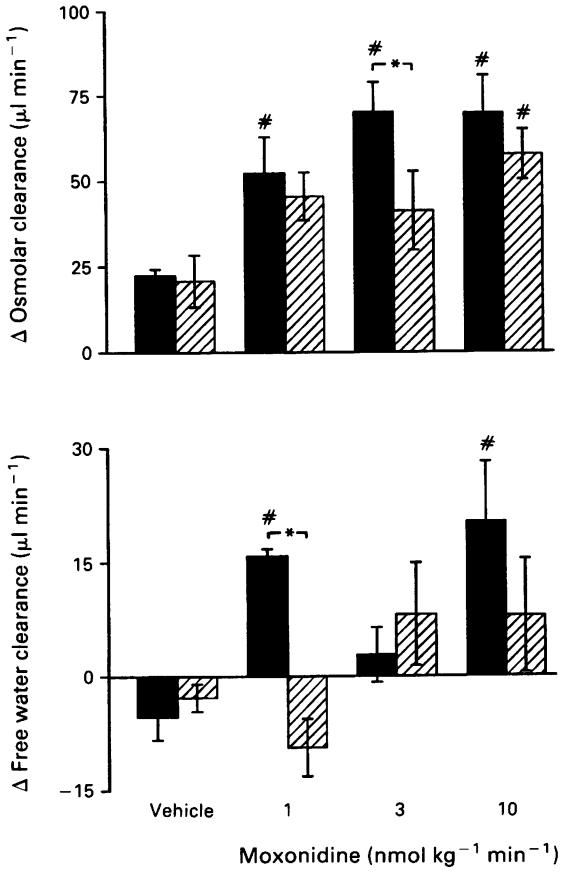


**Figure 6** The effects of moxonidine on free water clearance and osmolar clearance. The solid columns represent the 1K-sham rats and the hatched columns represent the 1K-1C hypertensive rats. *denotes $P<0.05$ between 1K-sham normotensive and 1K-1C hypertensive rats. #denotes $P<0.05$ as compared with the respective control group.

non-adrenoceptor, imidazoline receptor may also undergo similar regulation. An infusion of angiotensin II produced an up-regulation of $I_1$ sites (Ernsberger *et al.*, 1991). Similarly, although the receptor subtype was not described, Olmos *et al.* (1992) demonstrated that chronic treatment with idazoxan or cirazoline (imidazoline compounds) increased the density of non-adrenoceptor, imidazoline receptors in the brain of Wistar Kyoto and Sprague-Dawley rats but not spontaneously hypertensive rats. The altered non-adrenoceptor, imidazoline receptor number/activity may be secondary to increased agonist levels (clonidine-displacing substance) or a pathophysiological decrease in receptor activity. Recent studies in our laboratory have shown that [$^3$H]-idazoxan binding was decreased in kidneys of 1K-1C hypertensive rats compared with 1K-sham normotensive rats. Conversely [$^3$H]-rauwolscine binding was similar in 1K-1C hypertensive and 1K-sham normotensive rats (Li & Smyth, 1994). This decrease in sites labelled by [$^3$H]-idazoxan in 1K-1C kidneys would be consistent with the decrease in response to non-adrenoceptor, imidazoline agonists reported in the present study.

In summary, the renal effects of rilmenidine were completely attenuated and the renal response of moxonidine was significantly decreased in 1K-1C hypertensive rats compared with the response in 1K-sham normotensive rats. This attenuation indicated that the activity of renal $I_1$ non-adrenoceptor, imidazoline receptors was decreased. Whether the decrease in activity of this natriuretic $I_1$ non-adrenoceptor, imidazoline receptor contributes to the increase in blood pressure in this 1K-1C acquired model of hypertension remains to be determined.

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# Reversal of established responses to endothelin-1 *in vivo* and *in vitro* by the endothelin receptor antagonists, BQ-123 and PD 145065

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**1** Endothelin-1 binds almost irreversibly to its receptors and causes prolonged vasoconstrictions. Here we have studied the reversal of established responses to ET-1 *in vivo* and *in vitro* by BQ-123, an $ET_A$ receptor-selective antagonist, and/or PD 145065, an $ET_A/ET_B$ receptor non-selective antagonist.

**2** In anaesthetized rats pretreated with hexamethonium, infusion of ET-1 ($10^{-11}$ mol kg$^{-1}$ min$^{-1}$) increased the mean arterial pressure (MAP) from 93 ± 1.5 mmHg to 137 ± 2.4 mmHg after 70 min ($n = 29$). While the ET-1 infusion was continued an additional infusion of BQ-123 caused a gradual dose-dependent reduction in the pressor effect of ET-1. For instance, after a 60 min infusion of BQ-123 ($10^{-8}$ mol kg$^{-1}$ min$^{-1}$) the MAP was decreased by 29.3 ± 4.3 mmHg ($n = 4$).

**3** PD 145065 was a much weaker antagonist of the established pressor effects of ET-1. At $10^{-8}$ mol kg$^{-1}$ min$^{-1}$ it had no significant effect and even at $10^{-7}$ mol kg$^{-1}$ min$^{-1}$ the elevated blood pressure was only reduced by 11.8 ± 8.0 mmHg ($n = 5$) after 60 min. Co-infusion of BQ-123 and PD 145065 caused smaller reductions in the established response to ET-1 than infusion of BQ-123 alone.

**4** Sustained contractions of rat aortic rings induced by ET-1 ($3 \times 10^{-9}$ M) and mediated by $ET_A$ receptors were slowly reversed by addition of BQ-123 ($10^{-5}$ M) or PD 145065 ($10^{-5}$ M). For instance, after 40 min the elevated tone was reduced 85.8 ± 5.6% ($n = 6$) by PD 145065, and 77.1 ± 6.7% ($n = 6$) by BQ-123. Thus, on the rat aortic rings *in vitro* both antagonists were equally effective against established responses to ET-1.

**5** ET-1 increased the perfusion pressure of the rat isolated perfused kidney by 138.1 ± 7.6 mmHg ($n = 14$). Subsequent co-infusion of BQ-123 or PD 145065 reversed this increase with PD 145065 being more active. For instance, PD 145065 ($10^{-6}$ M) reversed the increase in perfusion pressure by 56.9 ± 8.8% ($n = 5$) and BQ-123 ($10^{-6}$ M) reversed it by 22.8 ± 8.0% ($n = 5$). This fits well with the vasoconstriction induced by ET-1 in the rat kidney being mediated by $ET_A$ and $ET_B$ receptors.

**6** Thus, sustained vasoconstrictions to ET-1 *in vitro*, mediated by either $ET_A$ or $ET_B$ receptors, may be reversed slowly by the subsequent application of receptor antagonists. Similarly, endothelin antagonists reverse the pressor effects of ET-1 *in vivo* although co-antagonism of $ET_A$ and $ET_B$ receptors or the co-administration of an $ET_A$ receptor antagonist, BQ-123, and a mixed antagonist, PD 145065 produces less reversal than the application of an $ET_A$ receptor-selective antagonist. This may be because PD 145065 also reduces vasodilatations induced by ET-1 *in vivo*, or could suggest that because of its peptide structure PD 145065 affects the elimination of ET-1.

**Keywords:** Endothelin-1; endothelin receptors; endothelin antagonists

## Introduction

The endothelins (endothelin-1, ET-1; ET-2; ET-3) are a family of structurally related peptides (Inoue *et al.*, 1989). Their effects are mediated by at least two receptors; the $ET_A$ receptor that has several hundred fold more affinity for ET-1 or ET-2 than ET-3, and the $ET_B$ receptor that accepts the endothelins with equal affinity (Arai *et al.*, 1990; Sakurai *et al.*, 1990). Either receptor may mediate constrictions to ET-1 in both vascular and non-vascular smooth muscle (Clozel *et al.*, 1992; Harrison *et al.*, 1992; Hay, 1992; Moreland *et al.*, 1992; Sumner *et al.*, 1992; Warner *et al.*, 1993a,b).

Studies with broken cell systems show that ET-1 binds almost irreversibly to its receptors with a dissociation half-life in excess of 30 h (Waggoner *et al.*, 1992), which may explain the prolonged nature of its effects *in vivo* and *in vitro* (Yanagisawa *et al.*, 1988; de Nucci *et al.*, 1988). Thus, receptor antagonists may act only weakly against established responses to ET-1. Here we have assessed the effectiveness of the endothelin receptor antagonists BQ-123 ($ET_A$ receptor-selective, Ihara *et al.*, 1992) and PD 145065 ($ET_A/ET_B$ receptor non-selective, Cody *et al.*, 1993; Doherty *et al.*, 1993) in reversing the *in vivo* pressor and *in vitro* vasoconstrictor effects of ET-1.

Some of these data have been presented to the British Pharmacological Society (Allcock *et al.*, 1993; 1994).

## Methods

### *Rat blood pressure*

Male Wistar rats (250–400 g) were anaesthetized with sodium thiopentone (Intraval, 120 mg kg$^{-1}$, i.p.). The trachea was cannulated to facilitate respiration and body temperature was maintained at 37°C by means of a rectal probe connected to a homeothermic blanket (Biosciences, Sheerness, Kent). The right carotid artery was cannulated and connected to a pressure transducer (Elcomatic type 750) for the measurement of arterial blood pressure which was recorded on a Graphtec Linearcorder (type WR3101). Mean arterial pressure (MAP) was calculated as the diastolic pressure plus one third of the pulse pressure. The left and right jugular veins were cannulated for the administration of drugs.

After surgery, animals were allowed to stabilize for 30 min

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before receiving a 5 min infusion of hexamethonium (10 mg $kg^{-1}$, i.v.). Twenty minutes later ET-1 was infused i.v. at a rate of $10^{-11}$ mol $kg^{-1}$ $min^{-1}$ to give a sustained increase in blood pressure. After 70 min, BQ-123 ($10^{-9}$–$10^{-8}$ mol $kg^{-1}$ $min^{-1}$) and/or PD 145065 ($10^{-8}$–$10^{-7}$ mol $kg^{-1}$ $min^{-1}$) and/or vehicle (saline plus 0.5% BSA) was additionally infused into the animals. The antagonist/vehicle infusion was stopped after 60 min and blood pressure recorded for a further 60 min. The infusion of ET-1 was maintained throughout.

In a further series of experiments hexamethonium-treated rats were given a bolus injection of $5 \times 10^{-10}$ mol $kg^{-1}$ ET-1, 5 min after a bolus injection of PD 145065 $5 \times 10^{-7}$ or $5 \times 10^{-6}$ mol $kg^{-1}$, or vehicle.

### *Rat thoracic aortic rings*

Male Wistar rats (250–400 g) were anaesthetized with sodium pentobarbitone (Sagatal, 120 mg $kg^{-1}$, i.p.) and then killed by exsanguination. Rings of rat aorta with endothelium removed (2–3 mm wide, resting tension 1 g) were suspended in isolated organ baths containing 10 ml of Krebs buffer. Responses of the tissues were detected by isometric transducers (Hugo Sachs Electronik, Germany) and displayed on a chart recorder (Graphtec WR3101). The Krebs solution, which was gassed with 95% $O_2$:5% $CO_2$ at a temperature of 37°C, contained bacitracin (3 mg $l^{-1}$), bovine serum albumin (50 mg $l^{-1}$), indomethacin ($5 \times 10^{-6}$ M), thiorphan ($10^{-6}$ M), captopril ($10^{-6}$ M) and bestatin ($10^{-6}$ M) to exclude indirect effects induced by the production of prostanoids and to minimize the possible local metabolism by tissue enzymes (Maggi *et al.*, 1989; Warner *et al.*, 1993a,b). After 1 h equilibration approximately 80% of maximum constrictor responses were induced in the rings with ET-1 ($3 \times 10^{-9}$ M) or phenylephrine ($3 \times 10^{-7}$ M). When responses had reached plateau, BQ-123 ($10^{-5}$ M), PD 145065 ($10^{-5}$ M) or vehicle was added to the organ baths.

### *Rat isolated perfused kidney*

Male Wistar rats (250–400 g) were anaesthetized with sodium pentobarbitone (Sagatal, 120 mg $kg^{-1}$, i.p.) and heparinised (500 u $kg^{-1}$, i.v.). The superior mesenteric artery was cannulated retrogradely and the cannula passed into the right renal artery and the kidney immediately perfused at a constant rate (5 ml $min^{-1}$) with warmed (37°C) and gassed (95% $O_2$:5% $CO_2$) Krebs buffer. The rat was killed by exsanguination and the kidney excised while being continuously perfused with Krebs buffer. The perfusion rate was then increased to 10 ml $min^{-1}$ and changes in perfusion pressure measured by a pressure transducer (Elcomatic type 750) linked to a chart recorder (Graphtec Linearcorder WR3101).

After an equilibration period of 30 min, bovine serum albumin (BSA) at a final concentration of 0.1% w/v was infused to reduce non-specific losses of low peptide concentrations into the perfusion apparatus. Approximately 80% of maximum vasoconstriction was then induced with ET-1 ($3 \times 10^{-10}$ M) or methoxamine ($2$–$2.5 \times 10^{-5}$ M). Cumulative concentrations of BQ-123 ($10^{-9}$ to $10^{-6}$ M) or PD 145065 ($10^{-9}$ to $10^{-6}$ M) or vehicle were then infused into the kidney. Changes in perfusion pressure were calculated when the responses to infusion reached a plateau level.

### *Materials*

The Krebs buffer had the following composition (mM): NaCl 118, KCl 4.7, $KH_2PO_4$ 1.2, $MgSO_4.7H_2O$ 1.17, $CaCl_2.6H_2O$ 2.5, $NaHCO_3$ 25, glucose 5.6. The trifluoroacetate salt of BQ-123 (cyclo-(D-Trp-D-Asp-Pro-D-Val-Leu-)) and the disodium salt of PD 145065 (Ac-D-Bhg-L-Leu-L-Asp-L-Ile-L-Ile-L-Trp; Bhg = 5H-dibenzyl[a,d]cycloheptene-10,11-dihydroglycine) were synthesized by Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company. Sodium pentobarbitone (Sagatal) and sodium thiopentone (Intraval) were obtained from May and Baker Ltd. (Dagenham, Essex). ET-1 was purchased from Peptide Institute (Osaka, Japan). BQ-123, PD 145065 and ET-1 were dissolved in 0.9% w/v saline containing 1% w/v bovine serum albumin and 10 mM sodium bicarbonate. Bovine serum albumin, bacitracin, thiorphan, captopril, bestatin, methoxamine, phenylephrine and acetylcholine were obtained from Sigma Chemical Co. (Poole, Dorset) and dissolved in either distilled water or saline. Indomethacin (from Sigma) was made as a stock solution in sodium bicarbonate (5%). The salts for the Krebs solution were obtained from BDH (Lutterworth, Leics.).

### *Statistics*

Statistical differences between points were determined by an unpaired or paired two tail Student's *t* test, or repeated measures or one-way ANOVA with *post hoc* Bonferroni *t* test, as appropriate. A $P<0.05$ was taken as significant. Statistical differences between curves were determined by two-way ANOVA and a $P<0.01$ taken as significant.

## Results

### *Rat blood pressure*

After the initial stabilisation period the MAP of the rats was $102 \pm 1.5$ mmHg ($n = 29$) which fell to $93 \pm 1.5$ mmHg ($n = 29$, $P<0.05$, Student's *t* test) after treatment with hexamethonium.

Infusion of ET-1 ($10^{-11}$ mol $kg^{-1}$ $min^{-1}$) elevated the MAP (Figures 1 and 2) to $137 \pm 2.4$ mmHg ($n = 29$) after 70 min and by a further $12.5 \pm 2.8$ mmHg ($n = 4$) over the next 60 min (Figure 2). The basal heart rate was $357 \pm 7$ beats per min (b.p.m.) ($n = 26$) which was not affected by hexamethonium ($345 \pm 6$ b.p.m., $n = 29$) but decreased to $320 \pm 7$ b.p.m. ($n = 29$, $P<0.05$, Student's *t* test) after infusion of ET-1 for 70 min.

BQ-123 ($10^{-9}$–$10^{-8}$ mol $kg^{-1}$ $min^{-1}$) caused significant ($P<0.01$, two-way ANOVA) dose-dependent reversals of the increases in MAP (Figures 2 and 3a). For instance, after 60 min of infusion BQ-123 at $10^{-9}$ mol $kg^{-1}$ $min^{-1}$ MAP decreased by $9.8 \pm 3.8$ mmHg ($n = 4$) from that at the beginning of the BQ-123 infusion. After 60 min infusion of BQ-123 ($10^{-8}$ mol $kg^{-1}$ $min^{-1}$), MAP was reduced by $29.3 \pm 4.3$ mmHg ($n = 4$). Neither dose of BQ-123 significantly affected the heart rate.

PD 145065 ($10^{-8}$ mol $kg^{-1}$ $min^{-1}$) did not significantly affect the increase in MAP induced by ET-1. For instance, 60 min after starting the antagonist infusion, MAP had increased by a further $5.1 \pm 2.0$ mmHg ($n = 5$). However, PD145065 at $10^{-7}$ mol $kg^{-1}$ $min^{-1}$ did inhibit the pressor effect of ET-1 ($P<0.01$, two-way ANOVA), such that at

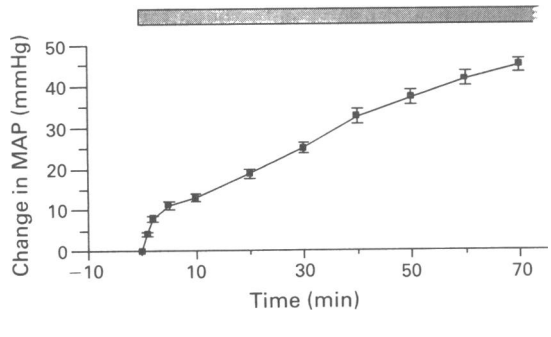



**Figure 1** Increase in mean arterial pressure induced by infusion of endothelin-1 (ET-1, $10^{-11}$ mol $kg^{-1}$ $min^{-1}$). Each point represents the mean (± s.e.mean) ($n = 29$). The hatched bar represents presence of the ET-1 infusion.

60 min MAP was decreased by 11.8 ± 8.0 mmHg ($n = 5$) (Figure 3b). There was no significant change in the heart rate following infusion of PD 145065.

Unlike BQ-123 ($10^{-9}$ mol kg$^{-1}$ min$^{-1}$) infused alone, BQ-123 ($10^{-9}$ mol kg$^{-1}$ min$^{-1}$) co-infused with PD 145065 ($10^{-8}$ mol kg$^{-1}$ min$^{-1}$) did not reduce the pressor effect of ET-1 (Figure 4a). Similarly, BQ-123 ($10^{-8}$ mol kg$^{-1}$ min$^{-1}$) caused a smaller, although still significant ($P < 0.01$, two-way ANOVA), reduction in MAP when infused with PD 145065 ($10^{-8}$ mol kg$^{-1}$ min$^{-1}$) than when infused alone (Figure 4b). Co-infusion of BQ-123 and PD 145065 did not affect the heart rate.

In all experiments the MAP returned to that of vehicle-treated animals within 60 min of ceasing BQ-123 and/or PD 145065 infusion (Figures 3a,b and 4a,b).

Bolus injection of ET-1 ($0.5 \times 10^{-10}$ mol kg$^{-1}$) caused a transient fall in blood pressure of 27 ± 6 mmHg ($n = 4$) followed by a prolonged pressor effect (maximum increase in MAP 42 ± 7 mmHg, $n = 4$). PD 145065 ($0.5 \times 10^{-7}$ and $0.5 \times 10^{-6}$ mol kg$^{-1}$) dose-dependently inhibited the depressor effect of ET-1 ($P < 0.05$, ANOVA) but did not significantly effect the secondary increase in MAP (Figure 5).

### *Rat thoracic aorta*

ET-1 ($3 \times 10^{-9}$ M) caused a sustained increase in tension (1.4 ± 0.1 g, $n = 18$) of the rings of rat aorta which was slowly reversed by additional application of BQ-123 ($10^{-5}$ M) or PD 145065 ($10^{-5}$ M) (Figure 6a,b). For instance, 20 and 40 min after addition of antagonist, the contraction was reduced by 52.8 ± 12.3% and 85.8 ± 5.6% respectively with PD 145065 ($n = 6$), and by 43.8 ± 12.2% and 77.1 ± 6.7% with BQ-123 ($n = 6$). Vehicle was without effect ($n = 6$, Figure 6a,b).

Phenylephrine ($3 \times 10^{-7}$ M) increased the tension of the rat aorta by 1.7 ± 0.1 g ($n = 15$). This constriction was unaffected by either BQ-123 ($10^{-5}$ M), PD 145065 ($10^{-5}$ M) or vehicle ($n = 5$ for each).

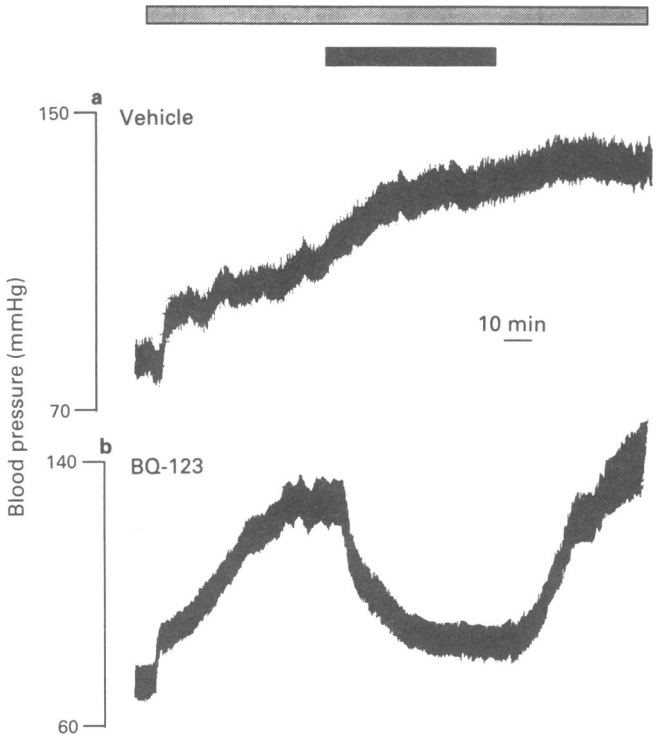



**Figure 2** Blood pressure traces from anaesthetized rats in two typical experiments. Infusion of endothelin-1 (ET-1, $10^{-11}$ mol kg$^{-1}$ min$^{-1}$, i.v., hatched bar) for 70 min increased the blood pressure of both animals. This increase was unaffected by additional i.v. infusion (solid bar) for 60 min of vehicle (a) whereas it was largely reversed by infusion of BQ-123 ($10^{-8}$ mol kg$^{-1}$ min$^{-1}$, b). When the BQ-123 infusion was removed the rise in blood pressure returned. Similar results were seen in at least 3 other experiments.

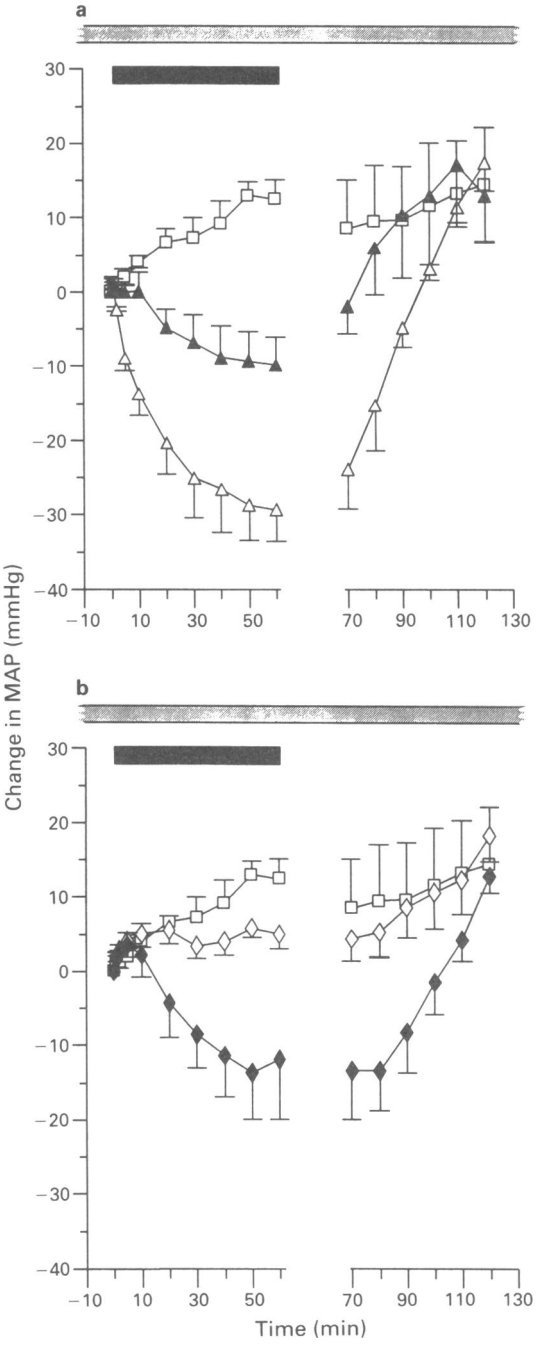



**Figure 3** (a) Reversal by BQ-123 of increases in mean arterial pressure induced by infusion of endothelin-1 (ET-1, $10^{-11}$ mol kg$^{-1}$ min$^{-1}$). Vehicle ($n = 4$), (□); BQ-123 ($10^{-9}$ mol kg$^{-1}$ min$^{-1}$, $n = 4$), (▲); BQ-123 ($10^{-8}$ mol kg$^{-1}$ min$^{-1}$, $n = 4$), (△). (b) Reversal by PD 145065 of increases in mean arterial pressure induced by infusion of ET-1 ($10^{-11}$ mol kg$^{-1}$ min$^{-1}$). Vehicle ($n = 4$), (□); PD 145065 ($10^{-8}$ mol kg$^{-1}$ min$^{-1}$, $n = 5$), (◇); PD 145065 ($10^{-7}$ mol kg$^{-1}$ min$^{-1}$, $n = 4$), (◆). The ordinate scale represents the change in ET-1-induced MAP starting from the infusion of antagonist. Results are calculated as the mean (± s.e.mean). The hatched bar represents presence of the ET-1 infusion, and the solid bar presence of antagonist/vehicle. In both panels the time axis is broken to separate the period of antagonist infusion (left side) from the period after antagonist was removed (right side).

*Rat isolated perfused kidney*

The basal perfusion pressure of the rat isolated kidney was 61.3 ± 2.9 mmHg ($n = 24$) which was increased by 138.1 ± 7.6 mmHg ($n = 14$) by infusion of ET-1 at $3 \times 10^{-10}$ M. Subsequent infusion of BQ-123 or PD 145065 (both $10^{-10}$ to $10^{-6}$ M) reversed the vasoconstriction with thresholds of approximately $10^{-8}$ M (Figure 7). PD 145065 was significantly more active ($P < 0.01$, two-way ANOVA), such that at $10^{-6}$ M it reduced the elevated perfusion pressure by 56.9 ± 8.8% ($n = 5$), whereas at the same concentration BQ-123 reduced it by 22.8 ± 8.0% ($n = 4$). Vehicle was without effect ($n = 5$, Figure 7).

Methoxamine ($2-2.5 \times 10^{-5}$ M) increased the perfusion pressure of the kidney by 159.3 ± 8.4 mmHg ($n = 10$) which was unaffected by BQ-123 ($10^{-10}$ to $10^{-6}$ M, $n = 3$), PD 145065 ($10^{-10}$ to $10^{-6}$ M, $n = 3$) or vehicle ($n = 4$).

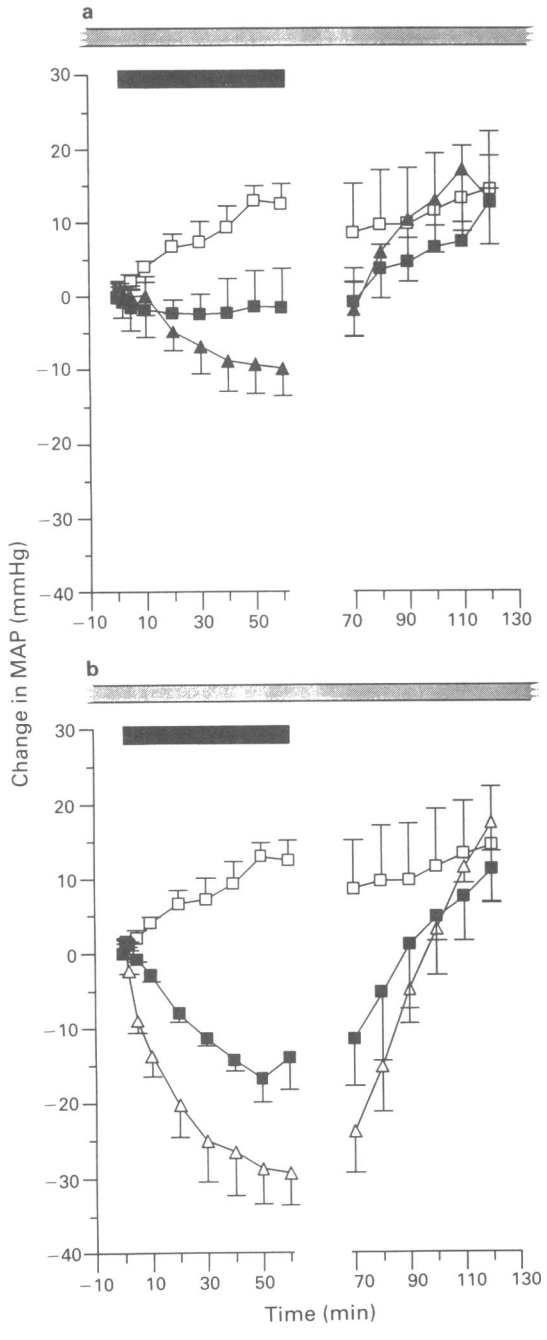



**Figure 4** Effect of PD 145065 on the ability of BQ-123 to reverse the increases in mean arterial pressure induced by infusion of endothelin-1 (ET-1, $10^{-11}$ mol kg$^{-1}$ min$^{-1}$). (a) Vehicle ($n = 4$), (□); BQ-123 ($10^{-9}$ mol kg$^{-1}$ min$^{-1}$, $n = 4$), (▲); BQ-123 ($10^{-9}$ mol kg$^{-1}$ min$^{-1}$) plus PD 145065 ($10^{-8}$ mol kg$^{-1}$ min$^{-1}$) ($n = 4$), (■). (b) Vehicle ($n = 4$), (□); BQ-123 ($10^{-8}$ mol kg$^{-1}$ min$^{-1}$, $n = 4$), (△); BQ-123 ($10^{-8}$ mol kg$^{-1}$ min$^{-1}$) plus PD 145065 ($10^{-8}$ mol kg$^{-1}$ min$^{-1}$) ($n = 4$), (■). The ordinate scale represents the change in ET-1-induced MAP starting from the infusion of antagonist. Results are calculated as the mean (± s.e.mean). The hatched bar represents presence of the ET-1 infusion, and the solid bar the presence of antagonist/vehicle. In both panels the time axis is broken to separate the period of antagonist infusion (left side) from the period after antagonist was removed (right side).

## Discussion

We have compared the abilities of two endothelin receptor antagonists to reverse established responses to ET-1 *in vivo* and *in vitro*. *In vivo*, BQ-123, a selective $ET_A$ receptor antagonist, is more active than PD 145065, a non-selective antagonist, in reversing existing pressor effects of ET-1. However, *in vitro*, PD 145065 was similar to, or more effective than BQ-123 in counteracting established vasoconstrictions to ET-1.

The much weaker anti-pressor effect of PD 145065 than BQ-123 *in vivo* is not due to PD 145065 being a weaker $ET_A$ receptor antagonist. For instance, contractions of the rabbit femoral artery or rat aorta induced by ET-1 and mediated by $ET_A$ receptors (Maggi *et al.*, 1988; Sumner *et al.*, 1992), are antagonized, by PD 145065 with a $pA_2$ of 6.5 (Cody *et al.*, 1993), and by BQ-123 with a $pA_2$ of 6.9 (Sumner *et al.*, 1992). In addition, in our *in vitro* experiments PD 145065 was as active as BQ-123 in causing slow reversals of constric-

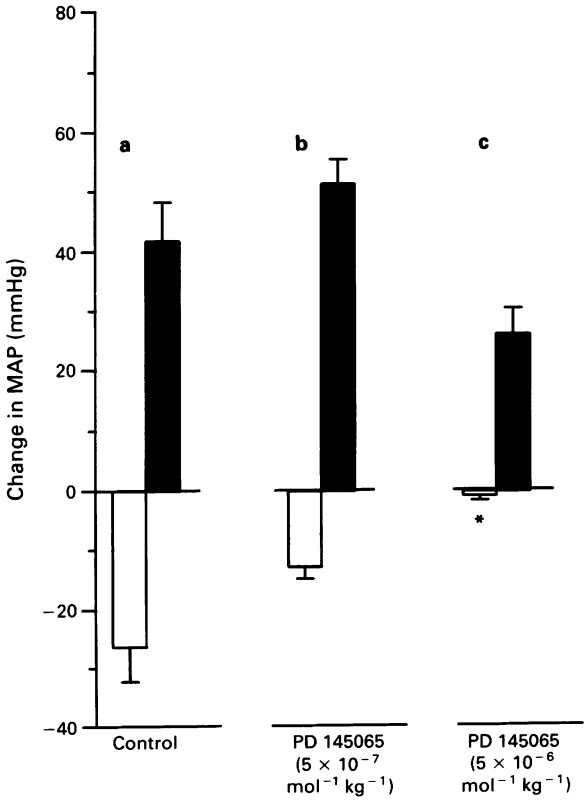
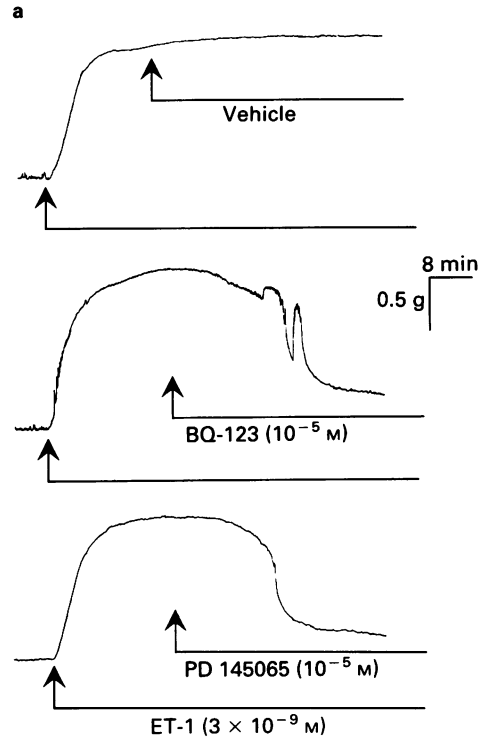



**Figure 5** Effect of PD 145065 on responses to a bolus injection of endothelin-1 (ET-1). Bolus injection of ET-1 ($5 \times 10^{-10}$ mol kg$^{-1}$, i.v.) caused an initial depressor response (open column) followed by a prolonged pressor effect (solid column). (a) Control responses; (b) + PD 145065 ($5 \times 10^{-7}$ mol kg$^{-1}$, i.v.); (c) + PD 145065 ($5 \times 10^{-6}$ mol kg$^{-1}$, i.v.). Results are calculated as the mean (± s.e.mean), ($n = 4$ for each). *Indicates significant difference from control.



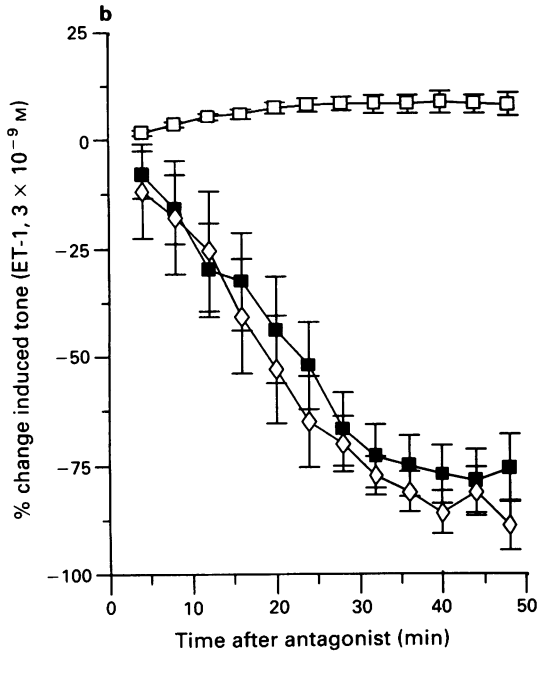




**Figure 6** (a) Endothelin-1 (ET-1, $3 \times 10^{-9}$ M) caused sustained constrictions of rings of rat thoracic aorta which were reversed by either BQ-123 ($10^{-5}$ M) or PD 145065 ($10^{-5}$ M). Each trace is representative of 6 experiments. (b) Mean data from experiments illustrated in (a). Data are expressed as the % reduction in tone induced by ET-1 ($3 \times 10^{-9}$ M). Vehicle ($n = 6$), (□); BQ-123 ($10^{-5}$ M, $n = 6$), (■); PD 145065 ($10^{-5}$ M, $n = 6$), (◇). Results are calculated as the mean (± s.e.mean, vertical bars).

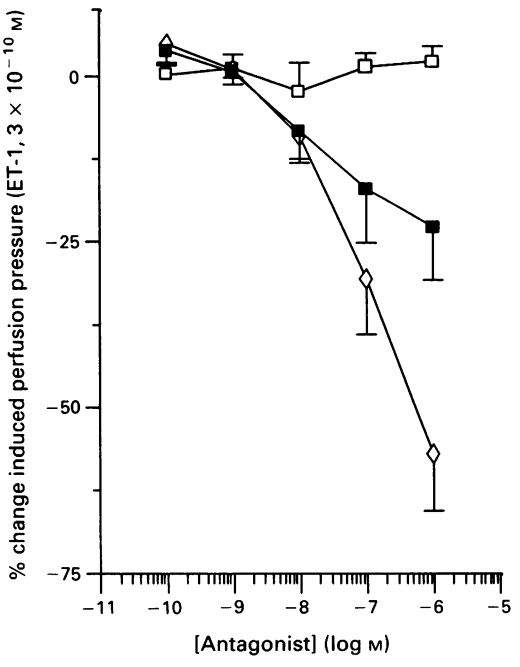



**Figure 7** Reduction by either BQ-123 or PD 145065 of sustained increases in perfusion pressure induced in the rat isolated perfused kidney by ET-1 ($3 \times 10^{-10}$ M). Vehicle ($n = 5$), (□); BQ-123 ($10^{-5}$ M, $n = 4$), (■); PD 145065 ($10^{-5}$ M, $n = 4$), (◇). Results are calculated as the mean (± s.e.mean).

tions of the rat aorta induced by ET-1. Similarly, in the rat isolated perfused kidney in which both $ET_A$ and $ET_B$ receptors mediate the vasoconstrictor effects of ET-1 (Cristol *et al.*, 1993; Warner *et al.*, 1993c), as they do the pressor effects of ET-1 *in vivo* (Cristol *et al.*, 1993; McMurdo *et al.*, 1993), PD 145065 was at least as effective as BQ-123 in reversing the vasoconstrictor effects of ET-1. Indeed, at the highest concentration employed, PD 145065 caused twice as great a reduction in induced pressure as BQ-123. Thus, vasoconstrictions mediated by a mixture of $ET_A$ and $ET_B$ receptors are more readily reversed by PD 145065 than BQ-123. This clearly suggests that the weakness of PD 145065 relative to BQ-123 as an antagonist in our *in vivo* experiments is due to effects at sites additional to the $ET_A$ and $ET_B$ receptors that mediate vasoconstriction.

Possibly this difference is due to PD 145065 *in vivo* blocking the indirect vasodilator effects of ET-1, mediated by $ET_B$ receptors present on the endothelium (Warner *et al.*, 1989; Takayanagi *et al.*, 1991) and mediated by an increased production of nitric oxide and prostacyclin (de Nucci *et al.*, 1988; Whittle *et al.*, 1989). This vasodilatation is revealed by a bolus injection of ET-1. In these experiments PD 145065 in doses 1000 or 10,000 greater than that of ET-1 had no significant effect on the pressor response to ET-1, but did greatly diminish the initial fall in blood pressure. Conversely, BQ-123 and related compounds either do not affect the depressor effect of the endothelins or potentiate it (Ihara *et al.*, 1992; McMurdo *et al.*, 1993). Thus, the anti-pressor effects of PD 145065 may be confounded by a concomitant decrease in the depressor response to ET-1. Similarly, antagonism by PD 145065 of the depressor effects of ET-1 may explain why co-infusion of BQ-123 and PD 145065 was less effective at reversing the pressor effects of ET-1 than infusion of BQ-123 alone.

An alternative explanation for the weaker effect of PD 145065 *in vivo* is that it increases the survival of ET-1 by inhibiting either its uptake and/or metabolism. For instance, if selective binding of ET-1 to its receptors is an important step in its elimination from the circulation, then PD 145056 could increase its circulating half-life (30–60 s, Ängård *et al.*, 1989). In addition, and perhaps more importantly, PD 145065 is a peptide and could be a substrate for ET-1 metabolizing enzymes, such as neutroendopeptidase. Indeed, this latter enzyme abolishes the activity of ET-1 by cleaving it between residues 18 and 19 (Sokolovsky *et al.*, 1990), and this site is contained within the structure of PD 145065.

Thus, PD145065 could inhibit ET-1 degradation by substrate competition.

In all our experiments we saw reversal of the effects of ET-1 following administration of the antagonists in 100–10,000 excess, which may be explained by the very tight binding of ET-1 to its receptors (Hirata *et al.*, 1988; Waggoner *et al.*, 1992). Indeed, in intact cells ET-1 appears only to become dissociated from its receptors following receptor internalisation with about 40% of receptors being recycled after 1 h. Thus, the slow reversal of the effects of ET-1 caused by the antagonists may be a function of their ability to prevent new binding of ET-1 following re-externalization of the receptors (Marsault *et al.*, 1993). Interestingly, it suggests that the endothelin receptor antagonists should be administered in high doses for prolonged periods when testing their effectiveness in models of pathological ET-1 production (Kon *et al.*, 1989; Watanabe *et al.*, 1990). Indeed, when administered as a bolus 10 mg $kg^{-1}$ BQ-123 equivalent to $1.4 \times 10^{-5}$ mol $kg^{-1}$, was needed to reduce the established pressor effects in conscious rats of an infusion of $10^{-11}$ mol $kg^{-1}$ $min^{-1}$ ET-1 (Bazil *et al.*, 1992). The dose of antagonist used, therefore was equivalent to the amount of ET-1 that would be administered in a period of 32 months.

Thus, established vasoconstrictions induced by ET-1 and mediated via $ET_A$ and/or $ET_B$ receptors *in vitro* may be reversed slowly by the subsequent application of receptor antagonists. *In vivo*, the anti-pressor effects of a non-selective receptor antagonist may be limited by its ability to decrease the vasodilator effects of ET-1 and/or reduce the elimination of ET-1. In the rat, therefore, BQ-123, a selective $ET_A$ receptor antagonist, is more active than PD 145065, a non-selective $ET_A/ET_B$ receptor antagonist, in reversing the established hypertensive effects of ET-1.

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# The renal functional responses to 5-$HT_{1A}$ receptor agonist, flesinoxan, in anaesthetized, normotensive rat

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**1** The present study was designed to examine the effects of a centrally acting 5-$HT_{1A}$ receptor agonist, flesinoxan, on the cardiovascular system and renal haemodynamics and excretory function.

**2** In chloralose-urethane anaesthetized Wistar rats, i.v. administration of bolus doses of flesinoxan, at 30, 100, 300 and 1000 $\mu g\,kg^{-1}$, caused significant, dose-dependent decreases in mean arterial pressure, of $33 \pm 2$ mmHg ($P<0.001$) and heart rate of $57 \pm 9$ beats $min^{-1}$ ($P<0.001$) at the highest dose used. Despite this substantial fall in perfusion pressure there were no meaningful changes in the renal excretion of water and sodium. In a second group of rats, reduction of renal perfusion pressure mechanically to the same values as observed in rats given flesinoxan (i.e. 100, 92, 84 and 76 mmHg) produced reductions in urine flow, absolute and fractional sodium excretions reaching a maximum of 74, 86 and 84% respectively (all $P<0.001$) at the lowest pressure. These reductions were significantly larger than those seen in the previous group of animals.

**3** In the group of rats subjected to renal denervation, flesinoxan produced changes in blood pressure and heart rate which were not different from those observed in intact animals. However, the reduction in pressure was accompanied by significant decreases in urine flow of 71%, absolute sodium excretion of 68% and fractional sodium excretion of 67% (all $P<0.001$) at the highest dose, which were all significantly greater than the changes seen in the innervated animals but were not different from those observed when renal perfusion pressure was reduced mechanically.

**4** The findings of this investigation showed that flesinoxan was effective in lowering blood pressure and heart rate in the anaesthetized rat, which was probably due to decreased sympathetic nerve activity. Renal excretion of water and sodium was well preserved in the face of the flesinoxan-induced hypotension. The maintenance of fluid excretion with flesinoxan appeared to be mediated via changes in renal nerve activity, since it did not occur when the kidney was denervated.

**Keywords:** 5-$HT_{1A}$ receptor agonist; flesinoxan; renal function

## Introduction

In recent years evidence has accumulated to show that central neural pathways utilising 5-hydroxytryptamine (5-HT) contribute towards the control of the sympathetic nervous system. Application of 5-HT to lateral ventricles of the cat brain was found to cause a fall in blood pressure, to reduce heart rate and inhibit efferent sympathetic nerve activity (Baum & Shropshire, 1975). In later studies several subtypes of receptors for 5-HT were identified in the brain (Peroutka, 1988) of which the 5-$HT_{1A}$ subtype appeared to mediate the reduction in sympathetic outflow as a result of centrally applied 5-HT (Göthert & Schlicker, 1990). Subsequently specific agonists of this receptor site were developed, such as 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) (Peroutka *et al.*, 1990), and used to determine the effects of 5-$HT_{1A}$ receptor stimulation on the degree of activation of the sympathetic nervous system and thereby the status of the cardiovascular system. There have been a number of reports in which agonists of this receptor site have been shown to reduce blood pressure and heart rate after intravenous (Gradin *et al.*, 1985; Dreteler *et al.*, 1990), intra-arterial (Dreteler *et al.*, 1991) and intracisternal administration (Wouters *et al.*, 1988). These responses appeared to be mediated by receptors located in dorsal raphe nucleus of the brain (Laubie *et al.*, 1989; Connor & Higgins, 1990). More recently Ramage and co-workers have provided evidence in the cat that centrally acting 5-$HT_{1A}$ receptor agonists produce peripheral sympatho-inhibition, with efferent renal nerve activity being most sensitive (Ramage *et al.*, 1988; Ramage & Wilkinson, 1989).

The kidney is very richly innervated with the renal sympathetic nerves supplying the renal vasculature, tubules and juxtaglomerular apparatus (Barajas *et al.*, 1992). It has been shown, both in studies utilising direct renal nerve stimulation as well as in denervation experiments, that the renal nerves can influence renal haemodynamics, tubular sodium reabsorption and renin release from juxtaglomerular cells (DiBona *et al.*, 1988; Johns, 1991). In terms of function, the electrical activity in the renal sympathetic nerves has been shown to change in response to such physiological stimuli as variations in arterial blood pressure (Deka-Starosta *et al.*, 1989) and intravascular volume (DiBona *et al.*, 1988) which consequently determines the level of renal haemodynamics and fluid excretion.

Given the important influence of the renal nerves upon sodium and water excretion and existing evidence that stimulation of central 5-$HT_{1A}$ receptors can decrease efferent renal sympathetic nerve activity, the aim of the present investigation was to examine the effects of the specific 5-$HT_{1A}$ agonist, flesinoxan, on the cardiovascular system and renal nerve-dependent change in renal haemodynamics and water and sodium excretion in anaesthetized, normotensive rats. A preliminary account of this work was presented to the Pharmacological Society meeting at Bradford in September 1993 (Chamienia & Johns, 1993).

## Methods

The experiments were performed on male Wistar rats (mean body weight 300 g) which had been fasted overnight. The animals were initially anaesthetized with 4% halothane in oxygen/nitrous oxide. A cannula was inserted into the right

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femoral vein and a bolus dose of α-chloralose/urethane mixture was given i.v. (initial dose 17.5 mg chloralose, 0.3 g urethane) and an infusion of 3 ml $h^{-1}$ normal saline started. The anaesthesia was maintained throughout the experiment with bolus doses of the same mixture as required and the animals breathed spontaneously throughout. After the induction of anaesthesia, tracheostomy was performed and catheters placed in the right carotid artery, for systemic pressure monitoring, and in the right femoral artery to allow measurements of renal perfusion pressure and arterial blood sampling. Both kidneys were approached retroperitoneally and their ureters cannulated. The left renal artery was then carefully cleared using a dissecting microscope and an electromagnetic flow probe (Carolina EP100 series, internal circumference 2–2.5 mm) placed around it for measuring renal blood flow. Silver wire electrodes were applied to the coeliac/aortico-renal ganglia and pulses of 15 V, 10 Hz, 0.2 ms were delivered for 10 s which caused a transient blanching of the kidney. If blanching did not occur, the kidney was treated as denervated and not included in the study. Both arterial catheters were connected to pressure transducers (Statham P23 I) and the signal fed to a custom built amplifier (Grayden, Birmingham). The flow meter probe was connected to a square wave electromagnetic flowmeter (Model FM501, Carolina Medical Instruments, U.S.A.). Blood pressure and renal blood flow signals were then fed via an I/O card to an Apple Macintosh computer running custom software written in LabVIEW (National Instruments, Austin, TX, U.S.A.) and displayed on the screen. Heart rate was derived from the carotid pressure wave signal on-line. Mean values for all variables were calculated for every 2 s and then averaged over each of 15 min clearance periods. Data were stored on the hard disk for later off-line analysis. On completion of the surgery, a priming dose of 2 ml inulin in saline (1.5 g 100 $ml^{-1}$) was given i.v. and isotonic saline infusion replaced by one containing inulin (1.5 g 100 $ml^{-1}$). Animals were then allowed 2 h to stabilize and reach equilibrium before starting the experiment.

### *Experimental protocols*

In this study, urine was collected from the ureters over 15 min and this was termed a clearance period. Five pairs of 15 min clearance periods were performed, with approximately 15 min intervals between each pair which allowed for blood sampling and for any drug to be flushed into the circulation via the venous cannula. Arterial blood samples (300 μl each) were taken before the first and then after the end of each pair of clearances. The blood samples were immediately centrifuged and plasma obtained, the red cells were resuspended in an equivalent volume of heparinized saline and reinfused into the animal within 5 min. Urine was collected in preweighed microcentrifuge capped tubes. Three groups of animals were studied:

*Group 1* ($n = 8$). The first pair of clearance periods provided basal values. The rats then received flesinoxan as a bolus i.v. dose 10 min before the start of remaining pairs of clearances at doses of 30, 100, 300 and 1000 μg $kg^{-1}$ in a cumulative fashion. Flesinoxan was injected slowly over 30 s in 300 μl of normal saline.

*Group 2* ($n = 6$). In this group of rats, a loop of surgical thread was placed around the aorta, between the renal arteries, and attached to a screw device to allow reduction of left kidney perfusion pressure when tightened. The first two clearances were completed at prevailing pressure and then the perfusion pressure was lowered in steps to the same values as observed in the Group 1 animals at each dose of flesinoxan, and 10 min later the next pair of clearances begun. The levels of perfusion pressure achieved were 100, 92, 84 and 76 mmHg respectively. Only left kidney function was studied in this group.

*Group 3* ($n = 6$). In this group of animals, in addition to the surgery described above, the left renal sympathetic nerves were identified, carefully dissected and cut. Only left kidney function was studied in this group. Rats received flesinoxan at the same doses as in group 1.

### *Chemical assays*

Urinary and plasma electrolyte concentrations were measured by flame photometry (Ciba Corning 410C). Plasma and urine samples inulin was measured as described previously (Chamienia & Johns, 1991).

### *Drugs and chemicals*

Flesinoxan was a gift from Solvay-Duphar B.V., Weesp, The Netherlands. Inulin and all other chemicals were purchased from Sigma, Poole, Dorset.

### *Statistical analysis*

All values are presented as means ± s.e.mean. The mean values of all variables were calculated for each pair of clearances. Statistical analysis was performed with repeated measures analysis of variance (using an Apple Macintosh computer and SuperANOVA software, Abbacus Concept, Berkeley, CA, U.S.A.). The effects were taken to be significant when $P < 0.05$.

## Results

Figure 1 presents the mean arterial pressure, renal perfusion pressure and heart rate in the three groups of experiments. In the first group, flesinoxan caused dose-dependent falls in both mean arterial pressure and renal perfusion pressure, of 33 ± 2 and 34 ± 2 mmHg respectively at the highest dose (both $P < 0.001$). This was accompanied by a progressive decrease in heart rate reaching some 57 ± 9 beats $min^{-1}$ ($P < 0.001$) at the highest dose of the drug. Renal blood flow (RBF) and glomerular filtration rate (GFR) did not change significantly throughout the experiment. Despite the profound fall in perfusion pressure, the left kidney urine flow (UV), urinary sodium excretion ($U_{Na}V$) and fractional sodium excretion ($FE_{Na}$) fell only slightly but not significantly by 28, 29 and 16% respectively, at the highest dose of drug. At the same time, right kidney UV, $U_{Na}V$ and $FE_{Na}$ were slightly, but not significantly, raised by 13, 14 and 11% respectively (Figure 2).

In the second group of animals, mean arterial pressure increased slightly by 8 ± 6 mmHg during stepwise reduction in left renal perfusion pressure. Levels of perfusion pressure obtained by applying aortic constriction were very similar to those observed in the animals in group 1 (Figure 1). Heart rate did not change at any time throughout the experiment in this group of rats, which was significantly different from the dose-related decreases in heart rate observed in the group 1 rats given flesinoxan ($P < 0.001$). RBF and GFR in this group were not different from those seen in group 1 and remained unchanged during the experiment. Urine flow decreased significantly (by 74% at the lowest pressure; $P < 0.001$), which was significantly less than the values observed for the right kidney in the previous group ($P < 0.05$). Absolute and fractional sodium excretions also decreased significantly (both $P < 0.001$) by 86 and 84%, respectively, at the lowest pressure. These falls in $U_{Na}V$ and $FE_{Na}$ were significantly different from the changes seen in group 1 ($U_{Na}V$: $P < 0.02$ and $P < 0.01$; $FE_{Na}$: $P < 0.02$ and $P < 0.001$ vs left and right kidneys of group 1 animals, respectively).

In the last group of animals, flesinoxan caused dose-dependent decreases in mean arterial pressure of 29 ± 4 mmHg, renal perfusion pressure of 30 ± 4 mmHg and heart rate of 47 ± 14 beats $min^{-1}$ (all $P < 0.001$) which were not

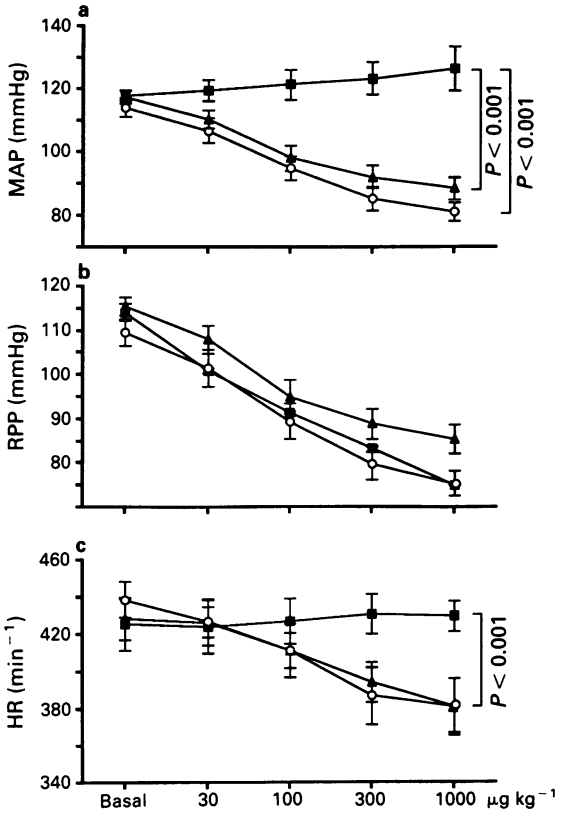


**Figure 1** This shows the mean arterial pressure (MAP) (a), renal perfusion pressure (RPP) (b) and heart rate (HR) (c) in three groups of animals. Each point represents the average value of the two 15 min clearance periods. Groups 1 (○) and 3 (▲) received flesinoxan; in Group 2 (■) left renal perfusion pressure was reduced by aortic constriction. The *P* values are generated in the comparison between the group 1 data and that of groups 2 or 3 using repeated measures ANOVA.

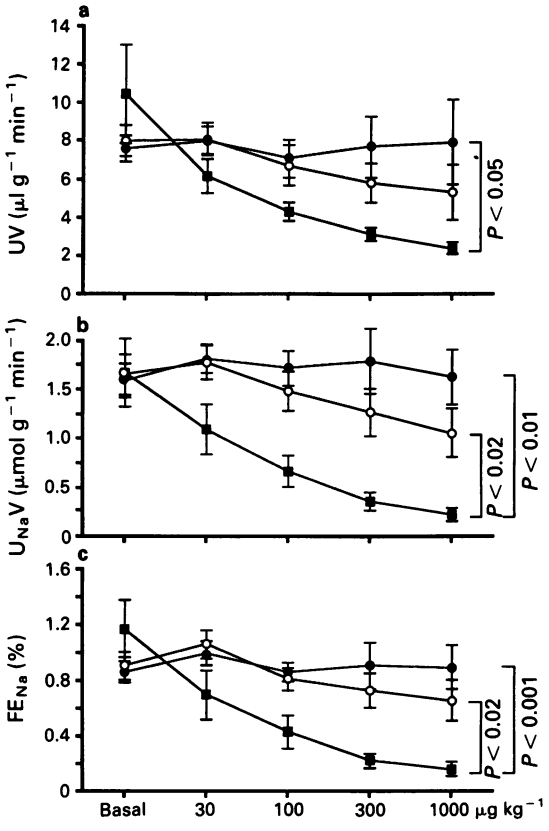


**Figure 2** This shows the urine flow (UV) (a), urinary sodium excretion ($U_{Na}V$) (b) and fractional sodium excretion ($FE_{Na}$) (c) of animals in group 1 left kidney (○), right kidney (●) and group 2 (■). The results for both kidneys of animals in group 1 are shown. Each point represents the average value of the two 15 min clearance periods. The *P* values are generated in the comparison between the group 1 data and that of group 2 or 3 using repeated measures ANOVA.

different from those observed in group 1. RBF fell slightly but not significantly and GFR did not change. Urine flow, absolute and fractional sodium excretions decreased significantly with increasing dose of flesinoxan (all $P<0.001$). These falls reached 71, 68 and 67% respectively at the highest dose and were not significantly different from those observed in the group 2, when perfusion pressure was reduced. However, these reductions in urine and sodium excretion in the denervated kidney were significantly different from those observed in group 1 (UV; $P<0.005$ and $P<0.001$; $U_{Na}V$: $P<0.05$ and $P<0.001$; $FE_{Na}$: $P<0.02$ and $P<0.001$ vs. left and right kidneys of animals in group 1 respectively). The urine and sodium excretions in this group of animals are presented graphically in Figure 3.

## Discussion

The purpose of the present study was to investigate the renal functional responses to 5-$HT_{1A}$ receptor stimulation by a specific agonist and to determine the role, if any, of the renal sympathetic nerves. The results from the normotensive Wistar rats showed that intravenous administration of flesinoxan caused dose-dependent decreases in blood pressure which were accompanied by falls in heart rate. This was in agreement with previously published results of studies in cats (Dreteler *et al.*, 1989) and rats (Dreteler *et al.*, 1990). It has been suggested that the hypotension induced by 5-$HT_{1A}$ receptor stimulation depends on inhibition of peripheral sympathetic tone with a resultant decrease in total vascular resistance. Dreteler *et al.* observed a reduction in vascular resistance in renal and cerebral circulations, but no change in cardiac output after flesinoxan administration in cats (Dreteler *et al.*, 1989) and spontaneously hypertensive rats (Dreteler *et al.*, 1991). An increase in vagal tone appears to contribute to the bradycardia since this effect was reported to be abolished by atropine and vagotomy in the cat (Ramage *et al.*, 1988).

In our experiments flesinoxan had little effect on glomerular filtration rate (GFR) or renal blood flow (RBF). This demonstrates that over this range of perfusion pressure both RBF and GFR were effectively autoregulated. However, despite the profound fall in blood pressure, renal excretion of urine and sodium was well preserved. This response was intriguing as it has been shown previously, that urine and sodium excretions depend critically on the level of renal perfusion pressure (pressure-natriuresis) (Roman & Cowley, 1985; Roman *et al.*, 1988) and therefore it might have been anticipated that urinary volume and sodium excretion would decrease in parallel with the hypotension. Indeed, in the study in which renal perfusion pressure was lowered by the means of aortic constriction to levels similar to those observed with flesinoxan, urine flow, absolute and fractional sodium excretions all fell significantly along with perfusion pressure. The mechanism by which renal excretory function was preserved in the presence of flesinoxan is not clear. Baum & Shropshire (1975) in early experiments were able to show an inhibition of efferent renal sympathetic nerve activity (ERSNA) due to a central action of 5-HT. More recently, Stein *et al.* (1987) and Montes & Johnson (1990) have shown

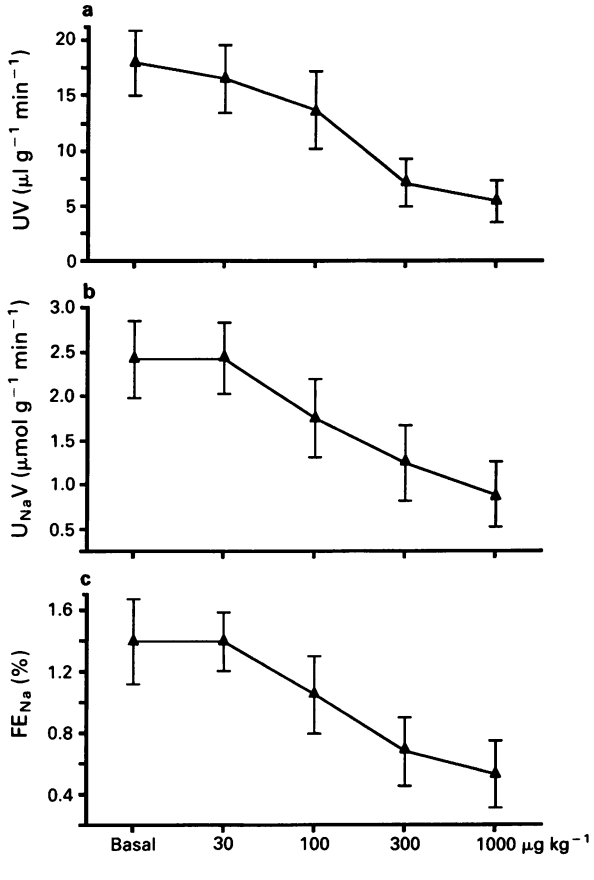


**Figure 3** This shows the urine flow (UV) (a), urinary sodium excretion ($U_{Na}V$) (b) and fractional sodium excretion ($FE_{Na}$) (c) of animals in group 3. Rats in this group had the kidney surgically denervated. Each point represents the average value of the two 15 min clearance periods. The *P* values are generated in the comparison between the group 1 data and those of group 2 or 3, using repeated measures ANOVA.

increased water and electrolyte excretion following intraventricular application of 5-HT in hydrated, conscious rats. The latter authors additionally found ERSNA to decrease after central administration of 5-HT. Clearly 5-HT will be acting on a range of different 5-HT receptors each having its own specific function but there is a recognition that activation of 5-$HT_{1A}$ receptors results in inhibition of sympathetic outflow. Ramage *et al.* (1988) and Ramage & Wilkinson (1989) have reported that intravenous and intraventricular injections of specific agonists of 5-$HT_{1A}$ receptor subtype, such as 8-OH-DPAT and flesinoxan, led to decreases in sympathetic activity recorded in cardiac and renal sympathetic nerves, furthermore, they found that these 5-$HT_{1A}$ agonists preferentially inhibited renal nerve activity in the anaesthetized cat.

The renal sympathetic nerves may influence renal haemodynamics under certain circumstances, like hard exercise, psychological stress or haemorrhage, but under normal conditions renal nerve activity is low, having minimal haemodynamic effect. Moreover, low levels of direct renal nerve stimulation have been shown to influence tubular water and sodium reabsorptions without any detectable changes in renal blood flow or glomerular filtration (DiBona, 1989). This view has been supported by experiments which showed that renal denervation could enhance excretion of water and sodium without changes in renal haemodynamics (Pelayo *et al.*, 1983; Bencsath *et al.*, 1985). Furthermore, such physiological stimuli as variations in arterial blood pressure (Deka-Starosta *et al.*, 1989) and intravascular volume (DiBona *et al.*, 1988) have been shown to cause reflex changes of ESRNA and renal nerve-dependent changes in water and sodium excretion. Consequently, in a physiological situation, a fall in blood pressure produces a reflex increase in ESRNA and increases the reabsorption of water and sodium. An increase in intravascular volume or blood pressure, on the other hand, results in a decrease in ESRNA, which in turn promotes water and sodium excretion.

Thus, one of the most likely explanations of our findings would be that flesinoxan via its action on central 5-$HT_{1A}$ receptors causes inhibition of ESRNA, which contributes to unchanged excretion of water and sodium. Although a decrease in ESRNA in itself could have been expected to cause a diuresis and natriuresis, a concomitant large fall in renal perfusion pressure was present, which would have an opposite effect. Hence the net result would be an unchanged excretion of urine and sodium in face of falling perfusion pressure. The data obtained in the last group of animals lend further support to the role of renal nerves. In those rats with denervated kidneys, i.v. flesinoxan caused a similar reduction in perfusion pressure to that observed in intact animals. However, when perfusion pressure decreased there were corresponding reductions in urine and sodium excretions, indeed in a manner similar to that present in animals of group 2. Taken together these results clearly support the role of renal nerves in the changes in renal excretory function induced by systemic administration of flesinoxan.

In contrast to the results reported by Stein *et al.* (1987) and Montes & Johnson (1990), we did not observe a positive diuresis or natriuresis. It is possible that the different routes of administration and different specificity of the agonist used may partly explain this discrepancy. It should also be noted that in both studies only minor falls in perfusion pressures (approx. 10 mmHg) were observed. Furthermore, both studies utilized conscious animals, while we used anaesthetized rats in our investigation. In addition, the animals in both reports cited above were hydrated with intravenous infusion of 6–12 ml $h^{-1}$ of fluid, which most probably induced a diuretic state.

Although the effects of flesinoxan in our study appear to be mediated via a central mechanism it is of interest that 5-$HT_{1A}$ receptors have recently been found in rat and human kidneys. Those receptors appear to be present on the basolateral membranes of the cells in medullary and cortical thick ascending limbs, distal convoluted tubules and initial portion of collecting tubules (Raymond *et al.*, 1993). The role of those receptors in the regulation of the renal function is not clear at the present time. However it is unlikely that they contributed to the responses observed in the present study as they would have been equally activated in both the innervated and denervated kidneys.

This study attempted to assess the effects of a novel 5-$HT_{1A}$ receptor agonist, flesinoxan, on renal function. We confirmed the findings in earlier reports that flesinoxan is effective in lowering blood pressure and heart rate in anaesthetized rat. Moreover we were able to establish that the renal excretion of urine and sodium is well preserved during flesinoxan-induced hypotension and this is a function of the renal nerves. It is likely that it reflects an inhibition of renal sympathetic outflow by flesinoxan leading to a partial denervation diuresis and natriuresis. This finding may suggest a potential role for this drug in the treatment of hypertension.

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# Differential effects of suramin on $P_2$-purinoceptors mediating contraction of the guinea-pig vas deferens and urinary bladder

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**1** The effect of the $P_2$-purinoceptor antagonist, suramin, was investigated on contractions of the guinea-pig vas deferens and urinary bladder induced by adenosine 5'-triphosphate (ATP) and by the other naturally occurring nucleoside triphosphates.

**2** ATP, guanosine 5'-triphosphate (GTP), cytidine 5'-triphosphate (CTP), inosine 5'-triphosphate (ITP) and uridine 5'-triphosphate (UTP) (0.1–500 μM) each contracted both the guinea-pig bladder and the guinea-pig vas deferens. In the vas deferens the order of potency of the nucleotides was ATP >> CTP > GTP ≥ UTP = ITP, and in the bladder it was ATP >> CTP = GTP > UTP = ITP, although maximal responses to these agonists were not achieved in either tissue.

**3** Suramin (30 μM–1 mM) dose-dependently inhibited ATP-induced contractions of the bladder in an apparently non-competitive manner, causing a reduction in the slope of the concentration-response curve to ATP. In contrast, suramin (5 μM–1 mM) had little inhibitory effect on ATP-induced contractions of the vas deferens, and indeed at concentrations of 100 μM and above markedly potentiated high concentrations of ATP (100–500 μM). The contractions induced by CTP, GTP, UTP and ITP (1–500 μM) were, however, abolished by suramin (1 mM) in each tissue.

**4** Desensitization of the $P_{2X}$ purinoceptors in the guinea-pig vas deferens with adenosine 5'-α,β-methylenetriphosphonate (AMPCPP) (300 μM) abolished contractions induced by ATP (1 μM–1 mM) in the absence of suramin. However, the contractions induced in the presence of suramin were unaffected by prior desensitization, indicating that they were not mediated by $P_{2X}$-purinoceptors.

**5** ATP (100 μM) was dephosphorylated by both isolated tissue preparations under the conditions of these experiments, breakdown products being detectable after 2 min, with the major breakdown product in the bladder being inosine whereas that in the vas deferens was adenosine. Approximately 35% of the ATP remained intact after incubation for 30 min with the bladder, and approximately 45% remained after incubation for 30 min with the vas deferens. In each tissue this degradation was inhibited by suramin (1 mM), so that after incubation of ATP (100 μM) in the presence of suramin for 30 min, approximately 50% remained in the case of the bladder and approximately 65% remained in the vas deferens. However, inhibition of the production of the inhibitory agonist, adenosine by suramin did not appear to be responsible for the potentiation observed in the vas deferens, as the $P_1$-purinoceptor antagonist 8-sulphophenyltheophylline (100 μM) did not reduce this potentiation.

**6** Chelation of divalent cations did not appear to account for the enhancement by suramin of ATP-induced contractions of the vas deferens, as the enhancement was still observed when $Mg^{2+}$ was omitted from the buffer or when its concentration (normally 1.2 mM) was increased ten fold to 12 mM, or when the concentration of $Ca^{2+}$ (normally 2.5 mM) was reduced to 0.83 mM. Even in the absence of $Mg^{2+}$ and with the $Ca^{2+}$ concentration reduced to 0.83 mM, no inhibition by suramin (1 mM) of ATP-induced contractions was observed.

**7** The most likely explanation for the potentiation by suramin of the ATP-induced contractions of the vas deferens is the co-existence of inhibitory $P_{2Y}$-purinoceptors. However, no consistent relaxations to ATP (1–100 μM) or to the more potent $P_{2Y}$-purinoceptor agonist 2-methylthioadenosine 5'-triphosphate (2-MeSATP) (0.01–100 μM) could be detected in the vas deferens precontracted with KCl (35 mM), even after desensitization of $P_{2X}$-purinoceptors with AMPCPP (300 μM). Similarly, ATP (1–100 μM) or 2-MeSATP (0.01–100 μM) added before KCl (35 mM), carbachol (10 μM) or noradrenaline (10 μM) did not reduce subsequent contractions to these agents.

**8** The differential effect of suramin on the contractions induced by ATP in the bladder and the vas deferens was unexpected, and shows that the receptor populations by which ATP acts in these tissues may not be identical. The failure of suramin to inhibit responses to ATP in the vas deferens suggests that this tissue, in addition to possessing $P_{2X}$-purinoceptors may also possess a suramin-insensitive contractile ATP receptor revealed in the presence of suramin.

**Keywords:** ATP; purinoceptors; nucleotides; vas deferens; bladder; suramin

## Introduction

Adenosine 5'-triphosphate (ATP) contracts the vas deferens and the urinary bladder of several species, and there is evidence that it acts as an autonomic transmitter or co-transmitter in these tissues (for review see Hoyle, 1992). The $P_2$-purinoceptors by which it acts in these tissues have been classified as belonging to the $P_{2X}$ subclass, and indeed these were two of the tissues on the basis of which the subdivision of $P_2$-purinoceptors into $P_{2X}$ (contractile) and $P_{2Y}$ (relaxant) was first proposed (Burnstock & Kennedy, 1985). This subdivision was made not only on the basis of the different effects observed, but also on the basis of different agonist potency orders. At the $P_{2X}$-purinoceptor adenosine 5'-α,β-methylenetriphosphonate (AMPCPP) is more potent than ATP which is equipotent with 2-methylthioadenosine 5'-

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triphosphate (2-MeSATP), whereas at the $P_{2Y}$-purinoceptor 2-MeSATP is more potent than ATP which is more potent than AMPCPP. Unfortunately no selective competitive antagonists exist which discriminate between these two subtypes and the best antagonist available so far, suramin, has equal activity in both the guinea-pig bladder ($P_{2X}$) and the guinea-pig taenia caeci ($P_{2Y}$) with an apparent $pA_2$ of approximately 5 (Hoyle *et al.*, 1990). Suramin has also been shown to inhibit the effects of AMPCPP in mouse vas deferens with a similar $pA_2$ value (Dunn & Blakeley, 1988; Von Kügelgen *et al.*, 1990), as well as the effect of ATP on $DDT_1$ MF-2 cells, which are derived from Syrian hamster vas deferens (Hoiting *et al.*, 1990). In addition, suramin inhibits the first phase of the neurogenic contractions in the mouse vas deferens (Von Kügelgen *et al.*, 1989) and the excitatory junction potentials in the guinea-pig vas deferens (Sneddon, 1992), both of which are thought to be due to released ATP. However, the effects of high concentrations of ATP in the mouse vas deferens were reported to be relatively resistant to inhibition by suramin, which caused small shifts in the concentration-response curve which were not dose-dependent. Concentrations of ATP up to 1 μM were however inhibited in an apparently competitive manner, giving a Schild slope close to unity with a $pA_2$ value for suramin again being approximately 5 (Von Kügelgen *et al.*, 1990).

Although the $P_2$-purinoceptors in the guinea-pig bladder and the guinea-pig vas deferens are both classed as $P_{2X}$ (Burnstock & Kennedy, 1985), the detailed structure-activity relationships for agonists are not identical in these two tissues. In particular, the L-enantiomers of ATP and some 2-substituted analogues are much less potent than the natural D- enantiomers in the vas deferens (Burnstock *et al.*, 1985), whereas the stereoselectivity is much less marked in the bladder (Burnstock *et al.*, 1983). Indeed in the case of adenylyl 5'-(β,γ-methylene)diphosphonate (AMPPCP) the L-enantiomer is more potent than the D-enantiomer in the bladder (Cusack & Hourani, 1984). The contractions of the guinea-pig vas deferens to ATP have been reported to be biphasic, with the second tonic phase, which is only apparent at very high (mM) concentrations of ATP, suggested to be due to cleavage of the phosphate chain with transfer of phosphate to the tissue (Fedan & Lamport, 1990a; Lamport-Vrana *et al.*, 1991). In addition, although some $Ca^{2+}$ is necessary for contraction, reducing the extracellular divalent cation concentration has been reported to potentiate responses to ATP (Morishita & Furukawa, 1989), and the active form of ATP in the guinea-pig vas deferens has been suggested to be $ATP^{4-}$ (Fedan *et al.*, 1990).

As well as acting at $P_2$-purinoceptors, ATP can be rapidly dephosphorylated by ectonucleotidases ultimately to adenosine, which has inhibitory effects via $A_2$ receptors on most smooth muscle preparations (White, 1988). This breakdown of ATP and of some of its analogues may influence their observed potency in tissues such as the guinea-pig bladder in which adenosine opposes the effect of ATP (Welford *et al.*, 1987), but may be less important in tissues such as the taenia caeci in which ATP and adenosine induce the same response (Welford *et al.*, 1986). However, in some tissues, such as the longitudinal muscle of the rat colon, the breakdown of ATP is so rapid that it appears to act entirely via its breakdown product, adenosine, which makes determination of the structure-activity relationships for $P_2$ purinoceptors very difficult (Bailey & Hourani, 1992). In addition to acting as a $P_2$-purinoceptor antagonist, high concentrations (1 mM and above) of suramin also inhibit the breakdown of ATP by ectonucleotidases in the guinea-pig bladder (Hourani & Chown, 1989), which may complicate its use as an antagonist.

Because of the differences between the stereoselectivity of ATP analogues in the guinea-pig vas deferens and bladder, it appeared possible that the $P_{2X}$-purinoceptors in these two tissues may not be identical, and we therefore compared in detail the effects of suramin on the responses to ATP in these tissues.

## Methods

### *Pharmacological studies*

Male guinea-pigs, 350–500 g, were killed by cervical dislocation and the urinary bladder and vas deferens were removed. The tissues were cleared of connective tissue, and the mucosal layer was removed from the bladder which was cut into strips approximately 5 mm wide. The tissues (15–20 mm long) were mounted in 3 ml organ baths and maintained at 35°C in Krebs solution of the following composition (mM): NaCl 120, KCl 5.9, $MgCl_2$ 1.2, $NaHCO_3$ 15.4, $NaHPO_4$ 1.2, $CaCl_2$ 2.5 and glucose 11.5, gassed with 95% $O_2$/5% $CO_2$. In some experiments the concentration of divalent cations in the buffer was modified, by omitting $MgCl_2$ or by increasing the concentration to 12 mM, or by reducing the concentration of $CaCl_2$ to 0.83 mM, the concentration reported to potentiate maximally ATP-induced contractions of the guinea-pig vas deferens (Morishita & Furukawa, 1989). A resting tension (1 g for the bladder, 0.5 g for the vas deferens) was applied to the tissues, and contractions were recorded isometrically with Grass FT03 transducers connected to Grass 79D polygraphs. The tissues were allowed to equilibrate for 1 h, suramin was added (or water for the controls) and the tissues were incubated for a further hour, after which non-cumulative concentration-response curves were constructed by giving increasing concentrations of agonists with at least 15 min between doses. Where 8-sulphophenyltheothylline (8-SPT) was used, this was added to the organ baths at the same time as suramin. In some experiments on the guinea-pig vas deferens the $P_{2X}$-purinoceptors were desensitized by a single addition of AMPCPP (300 μM), left in contact with the tissues for 10 min before washing out and incubating for 15 min with suramin (1 mM) or water as usual. This desensitization lasted for approximately 75 min, as assessed in the time-matched water controls, and so for these experiments only four points (randomized) on the concentration-response curve could be obtained on each tissue.

Experiments using suramin were carried out in parallel with control experiments, using paired tissues from the same animal, with only one concentration-response curve determined on each tissue. All contractions were expressed as a percentage of the contraction induced by ATP (500 μM) applied at the start of the experiment, before incubation of the tissues with suramin or 8-SPT, desensitization with AMPCPP or modification of the buffer.

### *Degradation studies*

The degradation of ATP (100 μM) in the presence and absence of suramin (1 mM) was followed by taking aliquots (50 μl) of the Krebs buffer from the organ bath at various times after the addition of ATP, in experiments carried out under identical conditions to the pharmacological studies except that the tissues were not washed after ATP was added. The aliquots were stored frozen at −20°C for later analysis by high performance liquid chromatography (h.p.l.c.) as described in detail elsewhere (Hourani *et al.*, 1991).

### *Drugs*

The naturally occurring nucleotides, AMPCPP, carbachol and noradrenaline were obtained from Sigma Chemical Co. (Poole), 8-SPT and 2-MeSATP were obtained from Research Biochemicals, Natick, MA (U.S.A.) and suramin was a generous gift from Bayer, UK. The purity of all nucleotides was checked before use by h.p.l.c. as above. All drugs were dissolved in water and stored frozen.

## Results

ATP contracted both the guinea-pig vas deferens and the guinea-pig bladder in a dose-dependent manner, although the

concentration-response curves did not reach a plateau in either tissue even at a concentration of 500 μM. Concentration-response curves for ATP (1–500 μM) were established on both tissues in the presence of various concentrations of suramin (5 μM–1 mM), but for clarity only the effects of 10 μM, 100 μM and 1 mM are shown, together with the effects of each concentration on responses induced by 30 and 500 μM ATP (Figure 1). Suramin at concentrations up to 10 μM had no effect on contractions of the guinea-pig bladder but was inhibitory at higher concentrations, causing a reduction in the slope of the concentration-response curves and an apparently reduced maximal response. In contrast, suramin did not consistently or dose-dependently inhibit ATP-induced contractions of the vas deferens even at low concentrations of ATP (e.g. 30 μM), and indeed at high concentrations of ATP (e.g. 500 μM) suramin at concentrations of 100 μM and above markedly potentiated the contractions (Figure 1). Guanosine 5′-triphosphate (GTP), cytidine 5′-triphosphate (CTP), inosine 5′-triphosphate (ITP) and uridine 5′-triphosphate (UTP) also each contracted the bladder and the vas deferens, and in the vas deferens the order of

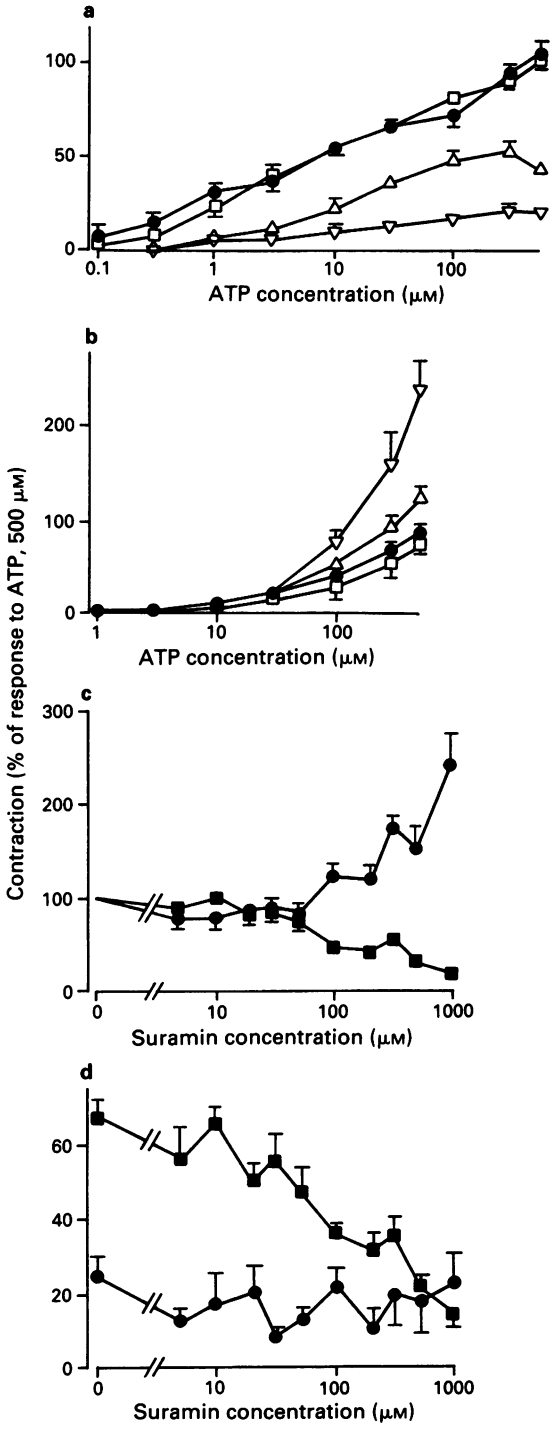


**Figure 1** Effect of suramin on ATP-induced contractions of the guinea-pig vas deferens and urinary bladder: contractions of the bladder (a) and vas deferens (b) are shown induced by ATP alone (●) or in the presence of 10 μM (□), 100 μM (△) or 1 mM (▽) suramin; (c) and (d) show the effect of suramin on contractions of the bladder (■) or vas deferens (●) induced by one concentration of ATP, 500 μM in (c), 30 μM in (d). All contractions are expressed as a percentage of the contraction induced by a control dose of ATP (500 μM) given at the start of the experiment. Each point is the mean of at least four determinations; s.e.mean are shown when they are larger than the symbols.

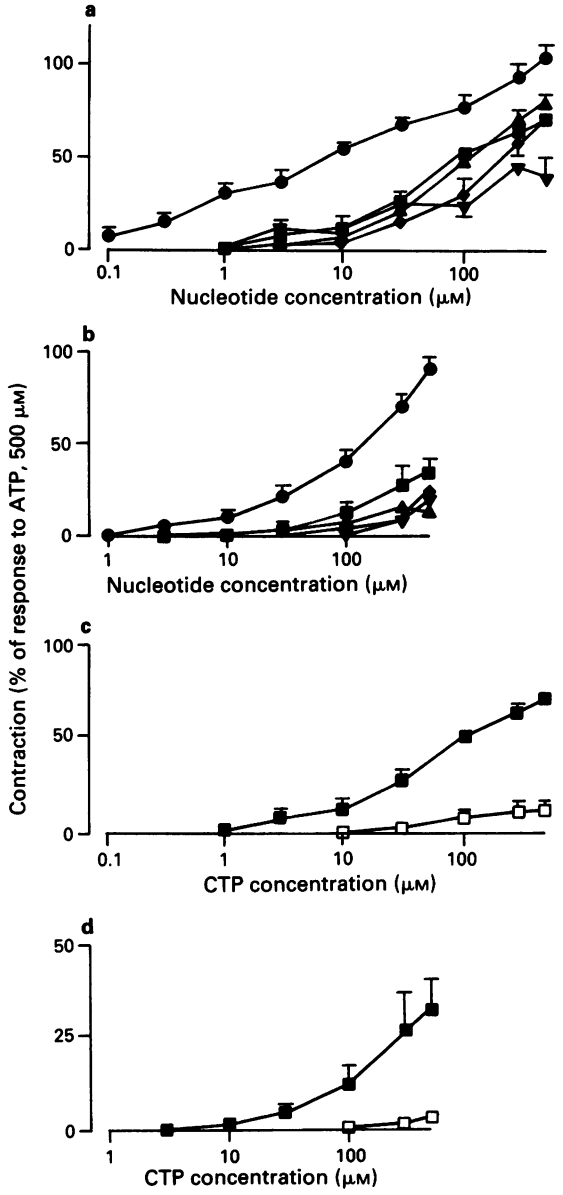


**Figure 2** Contractions of the guinea-pig urinary bladder (a) or the guinea-pig vas deferens (b) induced by ATP (●), CTP (■), GTP (▲), UTP (◆) or ITP (▼). The contractions induced by CTP alone (■) or in the presence of suramin (1 mM) (□) are shown for the bladder in (c) and for the vas deferens in (d). All contractions are expressed as a percentage of the contraction induced by a control dose of ATP (500 μM) given at the start of the experiment. Each point is the mean of at least four determinations; s.e.mean are shown when they are larger than the symbols.

potency was ATP >> CTP > GTP ≥ UTP = ITP, whereas in the bladder it was ATP >> CTP = GTP > UTP = ITP. Again, however, the concentration-response curves to these agonists did not plateau in either tissue (Figure 2a,b). In both tissues suramin (1 mM) abolished responses to CTP (Figure 2c,d) and also responses to GTP, UTP and ITP (results not shown).

Desensitization of the vas deferens with AMPCPP (300 μM, 10 min) abolished contractions induced by ATP in the absence of suramin, but contractions in the presence of suramin (1 mM) were unaffected by prior desensitization with AMPCPP (Figure 3).

8-SPT (100 μM) did not affect the ATP-induced contractions of the vas deferens, nor did it reduce the potentiation caused by suramin (1 mM) of these contractions (Figure 4).

ATP-induced contractions of the vas deferens were enhanced by removing $Mg^{2+}$ from the buffer, and reduced by increasing the concentration from the normal 1.2 mM to 12 mM. For example, in the absence of $Mg^{2+}$ the contractions induced by ATP (500 μM) increased to 156 ± 16%, whereas in the presence of 12 mM $Mg^{2+}$ the contractions were 44 ± 14%. However, in each case suramin enhanced the ATP-induced contractions in a similar manner (Figure 5a,b). Similarly, reducing the concentration of $Ca^{2+}$ in the buffer from 2.5 mM to 0.83 mM did not abolish the enhancement, although it reduced the contractions to ATP, with 500 μM ATP now only achieving 51 ± 3% (Figure 5c). When $Mg^{2+}$ was omitted and the concentration of $Ca^{2+}$ was reduced to 0.83 M the contractions were enhanced, with ATP (500 μM) causing 125 ± 4% contraction, but suramin (1 mM) still did not inhibit these contractions (Figure 5d).

Attempts to demonstrate directly any inhibitory effects of ATP or of 2-MeSATP in the vas deferens were unsuccessful

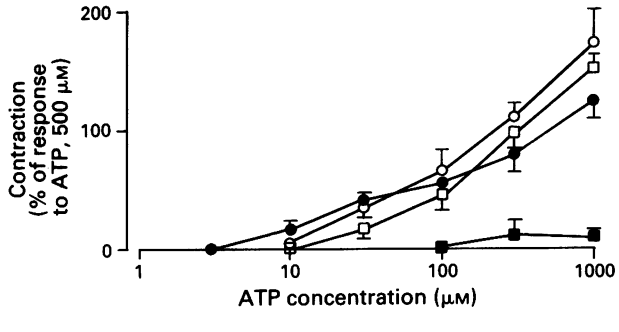


**Figure 3** Contraction of the guinea-pig vas deferens induced by ATP alone (●, ○) or after desensitization with adenosine 5'-α,β-methylenetriphosphonate (AMPCPP) (300 μM, 10 min) (■, □). Closed symbols show the responses in the absence of suramin, and open symbols the responses in the presence of suramin (1 mM). All contractions are expressed as a percentage of the contraction induced by a control dose of ATP (500 μM) given at the start of the experiment. Each point is the mean of at least four determinations; s.e.mean are shown when they are larger than the symbols.

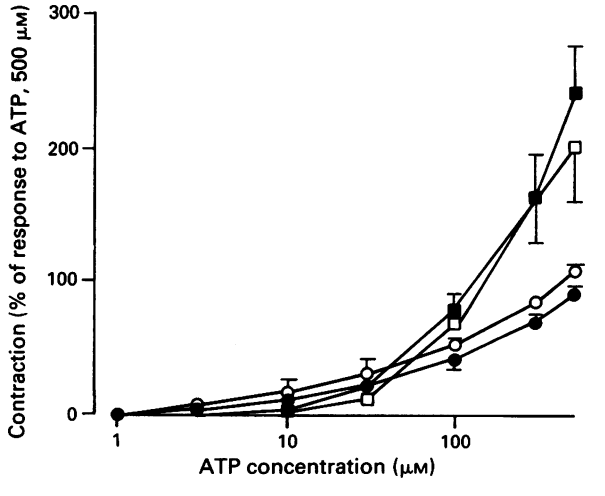


**Figure 4** Contraction of the guinea-pig vas deferens by ATP alone (●) or in the presence of 8-sulphophenyltheophylline (8-SPT) (100 μM) (○) or suramin (1 mM) (■) or 8-SPT (100 μM) plus suramin (1 mM) (□). All contractions are expressed as a percentage of the contraction induced by a control dose of ATP (500 μM) given at the start of the experiment. Each point is the mean of at least four determinations; s.e.mean are shown when they are larger than the symbols.

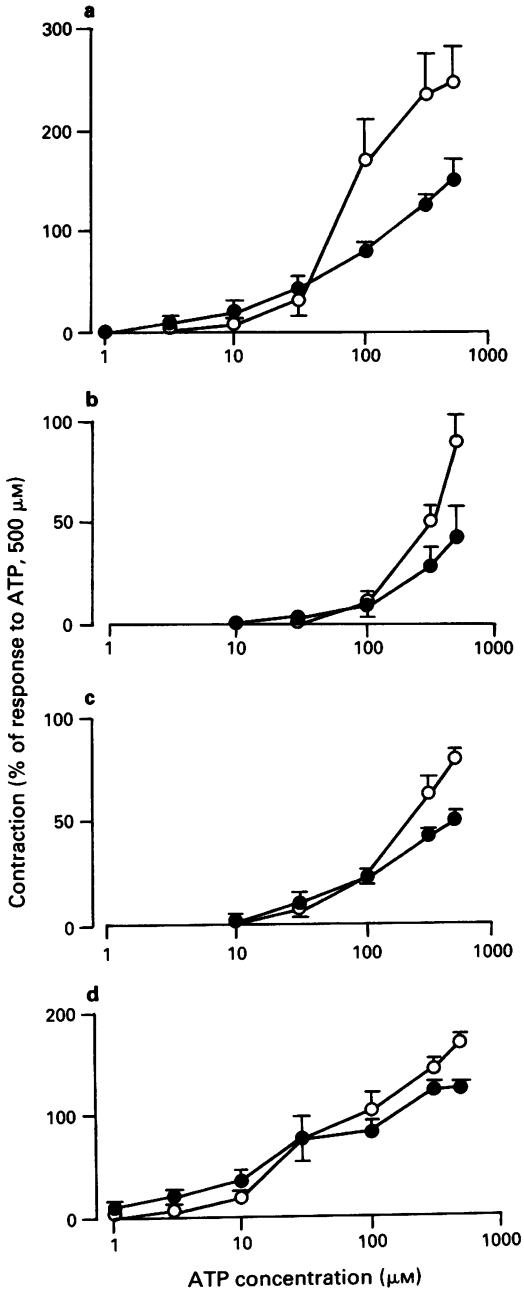


**Figure 5** Contraction of the vas deferens by ATP alone (●) or in the presence of suramin (1 mM) (○) with various concentrations of divalent cations in the buffer: (a) $Ca^{2+}$ 2.5 mM, no $Mg^{2+}$; (b) $Ca^{2+}$ 2.5 mM, $Mg^{2+}$ 12 mM; (c) $Ca^{2+}$ 0.83 mM, $Mg^{2+}$ 1.2 mM; (d) $Ca^{2+}$ 0.83 mM, no $Mg^{2+}$. All contractions are expressed as a percentage of the contraction induced by a control dose of ATP (500 μM) given at the start of the experiment. Each point is the mean of at least four determinations; s.e.mean are shown when they are larger than the symbols.

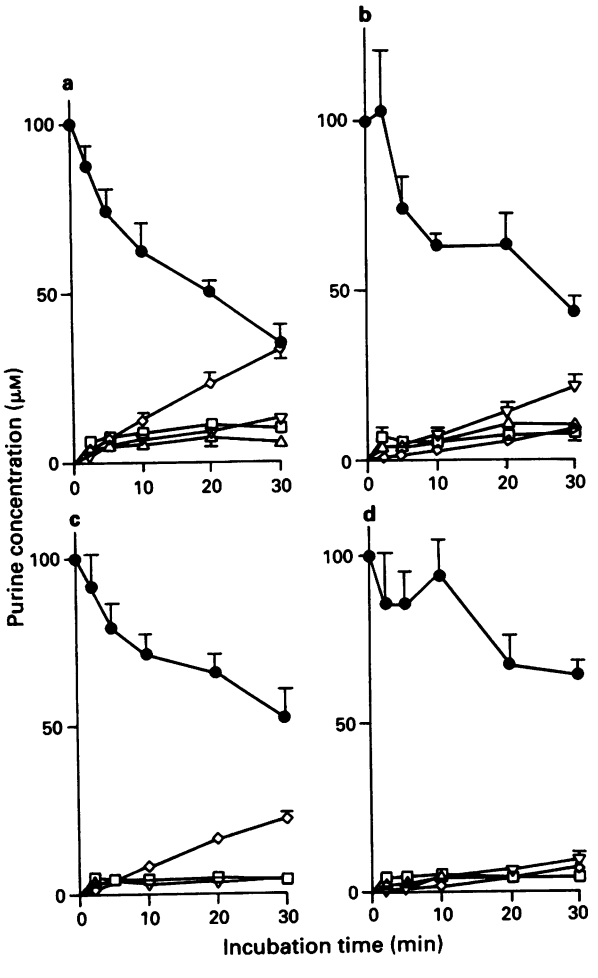


**Figure 6** Degradation of ATP (100 μM) (●) and production of ADP (□), AMP (△), adenosine (▽) and inosine (◇) by the guinea-pig bladder (a) or vas deferens (b) alone, or in the presence of suramin (1 mM) ((c) for the bladder, (d) for the vas deferens). Each point is the mean of four determinations; s.e.mean are shown when they are larger than the symbols.

(results not shown). Neither ATP (1–100 μM) nor 2-MeSATP (0.01–100 μM) consistently relaxed the vas deferens when precontracted with KCl (35 mM), either alone or after desensitization of $P_{2X}$-purinoceptors with AMPCPP (300 μM). In addition, neither ATP (1–100 μM) nor 2-MeSATP (0.01–100 μM) added before KCl (35 mM), carbachol (10 μM) or noradrenaline (10 μM) reduced subsequent contractions induced by these agents.

Under the conditions of these experiments, ATP (100 μM) was rapidly broken down by both the bladder and the vas deferens, with breakdown products being detectable after 2 min, the earliest time tested. However, whereas in the bladder the main degradation product was inosine, in the vas deferens it was adenosine. Approximately 35% of the ATP remained after incubation for 30 min with the bladder, and approximately 45% remained after incubation for 30 min with the vas deferens (Figure 6a,b). In each tissue this degradation was inhibited by suramin (1 mM), so that after incubation of ATP (100 μM) in the presence of suramin for 30 min, approximately 50% remained in the case of the bladder and approximately 65% in the case of the vas deferens (Figure 6c,d).

## Discussion

These results show that, as expected, suramin dose-dependently inhibited contractions of the guinea-pig bladder induced by ATP. This is in agreement with the reported inhibition by suramin of the effects of AMPCPP in this tissue (Hoyle *et al.*, 1990). These authors reported that the inhibition was non-competitive, as suramin caused a depression of the maximal response to AMPCPP, but derived an approximate $pA_2$ value of 4.7, with 10 μM suramin being ineffective. The inhibition by suramin of ATP-induced responses reported here is also apparently non-competitive, with a reduction in the slope of the concentration-response curves, and although a $pA_2$ value cannot be obtained from these data a value in the region of 4.7 would not be unreasonable, as again no inhibition was observed with concentrations of suramin of 10 μM or below. Apparently non-competitive inhibition by suramin has also been reported in other tissues, where although the maxima of the concentration-response curves were not obviously depressed, Schild analysis resulted in slopes greater than unity. In the case of the contractions of the rabbit ear artery mediated by $P_{2X}$-purinoceptors, this was attributed to a slow equilibration of the antagonist with the receptors (Leff *et al.*, 1990), whereas the non-competitive behaviour of suramin as an antagonist of ADP-induced platelet aggregation, an effect mediated by another class of $P_2$-purinoceptors, the $P_{2T}$ class, has been attributed to a non-selective inhibitory effect at high concentrations (Hourani *et al.*, 1992). In contrast, the inhibition by suramin of contractions of the mouse vas deferens to AMPCPP or to low concentrations of ATP was reported to be competitive, with Schild plot slopes not significantly different from unity (Von Kügelgen *et al.*, 1990).

The other naturally occurring nucleotides, GTP, CTP, UTP and ITP all caused contraction of the guinea-pig bladder, and of these CTP and GTP were the most potent, being approximately 10 fold less potent than ATP, although this is hard to quantify accurately owing to the shape of the concentration-response curves. The responses to all the nucleotides were abolished by suramin (1 mM), which suggests that they also act at the $P_{2X}$-purinoceptor by which ATP has its effect. This is in agreement with the findings of Lukacsko & Krell (1982), who also showed CTP and GTP to be approximately 10 fold less potent than ATP, and showed that there was cross-tachyphylaxis between these nucleotides and ATP, suggesting that they all act at the same receptor. Recently there has been a lot of interest in the pharmacological actions of pyrimidine nucleotides, in particular UTP, and the suggestion has been made that at some purinoceptors, ATP and UTP are equipotent agonists (O'Connor *et al.*, 1991). Indeed a G-protein-coupled $P_2$-purinoceptor ('$P_{2U}$') at which both ATP and UTP are active has recently been cloned from rat neuroblastoma cells (Lustig *et al.*, 1993). The effects of UTP in the guinea-pig bladder have not been reported before, but our results show that it is a very weak agonist, so the receptors here are not similar to this $P_{2U}$-purinoceptor.

In the vas deferens, the order of potency of the nucleotides was similar to that found in the bladder with CTP being the most potent apart from ATP, although GTP was less potent in the vas deferens than it was in the bladder. Again, however, the shape of the concentration-response curves makes it hard to quantify their potencies. GTP and ITP are very weak agonists here, in agreement with the results reported by Fedan *et al.* (1986). Again, the effect of UTP in this tissue had not previously been reported, but its lack of potency implies that there is not a contractile $P_{2U}$-purinoceptor on this tissue. The responses of all the naturally occurring nucleotides apart from ATP were inhibited by suramin suggesting that they were mediated by $P_{2X}$-purinoceptors, as in the bladder.

The most surprising finding of this study was that the effect of ATP in the vas deferens was not inhibited by suramin in the same way as the other nucleotides, and indeed at high concentrations of ATP (100 μM and above) was markedly enhanced. It should be pointed out that none of the concentrations of ATP used here were high enough to elicit the second, tonic phase of contraction reported by Fedan & Lamport (1990a) and Lamport-Vrana *et al.* (1991).

Even at lower concentrations of ATP such as 30 μM, where suramin did not enhance ATP-induced contractions, any inhibitory effect of suramin was weak and not dose-related. Von Kügelgen *et al.* (1990) also reported a suramin-resistant component of the effect of ATP on the mouse vas deferens, but in contrast to our findings did not observe any potentiation of ATP-induced responses and did report dose-related, competitive inhibition by suramin (Schild slope close to unity) of the effect of low concentrations of ATP. Suramin has however been reported to increase the maximal response to AMPCPP in both the rat and the mouse vas deferens, although it also shifted the concentration-response curves to the right indicating that it was acting as an antagonist (Blakely *et al.*, 1991; Mallard *et al.*, 1992).

Because it seemed possible that the enhancement of the ATP-induced contractions in the vas deferens was due to inhibition by suramin of ectonucleotidases which could result in the production of the inhibitory nucleoside adenosine, the breakdown of ATP and the appearance of its degradation products in both the vas deferens and the bladder was investigated. The breakdown measured in these studies probably underestimates the true breakdown in the vicinity of the receptor, as it is of course only possible to detect removal of ATP and appearance of breakdown products in the bathing solution. In spite of these technical limitations, breakdown was detected after 2 min incubation, the earliest time tested, and more importantly the pattern of breakdown was different for the two tissues. ATP was broken down somewhat faster in the vas deferens than in the bladder, and in the bladder its major degradation product after 30 min was inosine rather than adenosine. In previous studies of the degradation of ATP by guinea-pig urinary bladder (Cusack & Hourani, 1984; Welford *et al.*, 1987), the h.p.l.c. techniques used were not able to separate adenosine adequately from inosine, so this was not detected. Suramin (1 mM) did indeed slow the breakdown of ATP in both tissues, as had been reported for the guinea-pig bladder (Hourani & Chown, 1989), but did not inhibit it completely. It appeared unlikely from this that the differential effect of suramin in the bladder and the vas deferens could be attributed solely to a differential effect on the removal of ATP in the two tissues. In any case, the failure of suramin to enhance responses to the other nucleotides in the vas deferens would argue against this explanation, as in those tissues in which this has been investigated the ectonucleotidases do not discriminate between the naturally occurring nucleotides but degrade each of them at the same rate (Welford *et al.*, 1986; 1987). It remained possible that the adenosine produced from ATP could be a significant inhibitory factor in the vas deferens but not in the bladder, and that inhibition of its production by suramin could account for the potentiation observed. Postsynaptic inhibitory $A_2$ receptors have been found in the rat vas deferens (Hourani *et al.*, 1993), so it seemed possible that adenosine could also inhibit the response to ATP in the guinea-pig vas deferens. However, the adenosine antagonist 8-SPT (100 μM) did not enhance the contractions induced by ATP in the vas deferens nor reduce the ability of suramin to enhance these contractions, suggesting that adenosine does not play a role in limiting the responses to ATP in this tissue. In addition, this implies that the potentiation by suramin of ATP-induced contractions is not a consequence of its inhibition of ATP breakdown, but occurs by some other mechanism.

It has previously been reported that altering the divalent cation concentration in the buffer modifies responses to ATP, and it has been suggested that this is due to the active species being $ATP^{4-}$ (Morishita & Furukawa, 1989; Fedan *et al.*, 1990). In agreement with this, we also observed enhancement of ATP-induced contractions by omitting $Mg^{2+}$ from the buffer, and inhibition by increasing the $Mg^{2+}$ concentration ten fold, although in contrast to Morishita & Furukawa (1989) we found that reducing the $Ca^{2+}$ concentration to 0.83 mM reduced responses to ATP whereas these authors reported this to be the concentration at which ATP-induced contractions were maximal. It seemed possible that suramin, by virtue of its many sulphonate groups, might chelate divalent cations and thereby raise the concentration of the active species $ATP^{4-}$ and so enhance ATP-induced contractions and that this enhancement might mask antagonism at the $P_{2X}$-purinoceptor. However, no inhibition of ATP-induced contractions by suramin was observed even with these different concentrations of $Mg^{2+}$ and $Ca^{2+}$, which suggests that chelation of divalent cations is not the explanation.

Another possible explanation is that there may be inhibitory $P_{2Y}$-purinoceptors also present on the guinea-pig vas deferens, and that blockade of these by suramin may enhance ATP-induced contractions. We were unable to detect any inhibitory effects of either ATP itself or the more potent $P_{2Y}$-agonist, 2-MeSATP, even after desensitization of the $P_{2X}$ receptors with AMPCPP, but the possibility that they are present cannot be eliminated. $P_{2Y}$-purinoceptors have been reported to exist on the mouse urinary bladder and vas deferens (Boland *et al.*, 1992; 1993), and this may account for the increase in the maximal response to AMPCPP in the mouse vas deferens seen in the presence of suramin (Blakely *et al.*, 1991). An increase in the maximal response to AMPCPP in the presence of suramin was also reported in the rat vas deferens (Mallard *et al.*, 1992) and indeed in the guinea-pig bladder (Hoyle *et al.*, 1990), and in these tissues a similar explanation may apply. However, suramin is not selective for $P_{2Y}$ over $P_{2X}$ receptors (Hoyle *et al.*, 1990), and in each of these cases there was also the expected shift to the right of the concentration-response curve to AMPCPP. In contrast to this, we did not observe any inhibition by suramin of ATP-induced contractions in the guinea-pig vas deferens, so the situation in this tissue is not so simple. In our experiments, 1 mM suramin abolished contractions to the other nucleotides in the vas deferens, and abolished contractions to ATP in the bladder, suggesting that at this concentration $P_{2X}$ receptors were effectively blocked. It is hard therefore to attribute the contractions to ATP in the vas deferens in the presence of 1 mM suramin to an interaction with $P_{2X}$ receptors, even though concomitant blockade of inhibitory $P_{2Y}$ receptors might enhance any contractions occurring. In the absence of suramin, ATP clearly acts mainly via $P_{2X}$-purinoceptors, as shown by the desensitization with AMPCPP seen here and reported by Meldrum & Burnstock (1983), as well as by the inhibition of ATP-induced contractions by the photoaffinity reagent $ANAPP_3$, which has been suggested to be a selective inhibitor of $P_{2X}$-purinoceptors (Fedan *et al.*, 1985; Fedan & Lamport, 1990b). In the presence of suramin however, ATP must be acting via a different receptor, as although desensitization with AMPCPP abolished contractions induced by ATP in the absence of suramin, the contractions in the presence of suramin were unaffected, again suggesting that they cannot be due to an interaction with $P_{2X}$-purinoceptors. Suramin 1 mM therefore effectively overcomes the inhibition of ATP-induced contractions caused by AMPCPP, possibly by blocking inhibitory $P_{2Y}$ receptors, or by some other unknown mechanism.

Overall our results therefore suggest that the guinea-pig vas deferens, in addition to $P_{2X}$-purinoceptors also contains another contractile $P_2$-purinoceptor. The existence of this receptor is only revealed in the presence of suramin which enhances ATP-induced contractions. The mechanism of this enhancement is unclear, but may be because the effects of ATP are normally opposed by inhibitory $P_{2Y}$-purinoceptors, although we are unable to demonstrate the existence of such receptors. The structure-activity relationships of this suramin-resistant contractile $P_2$-purinoceptor are currently being investigated in our laboratory. Whatever the mechanism of the enhancement by suramin of ATP-induced contractions of the vas deferens, the difference in its effects on two contractile tissues from the guinea-pig which have both been assumed to contain only $P_{2X}$-purinoceptors indicates that purinoceptor

pharmacology is still an area in which much remains to be elucidated.

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# Effects of naftidrofuryl oxalate on microsphere embolism-induced decrease in regional blood flow of rat brain

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**1** The purpose of the present study was to determine whether naftidrofuryl oxalate (naftidrofuryl), a vasodilator, is capable of improving brain regional blood flow of animals in sustained ischaemia.

**2** Cerebral ischaemia was induced by injecting 900 microspheres (48 μm in diameter) into the right internal carotid artery of rats. Cerebral blood flow of brain regions was measured by a hydrogen clearance method on the 3rd, 7th and 28th days after the onset of ischaemia. Ischaemic animals were treated with naftidrofuryl, 15 mg $kg^{-1}$ $day^{-1}$ i.p., from the first to 28th day.

**3** Microsphere-embolism caused a sustained decrease in cortical and striatal blood flow over a period of 28 days, whereas hippocampal blood flow was decreased on the 3rd day but not on the 7th or 28th day. On the 3rd day, the striatal and hippocampal but not cortical blood flow of naftidrofuryl-treated, microsphere-embolized rats was higher than untreated rats. On the 7th and 28th days, the cortical and striatal blood flow of the treated and untreated animals did not differ.

**4** Brain slices from microsphere-embolized rats contained areas, which were not stained with triphenyltetrazolium chloride (TTC), to a similar degree on the 3rd, 7th and 28th days, indicating the genesis of cerebral infarction. TTC-unstained areas of microsphere-embolized rats that had received naftidrofuryl treatment were smaller than those of untreated rats on the 3rd and 7th days, but not on the 28th day.

**5** The results suggest that naftidrofuryl improves cerebral circulation impaired by microsphere-induced ischaemia and this higher level of cerebral blood flow of the treated animal may account for the delayed development of cerebral infarction.

**Keywords:** Cerebral blood flow; cerebral cortex; hippocampus; microsphere-embolism; naftidrofuryl; striatum

## Introduction

Naftidrofuryl oxalate, [2-(diethylamino)ethyltetrahydro-α-(1-nafthylmethyl)-2-furanpropionate ester oxalate], (naftidrofuryl), is a cerebral and peripheral vasodilator (Admani, 1978), and has been found to increase cerebral blood flow in anaesthetized animals (Hagiwara *et al.*, 1973; Young *et al.*, 1983; Hiramatsu *et al.*, 1988) and man (Ohtomo *et al.*, 1986). However, the mechanism by which this agent induces its beneficial effect on brain function under pathophysiological conditions is poorly understood. In the present study we attempted to determine whether naftidrofuryl oxalate increased the blood flow of brain regions subjected to sustained ischaemia. For this purpose, we induced cerebral ischaemia by microsphere-embolism. Cerebral embolism with microspheres has been shown to induce biochemical, electrophysiological, neurological and morphological abnormalities (Siegel *et al.*, 1972; Kogure *et al.*, 1974; Bralet *et al.*, 1979; Sugi *et al.*, 1984; Kiyota *et al.*, 1985; Narumi *et al.*, 1986). The microspheres (diameters 15–50 μm) are considered to produce widespread pre-capillary occlusion, resulting in a multi-focal, long-lasting cerebral ischaemia and infarction in the brain. Using this ischaemic model, we examined the effects of naftidrofuryl on the cerebral blood flow of a number of brain regions, cerebral cortex, striatum and hippocampus, which are vulnerable to ischaemia (Kirino, 1982; Pulsinelli *et al.*, 1982; Smith *et al.*, 1984).

## Methods

Male Wistar rats weighing 200–220 g (Charles River Japan Inc., Atsugi, Japan) were used in the present study. The animals were maintained under artificial conditions at 23 ± 1°C, with a constant humidity of 55 ± 5%, a cycle of 12 h of light and 12 h of dark and free access to food and tap water, according to the Guidelines of Experimental Animal Care issued by the Prime Minister's Office of Japan. Microsphere-induced cerebral embolism was conducted by the methods previously described (Miyake *et al.*, 1989). Briefly, rats were anaesthetized with sodium pentobarbitone, 35 mg $kg^{-1}$ i.p. After ligation of the right external carotid and pterygopalatine arteries, 900 microspheres (47.5 ± 0.5 μm in diameter; NEN-005, New England Nuclear Inc., Boston, Mass, U.S.A.), suspended in 20% dextran solution, were injected into the right internal carotid artery through a polyethylene catheter (3 French size, 1.0 mm in diameter, Atom Co., Tokyo, Japan) inserted into the right common carotid artery. Rats that underwent sham operation were injected with the same volume of vehicle without microspheres, and their right common carotid arteries were ligated. The control group comprised non-operated rats and were used to examine normal levels of haemodynamic and histological variables of the intact animal. Fifteen hours after the operation, the animal behaviour was scored on the basis of paucity of movement, truncal curvature and forced circling during locomotion, which are considered to be typical symptoms of stroke in rats (Furlow & Bass, 1976; McGraw, 1977). Each symptom was scored from 3 to 0, 3 very severe, 2 severe, 1 moderately severe, 0 little or no symptoms. The sum of these scores was evaluated as a marker of neurological deficits. Rats with 7–9 points were considered type A; 4–6 points, type B; and 3–0 points, type C. The type A animals were used in the following experiment. The animal's behaviour was examined at 09 h 00 min every day throughout the experiment.

### *Treatment of microsphere-injected and sham-operated rats with naftidrofuryl*

After ensuring their stroke-like symptoms, the microsphere-injected and sham-operated rats were treated with naftidro-

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furyl 15 mg kg$^{-1}$ day$^{-1}$ i.p., from the first to the 28th day after the operation. The dose used in the present study was shown to be effective in restoring biochemical parameters of microsphere-injected rats in previous studies (Miyake *et al.*, 1989; Takeo *et al.*, 1991).

### *Measurements of cerebral blood flow*

Cerebral blood flow was measured in control, microsphere-injected and sham-operated rats with and without naftidrofuryl-treatment. The animals were anaesthetized with intraperitoneal administration of sodium pentobarbitone 35 mg kg$^{-1}$. Arterial blood pressure was monitored via a cannula inserted into the right femoral artery and measured by means of a pressure transducer (model AP-601G, Nihonkohden Co., Tokyo, Japan) throughout the experiment. The heart rate measurement was triggered through the ECG (lead II) and counted by means of a heart rate counter (model AT-601G, Nihonkohden Co., Tokyo, Japan) throughout the experiment. The rectal temperature of the rat was maintained at 36.5 ± 0.5°C by warming the animal with an electronic panel-heater placed underneath it. Tissue blood flow of brain regions was measured by hydrogen clearance using a hydrogen clearance flow meter (model UPS-400, Unique Medical Inc., Tokyo, Japan). The head of the rat was fixed in a head holder (model SR-5, Narishige Instrument Co., Tokyo, Japan). Three small bar holes were made with an electric mini-drill (model 28400, Proxxton, Germany) in the skull of each side. Two Teflon-coated platinum electrodes, 100 μm in diameter, with a 1-mm uncoated portion at their tips and plated with black platinum, were positioned at a depth of 2 mm, 2 mm posterior and 2 mm lateral to the bregma for measurement of blood flow of the cortex, at a depth of 4 mm, 2 mm anterior and 3 mm lateral to the bregma for that of the striatum, and at a depth of 5 mm, 6 mm posterior and 5 mm lateral to the bregma for that of the hippocampus of each hemisphere according to the *Atlas of Anatomy of Rat Brain* (Paxinos & Watson, 1986), using a stereotactic apparatus (model SR-5, Narishige Instrument Co., Tokyo, Japan). The reference Ag-AgCl electrode was inserted under the skin of the neck. The animals inhaled a gas mixture of 90 ml min$^{-1}$ air and 10 ml min$^{-1}$ hydrogen through a mask loosely fitted to their nose. After ensuring the equilibration of the brain tissue with hydrogen gas, the cerebral blood flow was measured. At about 30 min intervals, the blood flow of the other brain regions was similarly determined.

In a separate set of experiments, blood gas analysis of control and sham-operated and microsphere-injected rats were performed on the 3rd day after embolism under the present experimental conditions to ensure the validity of these experimental conditions. The animals were anaesthetized with sodium pentobarbitone, 35 mg kg$^{-1}$, as described above. A cannula was inserted into the right femoral artery for blood sampling. The animals inhaled air containing 10% hydrogen gas as described above. Blood (0.2 ml) was sampled before inhalation (0 min), and three times after equilibration of hydrogen gas in the tissue (30, 60 and 90 min). The blood was immediately analysed with a blood gas analyzer (model 288, Ciba-Corning Japan, Tokyo, Japan).

### *Measurement of infarct areas*

To determine the infarct area, microsphere-injected and sham-operated animals with and without naftidrofuryl-treatment were lightly anaesthetized with ether and decapitated at different time intervals (the 3rd, 7th and 28th days) after the operation. Rats that did not undergo any operation (controls) were also decapitated. The brains were rapidly isolated; after cooling in a stainless-steeel container surrounded with ice, the brain was positioned on a brain holder and coronally sectioned with razors, 3, 5, and 7 mm from the frontal pole. The sections of brain tissue were incubated at 37°C for 30 min with 2% of 2,3,5-triphenyltetrazolium chloride (TTC) in physiological saline (Benderson *et al.*, 1986). The slices were briefly immersed in a 10% formalin solution and then photographed. The sum of TTC-stained, and TTC-unstained (including weakly stained) areas of three brain slices were estimated by a planimetric method.

### *Statistics*

The results are expressed as mean ± s.e.mean. Statistical significance between drug-treated and untreated groups was evaluated by Student's *t* test. Analysis of variance followed by Dunnett's *t* test was employed for evaluation of significance of the time course of changes in variables. The Wilcoxon rank sum test was used for comparison of changes in behavioural scores with and without naftidrofuryl oxalate treatment. A confidence value of >95% was considered significant ($P<0.05$).

## Results

The animals (200 rats) were inspected 15 h after surgery. Forty-two rats (21%) died within 24 h of the embolism. One hundred and thirty embolized rats (65%) showed type A symptoms, 20 rats (10%) type B, and 8 rats (4%) type C, which was similar to previous results (Miyake *et al.*, 1989; 1993; Takeo *et al.*, 1991). Among the type A rats, 20 rats died within 3 days of the embolism. Consequently, 110 type A rats were used in the following experiment. No neurological deficits were seen in 104 sham-operated rats.

In the first set of experiments, we performed blood gas analysis to ascertain the physiological profile of control, sham-operated and microsphere-injected rats under the same conditions as those undergoing determination of cerebral blood flow according to the hydrogen gas clearance method. The microsphere-injected and sham-operated rats on the 3rd day after the operation were chosen as typical samples in this series of experiment. As shown in Table 1, there were no differences in pH, $P_{O_2}$ and $P_{CO_2}$ values of the control, sham-operated and microsphere-injected rats during the entire experimental period (0–90 min).

Mean arterial blood pressure and heart rate of microsphere-injected and sham-operated rats were routinely monitored before and during measurements of cerebral blood flow at different time periods after microsphere-embolism. Table 2 shows mean arterial pressure and heart rate of sham-

**Table 1** Blood gas analysis of rats under the same conditions as those for measurements of regional cerebral blood flow

| | *Time after the start of experiment* | | | |
|---|---|---|---|---|
| | 0 | 30 | 60 | 90 min |
| *Control* | | | | |
| pH | 7.37 ± 0.01 | 7.40 ± 0.01 | 7.39 ± 0.01 | 7.40 ± 0.01 |
| $P_{O_2}$ | 90.7 ± 2.3 | 93.6 ± 2.4 | 96.5 ± 3.0 | 97.7 ± 2.7 |
| $P_{CO_2}$ | 40.1 ± 1.5 | 40.0 ± 1.2 | 40.5 ± 0.8 | 38.2 ± 1.3 |
| *Sham-operated* | | | | |
| pH | 7.44 ± 0.01 | 7.49 ± 0.03 | 7.44 ± 0.03 | 7.46 ± 0.02 |
| $P_{O_2}$ | 95.6 ± 2.3 | 95.4 ± 2.4 | 94.3 ± 1.5 | 97.1 ± 1.3 |
| $P_{CO_2}$ | 40.1 ± 2.1 | 41.0 ± 1.7 | 40.2 ± 4.0 | 37.5 ± 2.7 |
| *Microsphere-injected* | | | | |
| pH | 7.40 ± 0.01 | 7.43 ± 0.01 | 7.42 ± 0.02 | 7.45 ± 0.01 |
| $P_{O_2}$ | 94.2 ± 1.5 | 94.3 ± 0.9 | 94.9 ± 1.2 | 95.3 ± 1.5 |
| $P_{CO_2}$ | 40.7 ± 1.0 | 40.8 ± 0.4 | 41.3 ± 1.4 | 41.3 ± 0.7 |

Blood samples were taken from animals anaesthetized with sodium pentobarbitone. Values for $P_{O_2}$ and $P_{CO_2}$ are expressed as mmHg. Each value represents the mean ± s.e.mean of 6 (control), 4 (sham-operated) and 4 (microsphere-injected) experiments.

**Table 2** Mean arterial blood pressure and heart rate of microsphere-injected and sham-operated rats with and without naftidrofuryl oxalate treatment

| | *Mean arterial pressure* (mmHg) *Time after the start of experiment* | | | | *Heart rate* (beats $min^{-1}$) *Time after the start of experiment* | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 30 | 60 | 90 min | 0 | 30 | 60 | 90 min |
| *Control* | 103 ± 2 | 102 ± 2 | 104 ± 2 | 105 ± 2 | 421 ± 17 | 422 ± 10 | 425 ± 9 | 420 ± 11 |
| *Sham-operated* | | | | | | | | |
| Untreated | 100 ± 4 | 106 ± 4 | 110 ± 5 | 111 ± 6 | 412 ± 17 | 419 ± 13 | 425 ± 14 | 433 ± 12 |
| Treated | 105 ± 8 | 108 ± 7 | 107 ± 7 | 105 ± 3 | 436 ± 26 | 437 ± 29 | 462 ± 29 | 425 ± 6 |
| *Microsphere-injected* | | | | | | | | |
| Untreated | 105 ± 2 | 106 ± 5 | 107 ± 2 | 101 ± 5 | 411 ± 13 | 407 ± 15 | 403 ± 12 | 422 ± 11 |
| Treated | 103 ± 3 | 101 ± 5 | 105 ± 6 | 103 ± 5 | 407 ± 7 | 408 ± 9 | 418 ± 10 | 411 ± 22 |

Measurements of blood pressure and heart rate were conducted in rats under sodium pentobarbitone anaesthesia. Values are shown only in sham-operated and microsphere-injected rats at the 3rd day after the operation. Mean arterial blood pressure and heart rate were similar in the operated and sham-operated rats irrespective of time and treatment. Each value represents the mean ± s.e.mean of 6 to 10 experiments.

operated and microsphere-injected rats on the 3rd day after the operation with and without naftidrofuryl oxalate treatment. There were no significant differences in either parameter between microsphere-injected and sham-operated rats with and without naftidrofuryl oxalate treatment on any day after the operation. Likewise, no appreciable changes were observed in either parameter of microsphere-injected animals throughout the measurements of regional blood flow. Similarly, there were no changes in mean arterial blood pressure and heart rate of microsphere-embolized and sham-operated animals examined on the 7th and 28th days after the operation.

The effects of naftidrofuryl on cortical, striatal and hippocampal blood flow of the right hemispheres of microsphere-injected, and sham-operated rats are shown in Figure 1. On the 3rd day after the operation, cortical, striatal and hippocampal blood flow was significantly decreased after microsphere-embolism. Treatment with naftidrofuryl significantly attenuated the decrease in blood flow of striatal and hippocampal, but not cortical, regions of the right hemisphere. On the 7th and 28th days after the operation, cortical and striatal blood flow was still lower than control. Naftidrofuryl did not attenuate the decrease in blood flow of cortical and striatal regions on the 7th and 28th days. In sham-operated animals, there were no significant reductions in the blood flow of these brain regions of the right hemisphere on the 3rd, 7th and 28th days after the operation. Likewise, naftidrofuryl had no significant effect on the blood flow of the three brain regions of sham-operated rats throughout the experiment. In the left hemisphere, there were no significant alterations in the blood flow of these brain regions at any period of the experiment examined.

The brains of microsphere-injected and sham-operated rats were isolated on the 3rd, 7th and 28th days after the operation and used for gross observation and TTC-staining. On the 3rd day, cerebral vasodilatation, oedema, and haemorrhage were seen in the right hemisphere of microsphere-injected rats. There was a marked degeneration and/or atrophy of the right hemisphere of microsphere-injected rats on the 28th day after the operation. No such abnormalities were seen in the left hemisphere of microsphere-injected rats or either hemisphere of sham-operated rats throughout the experiment.

TTC-staining of brain slices was performed to determine cerebral infarct area (Figure 2). The TTC-unstained areas (including weakly stained areas) of the right hemisphere of microsphere-injected rats on the 3rd day were approximately 79% of the corresponding sham-operated animals. The TTC-stained areas of the right hemisphere on the 7th and 28th days were almost the same as those on the 3rd day. Treatment with naftidrofuryl resulted in a significant attenuation of the development of TTC-unstained area on the 3rd and 7th days, but not 28th day, after the operation. No TTC-unstained area was seen in the brain slices of either hemisphere of sham-operated rats, even though the right common carotid arteries of all animals were permanently ligated.

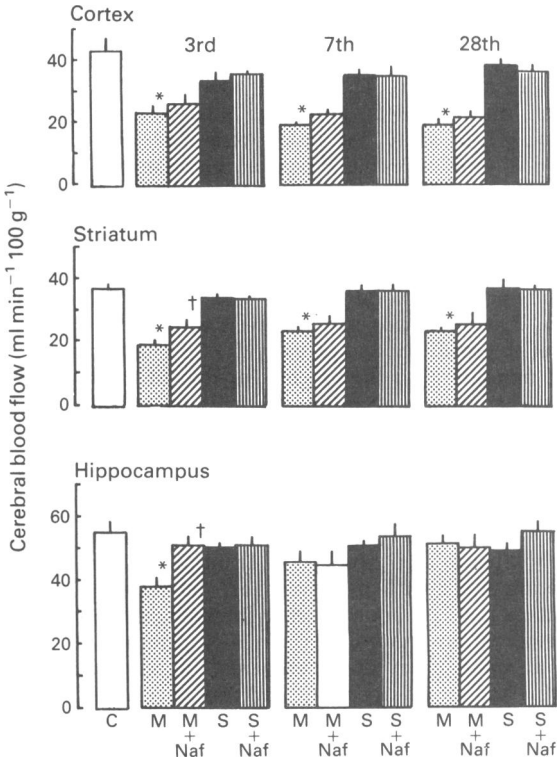


**Figure 1** The effects of naftidrofuryl oxalate treatment on blood flow of the cerebral cortex, striatum and hippocampus of the right hemisphere of microsphere-injected (M and M + Naf), sham-operated (S and S + Naf) rats with (+Naf) and without naftidrofuryl oxalate treatment, and control rats (C), respectively. Measurement of cerebral blood flow was carried out under sodium pentobarbitone anaesthesia. Control values of cortical, striatal and hippocampal blood flow of the right hemisphere were 42.9 ± 4.7, 36.4 ± 1.3 and 54.6 ± 3.1 ml $min^{-1}$ 100 $g^{-1}$ tissue, and those of the left hemisphere 44.4 ± 4.3, 38.1 ± 1.8 and 54.3 ± 3.2 ml $min^{-1}$ 100 $g^{-1}$ tissue, respectively ($n = 6$). Each value represents the mean ± s.e.mean of 6 (control), and 8 to 10 (sham and microsphere embolism) experiments. There were no significant alterations in cerebral blood flow of the left hemisphere of microsphere-injected rats on the 3rd, 7th and 28th days after the operation regardless of treatment with or without naftidrofuryl. *Significantly different from the corresponding sham-operated group and †significantly different from microsphere-injected group ($P < 0.05$).

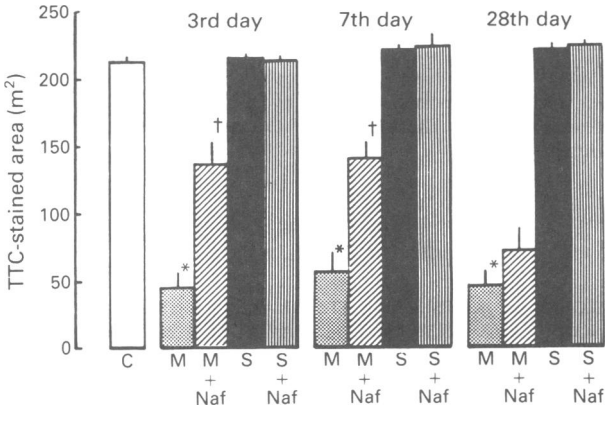


**Figure 2** The sum of triphenyltetrazolium chloride (TTC)-stained areas of three brain slices from the right hemisphere of microsphere-injected rats (M and M + Naf) and those of sham-operated rats (S and S + Naf) with (+Naf) and without naftidrofuryl treatment on the 3rd, 7th and 28th days after the operation. Values are expressed as the mean ± s.e.mean of 6 (control) and 8 to 9 (sham and microsphere-embolism) experiments. The TTC-stained areas of the right and left hemispheres of control animals were 214.5 ± 0.9 and 214.1 ± 0.3 mm², respectively ($n = 6$). There were no significant alterations in the TTC-strained areas of the left hemisphere of microsphere-injected and sham-operated rats regardless of treatment with or without naftidrofuryl. *Significantly different from the sham-operated groups and †significantly different from the untreated group ($P < 0.05$).

Animal behaviour was examined in rats employed for the studies on cerebral blood flow and TTC staining areas throughout the experiments; the initial numbers of the untreated and treated animals were 52 and 54, respectively. The behavioural scores of untreated, microsphere-injected animals on the 1st, 3rd, 7th and 28th days after the operation were 7.6 ± 0.4, 6.7 ± 0.6, 2.8 ± 0.6 and 0.3 ± 0.3, while those of naftidrofuryl oxalate-treated, microsphere-injected animals were 7.6 ± 0.2, 5.6 ± 0.5, 2.6 ± 0.4 and 0.2 ± 0.2, respectively. Although a slight improvement of the behavioural scores was observed in the treated animals from the 3rd and 7th day after the operation, there was no significant difference in the scores between the treated and untreated animals throughout the experiments.

## Discussion

In the present study we observed variable changes in blood flow of the brain regions: that is, a sustained decrease in cortical and striatal blood flow, and a decrease in hippocampal blood flow which was reversed 7 days after the embolism. The findings provide direct evidence that microsphere-embolism induced sustained ischaemia or oligaemia in some brain regions of the cerebral hemisphere into which microspheres were introduced. Variable reversal of blood flow of brain regions has also been documented in rats with middle cerebral artery occlusion; relatively better recovery of blood flow in the anterior cingulate gyral and hippocampal regions and very little recovery of blood flow in the frontal cortical and dorsal caudate regions, 48 h after the occlusion (Hakim *et al.*, 1992).

Reduction in blood flow of brain regions after microsphere-embolism can be predicted from the findings of our previous studies. These included decreases in tissue high-energy phosphates (Takeo *et al.*, 1992) and acetylcholine (Taguchi *et al.*, 1993), and increases in tissue glucose and lactate (Takeo *et al.*, 1991; 1992). These changes are consistent with an ischaemic state (Siesjö *et al.*, 1976; Welsh *et al.*, 1977; Gibson *et al.*, 1981). From both circulatory and biochemical observations, it could be concluded that microsphere-embolism induces a sustained decrease in cerebral blood flow of the cerebral cortex and striatum, which results in a profound metabolic disturbance in these regions.

We also observed variable effects of naftidrofuryl on the decreased levels of blood flow of the brain regions. That is, the decrease in striatal blood flow was partially, but significantly, attenuated by treatment with naftidrofuryl.

Furthermore, the decrease in hippocampal blood flow was attenuated almost completely by treatment with naftidrofuryl, whereas treatment did not restore the decreased cortical blood flow on the 3rd day. Treatment with naftidrofuryl did not result in a significant recovery of the blood flow of these brain regions thereafter. The results suggest that these cortical regions are severely damaged following microsphere-embolism and naftidrofuryl treatment is capable of improving cerebral blood flow of certain brain regions, which are damaged to a relatively lesser degree, but only at an early stage (3 day) of cerebral ischaemia. This implies that naftidrofuryl substantially retards, but may not prevent, the development of ischaemic damage to brain regions.

The TTC-staining method is a convenient method of determining cerebral infarct area and a close correlation with evaluation of infarct size by haematoxylin-eosin has been described in the literature (Benderson *et al.*, 1986; Isayama *et al.*, 1991). Our results show the formation of widespread TTC-unstained or weakly stained areas in the brain slices of the right hemisphere from microsphere-injected animals from 3 to 28 days after the operation, which demonstrated the development of cerebral infarction in the brain. In accordance with this, in a previous microscopic study (Miyake *et al.*, 1993), we observed areas sparsely stained with haematoxylin-eosin in the right hemisphere, which suggests a widespread degeneration of the microsphere-injected hemisphere. Necrotic areas, variable in size and shape, with small haemorrhagic foci were also widely observed in various brain regions. This is indicative of multi-focal necrosis. These morphological abnormalities were partially reduced by treatment with naftidrofuryl (Taguchi *et al.*, 1994). This is in good agreement with our present findings of the delayed development of TTC-unstained areas in the microsphere-embolized, naftidrofuryl-treated rat brain slices. In contrast, there are discrepancies in the results regarding the effects of the agent on the development of infarct areas and cerebral blood flow in the present study. That is, the effects of naftidrofuryl on the cerebral blood flow were seen only on the 3rd day and the effects on the TTC-unstained areas were observed both on the 3rd and 7th days after the operation. It is frequently observed in an ischaemic animal that biochemical alterations precede histological alterations. Actually, in this microsphere-embolized model, metabolic abnormalities in the ipsilateral brain hemisphere were observed immediately or one day after the microsphere-embolism, whereas TTC-unstained areas were poorly developed 12–24 h after the embolism (Takeo *et al.*, 1992; Miyake *et al.*, 1992; Taguchi *et al.*, 1993). Thus, it is conceivable that the influence of naftidrofuryl-induced improvement of cerebral circulation may retard the development of morphological damage for a few days.

In the present study, the behavioural scores of the microsphere-embolized animal which are considered to be stroke-like symptoms in rats (Furlow & Bass, 1976; McGraw, 1977) improved only to a limited degree, despite improvement of cerebral blood flow of the right hemisphere of microsphere-embolized rats 3 days after the operation by treatment with naftidrofuryl. One could not argue, however, that the two results are inconsistent because of uncertainty concerning the precise area that controls animal behaviour and the extent of restoration of cerebral blood flow necessary for improvement of animal behaviour. It would also be unreasonable to correlate directly alterations of behavioural score, a qualitative parameter, with changes in cerebral blood flow, a quantitative parameter.

In summary, we found that naftidrofuryl improved striatal and hippocampal blood flow at an early stage of cerebral ischaemia following microsphere-embolism. This was assoc-

iated with a delayed development of cerebral infarction as shown by TTC-staining. This improvement in cerebral blood flow may play a role in the naftidrofuryl-induced improvement of metabolic disturbances, such as depletion of tissue high-energy phosphates (Takeo *et al.*, 1991), decreases in acetylcholine and neurotransmitter amino acids (Taguchi *et al.*, 1994), and increases in tissue glucose and lactate (Takeo *et al.*, 1991).

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# Modulatory effects of NMDA on phosphoinositide responses evoked by the metabotropic glutamate receptor agonist 1S,3R-ACPD in neonatal rat cerebral cortex

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**1** The effect of NMDA-receptor stimulation on phosphoinositide signalling in response to the metabotropic glutamate receptor agonist 1-aminocyclopentane-1S,3R-dicarboxylic acid (1S,3R-ACPD) has been examined in neonatal rat cerebral cortex slices.

**2** Total [$^3$H]-inositol phosphate ([$^3$H]-Ins$P_x$) accumulation, in the presence of 5 mM LiCl, in [$^3$H]-inositol pre-labelled slices was concentration-dependently increased by 1S,3R-ACPD ($EC_{50}$ 16.6 μM) and, at a maximally effective concentration, 1S,3R-ACPD (300 μM) increased [$^3$H]-Ins$P_x$ accumulation by 12.8 fold over basal values.

**3** [$^3$H]-Ins$P_x$ accumulation stimulated by 1S,1R-ACPD was enhanced by low concentrations of NMDA (3–30 μM), but not by higher concentrations (> 30 μM). [$^3$H]-Ins$P_x$ accumulations stimulated by 1S,3R-ACPD in the absence or presence of 10 μM NMDA were linear with time, at least over the 15 min period examined; however, in the presence of 100 μM NMDA the initial enhancement of 1S,3R-ACPD-stimulated phosphoinositide hydrolysis progressively decreased with time.

**4** In the presence of a maximal enhancing concentration of NMDA (10 μM), the response to 1S,3R-ACPD (300 μM) was increased 1.9 fold and the $EC_{50}$ for agonist-stimulated [$^3$H]-Ins$P_x$ accumulation decreased about 4 fold. The enhanced response to the metabotropic agonist was concentration-dependently inhibited by competitive and uncompetitive antagonists of NMDA-receptor activation.

**5** 1S,3R-ACPD also stimulated inositol 1,4,5-trisphosphate (Ins(1,4,5)$P_3$) mass accumulation with an initial peak response (5–6 fold over basal) at 15 s decaying to a smaller (2 fold), but persistent elevated accumulation (1–10 min).

**6** Co-addition of 10 or 100 μM NMDA enhanced the initial peak Ins(1,4,5)$P_3$ response to 1S,3R-ACPD. However, the enhancing effect was only maintained over 10 min in the presence of 10 μM NMDA, whilst in contrast, 100 μM NMDA ceased to cause a significant enhancement of the metabotropic response by 5 min and completely suppressed 1S,3R-ACPD-stimulated Ins(1,4,5)$P_3$ accumulation at 10 min.

**7** Both basal and 1S,3R-ACPD-stimulated Ins(1,4,5)$P_3$ accumulations were reduced when slices were incubated in nominally $Ca^{2+}$-free medium. Under these conditions only a concentration-dependent enhancement of the response was observed ($EC_{50}$ for NMDA facilitation of 1S,3R-ACPD-stimulated Ins(1,4,5)$P_3$ accumulation of 32 μM).

**8** These experiments have revealed that at low concentrations, NMDA can dramatically potentiate 1S,3R-ACPD-stimulated phosphoinositide hydrolysis, probably by a $Ca^{2+}$-dependent facilitation of agonist-stimulated phosphoinositide-specific phospholipase C activity. Higher concentrations of NMDA result in time-dependent inhibition of the metabotropic agonist-stimulated response. We believe the former effect could be fundamental in glutamate receptor 'cross-talk', whereas the latter may reflect a $Ca^{2+}$-dependent neurotoxic effect of NMDA on the neonatal cerebral cortex slices.

**Keywords:** Metabotropic glutamate receptor; NMDA-receptor; phosphoinositide turnover; inositol 1,4,5-trisphosphate; cerebral cortex (neonatal rat)

## Introduction

Glutamate is now recognised as the major excitatory neurotransmitter in the mammalian central nervous system. Glutamatergic transmission has been implicated in a variety of brain functions and is believed to be the principal neurotransmitter involved in the processes of information storage and retrieval (Madison *et al.*, 1991; Bliss & Collingridge, 1993). In tandem with the rapid development of our understanding of the physiological neurotransmitter roles of glutamate, has been the realization that glutamate can also act as a neurotoxin. Thus, under adverse conditions, where the stringent regulation of extracellular glutamate concentration cannot be maintained, glutamate receptor activation can lead to a massive disturbance in neuronal $Ca^{2+}$ and $Na^+$ homeostasis which can eventually lead to irreversible cell damage and neuronal death (Choi, 1988; Beal, 1992; Randall & Thayer, 1992; Frandsen & Schousboe, 1993).

Considerable progress has been made towards identifying and classifying glutamate receptors based on molecular biological, as well as pharmacological, criteria. Two broad categories of glutamate receptor have been defined; the 'ionotropic' N-methyl-D-aspartate (NMDA)/α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA)-kainate receptors which are multimeric proteins that function as ligand-gated ion channels (Gasic & Hollmann, 1992; Nakanishi, 1992), and the 'metabotropic' receptors which, via guanine nucleotide binding proteins, are either positively coupled to phosphoinositide-specific phospholipase C (PI-PLC) or negatively coupled to adenylyl cyclase (Abe *et al.*, 1992; Aramori & Nakanishi, 1992; Nakanishi, 1992; Tanabe *et al.*, 1992).

A common finding of many studies investigating the physiological roles of glutamatergic signalling has been the com-

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plex interplay between the different receptor subtypes. Thus, different classes of glutamate receptor may be co-expressed in single neurones or homogeneous neuronal populations (Bekkers & Stevens, 1989; Jones & Baughman, 1991; Courtney & Nicholls, 1992; Irving et al., 1992; Martin et al., 1992), or coordinately located pre- and post-synaptically (Baskys & Malenka, 1991; Martin et al., 1992; Nicholls, 1992). With respect to ionotropic/metabotropic interactions, activation of metabotropic glutamate receptors using a selective agonist (e.g. 1-aminocyclopentane-1S,3R-dicarboxylic acid (1S,3R-ACPD)) can potentiate NMDA receptor-mediated responses (Bleakman et al., 1992; Harvey & Collingridge, 1993), and co-activation of metabotropic and NMDA receptors has been implicated as a necessary requirement for induction and maintenance of long-term potentiation (Zheng & Gallagher, 1992; Bashir et al., 1993; Behnisch & Reymann, 1993).

Further biochemical analysis of glutamate ionotropic/metabotropic interactions may provide clues to mechanisms underlying these fundamental adaptive changes at central synapses. Here we have used neonatal rat cerebral cortex slices, in which metabotropic glutamate-receptor agonists produce large phosphoinositide responses, to reveal dramatic stimulatory and inhibitory effects of agonist occupation of NMDA-receptors upon metabotropic-receptor agonist-stimulated phosphoinositide responses. A preliminary account of this work has been communicated to the British Pharmacological Society (Challiss et al., 1993).

## Methods

### *Incubation methods*

Cerebral cortex from 7–8 day old neonatal rats (Wistar strain, either sex) was cross-chopped (350 × 350 μm, McIlwain tissue chopper) and slices dispersed into HEPES-buffered Krebs-Henseleit solution (KHB, composition in mM: NaCl 118, $NaHCO_3$ 25, KCl 4.7, $CaCl_2$ 1.3, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, HEPES 5, glucose 10, pH 7.4 following equilibration with $O_2/CO_2$ (19:1) at 37°C). Following washing with multiple buffer changes, slices were incubated in 50 ml KHB for 60 min at 37°C, with buffer replacement every 15 min. Where inositol phospholipids were labelled with [$^3$H]-inositol, slices were allowed to sediment and 25 μl aliquots transferred to 250 μl KHB containing 0.5 μCi [$^3$H]-inositol in flat-bottomed polypropylene vials. Samples were incubated at 37°C for a period of 60 min during which samples were regularly purged with $O_2/CO_2$ (19:1). At the end of this period, LiCl (5 mM final concentration) was added. For Ins(1,4,5)$P_3$ mass studies, the labelling period and LiCl addition were omitted from the protocol.

In the latter studies where nominally $Ca^{2+}$-free incubation conditions were required, slices were extensively washed with multiple changes of $Ca^{2+}$-free KHB (6 × 20 ml) at 37°C for 15 min.

In some experiments, slices were exposed to NMDA prior to inositol phospholipid labelling or determination of Ins (1,4,5)$P_3$ responses to agonist challenge. In these cases, slices were incubated 'in bulk' in the absence or presence of 100 μM NMDA for 15 min. After this period, slices were extensively washed with KHB (8 × 20 ml KHB over a period of 15–20 min) before dispensing 25 μl aliquots into vials containing 250 μl KHB (± [$^3$H]-inositol) and performing experiments as described above.

### *Preparation of samples for [$^3$H]-InsP$_x$ and Ins(1,4,5)$P_3$ measurement*

In all cases, incubations were terminated by addition of 300 μl ice-cold 1 M trichloroacetic acid (TCA) and immediately transferred to an ice-bath. Following intermittent vortex-mixing for 20 min, samples were centrifuged (4000 *g*, 20 min, 4°C) and the supernatant fraction extracted with 4 × 3 vol water-saturated diethylether. For recovery of the total [$^3$H]-inositol phosphate fraction ([$^3$H]-InsP$_x$), 150 μl 60 mM $NaHCO_3$ was added to 450 μl of the diethylether-extracted sample (to bring the pH to 7) and the neutral extract washed onto a 0.5 ml bed-volume Dowex 1-X8 ($Cl^-$ form) column with 20 ml $H_2O$. The column was washed with 12 ml 25 mM formate and then [$^3$H]-InsP$_x$ eluted in 10 ml 1 M HCl, with a 2 ml fraction being taken for scintillation counting. For Ins(1,4,5)$P_3$ mass assay, 50 μl 30 mM EDTA and 50 μl 60 mM $NaHCO_3$ were added to 200 μl of diethylether-extracted sample. Mass assay of Ins(1,4,5)$P_3$ was performed as described prevously (Challiss et al., 1988). In general, the slice pellets were washed with 2 ml 0.9% NaCl and then digested in 0.5 M NaOH for subsequent protein determination by the Lowry method. Alternatively, slice pellets were sequentially washed with 5% TCA/1 mM EDTA and $H_2O$ before phospholipid extraction as described by Downes & Wusteman (1983) and determination of total [$^3$H]-inositol phospholipids ([$^3$H]-PtdIns(P$_x$)) in the chloroform phase.

### *Materials*

*Myo*-[2-$^3$H]-inositol (17–20 Ci mmol$^{-1}$) and [$^3$H]-inositol 1,4,5-trisphosphate (Ins(1,4,5)$P_3$; 17–20 Ci mmol$^{-1}$) were obtained from DuPont NEN (DuPont U.K. Ltd., Stevenage, Herts.). D-Ins(1,4,5)$P_3$ was purchased from Rhode Island University (Kingston, Rhode Island, U.S.A.). 1-Aminocyclopentane-1S,3R-dicarboxylic acid (ACPD) was obtained from Tocris Neuramin (Langford, Bristol). N-methyl-D-aspartate (NMDA), D- and L-2-amino-5-phosphonopentanoate (D- and L-AP5), and Dowex anion exchange resin (AG1-X8, 100–200 mesh, $Cl^-$ form) were from Sigma Chemical Co. Ltd. (Poole, Dorset). MK-801 ((+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]-cyclohepten-5,10-imine maleate) was a gift from Merck Sharp & Dohme Laboratories (Harlow, Essex).

### *Data analysis*

All values are expressed as means ± s.e.mean for the indicated number of separate experiments. The computer programme GraphPad (ISI) was used to generate $EC_{50}/IC_{50}$ values from concentration-response data; $EC_{50}/IC_{50}$ values are given as means with 95% confidence limits in parentheses. Statistical comparisons were performed by use of Student's *t* test for unpaired observations.

## Results

### *[$^3$H]-phosphoinositide hydrolysis*

In initial experiments the effects of NMDA on [$^3$H]-InsP$_x$ accumulation stimulated by sub-maximal (10 μM) or maximal (300 μM) concentrations of 1S,3R-ACPD were investigated in neonatal rat cerebral cortex slices. In the presence of a concentration of LiCl (5 mM) sufficient to block inositol monophosphate dephosphorylation, 300 μM 1S,3R-ACPD caused a 12.8 ± 0.9 fold increase in [$^3$H]-InsP$_x$ accumulation over basal levels in the 15 min period of agonist exposure (Figure 1), whilst 10 μM 1S,3R-ACPD elicited about 40% of this response. Co-addition of low concentrations of NMDA caused a significant enhancement of [$^3$H]-InsP$_x$ accumulation elicited by 300 μM 1S,3R-ACPD (by 1.87 ± 0.15 fold ($P < 0.001$) at 10 μM NMDA), however, the enhancement became less pronounced at higher NMDA concentrations and was insignificant at 300 μM NMDA (Figure 1). Similar 'bell-shaped' patterns of modulation by NMDA were observed in the absence of LiCl, or when [$^3$H]-InsP$_x$ accumulation was stimulated by a sub-maximal concentration of 1S,3R-ACPD (Figure 1). Furthermore, supplementation of the incubation medium with 10 μM glycine, an established co-agonist necessary for activation of the NMDA receptor by glutamate

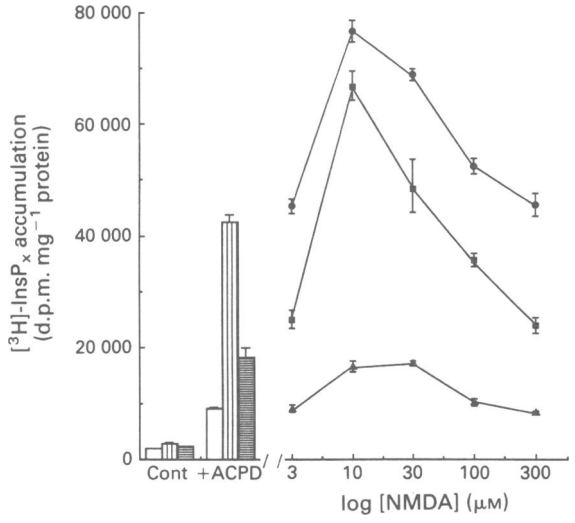


**Figure 1** Concentration-dependence of NMDA effect on 1S,3R-ACPD-stimulated [$^3$H]-InsP$_x$ accumulation: cerebral cortex slices were incubated with [$^3$H]-inositol (0.5 μCi per vial) for 60 min. After the labelling period, experiments were performed in the absence or presence of LiCl (5 mM final concentration). Slices were stimulated by addition of the indicated concentration of NMDA and either 10 μM (■) or 300 μM (▲, ●) 1S,3R-ACPD in the absence (▲) or presence (●) of LiCl. The effect of vehicle (Cont) or 1S,3R-ACPD (+ ACPD) additions alone are indicated as: + 300 μM 1S,3R-ACPD–LiCl (open columns); ± 10 μM 1S,3R-ACPD + LiCl (horizontally lined columns); ± 300 μM 1S,3R-ACPD + LiCl (vertically lined columns), in the histogram on the left of the figure. Incubations were terminated 15 min after 1S,3R-ACPD/NMDA challenge and samples processed for analysis of [$^3$H]-InsP$_x$ accumulation as described in the Methods section. Values are presented as means ± s.e.mean for at least three separate experiments performed in triplicate.

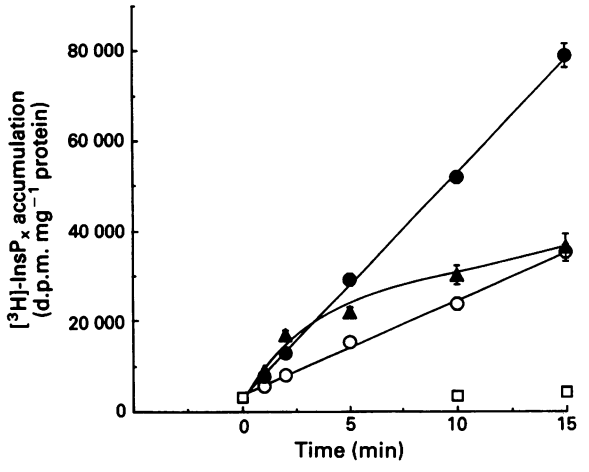


**Figure 2** Time course of 1S,3R-ACPD-stimulated [$^3$H]-InsP$_x$ accumulation in the absence or presence of different concentrations of NMDA: cerebral cortex slices were incubated with [$^3$H]-inositol (0.5 μCi per vial) for 60 min. LiCl (5 mM final concentration) was added and slices stimulated by addition of 300 μM 1S,3R-ACPD in the absence (○) or presence of 10 μM (●) or 100 μM (▲) NMDA. The effect of vehicle additions are also shown (□). Incubations were terminated after the indicated periods of agonist challenge and samples processed for analysis of [$^3$H]-InsP$_x$ accumulation as described in the Methods section. Values are presented as means ± s.e.mean for at least three separate experiments performed in triplicate.

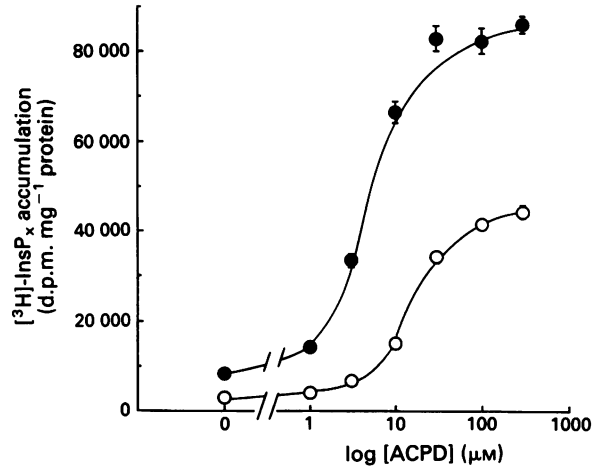


**Figure 3** Concentration-dependence of 1S,3R-ACPD-stimulated [$^3$H]-InsP$_x$ accumulation in the absence and presence of NMDA: cerebral cortex slices were incubated with [$^3$H]-inositol (0.5 μCi per vial) for 60 min. LiCl (5 mM final concentration) was added and slices stimulated by addition of various concentrations of 1S,3R-ACPD in the absence (○) or presence (●) of 10 μM NMDA. Incubations were terminated after 15 min and samples processed for analysis of [$^3$H]-InsP$_x$ accumulation as described in the Methods section. Values are presented as means ± s.e.mean for at least three separate experiments performed in triplicate.

(Johnson & Ascher, 1987; Kemp & Leeson, 1993), had no effect on either the magnitude or the concentration-dependency of the NMDA modulation of the 1S,3R-ACPD-stimulated response (data not shown) suggesting that there is sufficient endogenous glycine to saturate this site in the neonatal rat cerebral cortex slice preparation.

The time dependency of the NMDA modulatory action upon 1S,3R-ACPD-stimulated [$^3$H]-InsP$_x$ accumulation was investigated. 1S,3R-ACPD (300 μM) caused a linear accumulation of [$^3$H[-InsP$_x$ over a 15 min period of agonist exposure (Figure 2). In the presence of 10 μM NMDA, an enhancement of 1S,3R-ACPD-stimulated [$^3$H]-InsP$_x$ accumulation was immediately apparent (at 1 min time-point: + 1S,3R-ACPD, 2439 ± 188; + 1S,3R-ACPD + NMDA, 4447 ± 287 d.p.m. over basal mg$^{-1}$ protein; $P<0.01$) and was sustained throughout the 15 min time-course. In contrast, co-addition of 100 μM NMDA initially enhanced 1S,3R-ACPD-stimulated [$^3$H]-InsP$_x$ accumulation to a greater extent than did 10 μM NDMA; however, the rate of accumulation decreased throughout the time-course such that by 15 min after 1S,3R-ACPD/NMDA challenge [$^3$H]-InsP$_x$ accumulation was not significantly different from that observed in the presence of 1S,3R-ACPD alone (Figure 2). Analysis of slice total [$^3$H]-inositol phospholipids after 15 min of 1S,3R-ACPD/NMDA challenge revealed only minor differences in labelling (+ 1S,3R-ACPD, 147033 ± 4944; + 1S,3R-ACPD/10 μM NMDA, 174594 ± 4610; + 1S,3R-ACPD/100 μM NMDA, 129161 ± 6396 d.p.m./25 μl slices, data for four triplicate experiments) suggesting that NMDA is very unlikely to exert a modulatory action through effects on cellular inositol phospholipid pools.

The effect of 10 μM NMDA on the concentration-dependent stimulation of [$^3$H]-InsP$_x$ accumulation by 1S,3R-ACPD is shown in Figure 3. In agreement with previous studies in striatal (Sladeczek *et al.*, 1985) and cerebral cortical (Godfrey *et al.*, 1988; Baird & Nahorski, 1991), but not hippocampal (Nicoletti *et al.*, 1986; Schoepp & Johnson, 1988) preparations, NMDA *per se* caused a small, but highly significant, increase in [$^3$H]-InsP$_x$ accumulation (basal, 3067 ± 133; + 10 μM NMDA, 8394 ± 271 d.p.m. mg$^{-1}$ protein; $P<0.001$). In the absence of NMDA, the EC$_{50}$ for 1S,3R-ACPD-stimulated [$^3$H]-InsP$_x$ accumulation was 16.6 (13.4–20.5) μM, whereas in the presence of 10 μM NMDA the maximal response was enhanced almost two fold and the EC$_{50}$ decreased to 4.9

(4.2–5.7) μM. Such modulation by NMDA combined to produce a very dramatic effect at sub-maximal concentrations of 1S,3R-ACPD, thus at 10 μM 1S,3R-ACPD, NMDA caused a 3.8 fold increase in [$^3$H]-InsP$_x$ accumulation (Figure 3). This 'window' was exploited to investigate the effects of NMDA-receptor/channel antagonists on the modulatory action of NMDA on the 1S,3R-ACPD-stimulated response (see Figure 4a).

The uncompetitive antagonist, MK-801, caused a concentration-dependent inhibition of [$^3$H]-InsP$_x$ accumulation stimulated by 10 μM 1S,3R-ACPD and 10 μM NMDA (Figure 4b). Complete inhibition of the NMDA-enhanced component of the response was achieved in the presence of 1 μM MK-801. The $IC_{50}$ for MK-801 inhibition of the NMDA-enhanced response was 64.3 (44.0–93.9) nM. Similar inhibitory effects could be achieved with the competitive antagonist D-AP5 (Figure 4b). D-AP5 completely inhibited the NMDA-enhanced component of the response ($IC_{50}$ 39.0 (32.5–46.8) μM), whilst the L-enantiomer caused only a 14.8 ± 4.2% inhibition at 1 mM (Figure 4b). Concentrations of MK-801 (1 μM) and D-AP5 (1 mM), which caused maximal inhibitions of the 1S,3R-ACPD/NMDA response, had no effect on [$^3$H]-InsP$_x$ accumulation stimulated by 1S,3R-ACPD alone (data not shown).

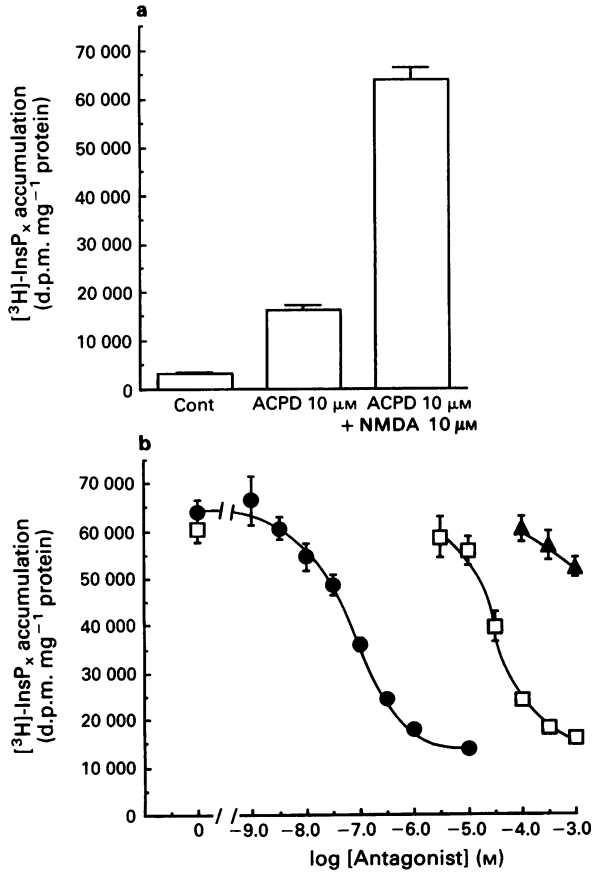


**Figure 4** Inhibition of the NMDA-enhanced [$^3$H]-InsP$_x$ response to 1S,3R-ACPD by MK-801 and D- or L-2-amino-5-phosphonopentanoate: cerebral cortex slices were incubated with [$^3$H]-inositol (0.5 μCi per vial) for 60 min. Following addition of LiCl (5 mM final concentration), the indicated concentrations of MK-801 (●), D-AP5 (□) or L-AP5 (▲) were added 15 min prior to simultaneous addition of 10 μM 1S,3R-ACPD and 10 μM NMDA (b); the basal [$^3$H]-InsP$_x$ accumulation (Cont) and the enhancement of 1S,3R-stimulated [$^3$H]-InsP$_x$ accumulation caused by co-addition of NMDA are shown in (a). Incubations were terminated 15 min after ACPD/NMDA addition as described in the Methods section. Values are presented as means ± s.e.mean for at least three separate experiments performed in triplicate. MK-801 (10 μM), D-AP5 (1 mM) or L-AP5 (1 mM) had no effect upon basal or 10 μM 1S,3R-ACPD-stimulated [$^3$H]-InsP$_x$ accumulation (data not shown).

### *Inositol 1,4,5-trisphosphate accumulation*

Further experiments investigated the ability of 1S,3R-ACPD to stimulate Ins(1,4,5)P$_3$ accumulation in neonatal rat cerebral cortex slices, and whether the NMDA modulatory effects on agonist-stimulated phosphoinositide turnover were also observed at the level of pathway second messenger generation. The time-course of Ins(1,4,5)P$_3$ accumulation stimulated by 1S,3R-ACPD is shown in Figure 5. A prompt 5–6 fold increase in Ins(1,4,5)P$_3$ accumulation was observed by 15 s after addition of 300 μM 1S,3R-ACPD, which decreased over the subsequent 10 min of agonist exposure. Despite the rapid decline from the early peak value, the Ins(1,4,5)P$_3$ accumulation was still significantly elevated over basal levels at 10 min (control, 22.7 ± 1.1; + 1S,3R-ACPD, 46.0 ± 4.0 pmol mg$^{-1}$ protein; $P<0.01$). When experiments were performed in nominally $Ca^{2+}$-free KHB ($[Ca^{2+}]_o$ approx 2–5 μM), the temporal changes in Ins(1,4,5)P$_3$ accumulation were similar to the control response although the magnitude was reduced (Figure 5). However, when the decrease in basal Ins(1,4,5)P$_3$ accumulation (about 40–50%) was taken into account, the relative changes over basal evoked by 1S,3R-ACPD were similar.

The modulatory effects of NMDA upon the time-course of Ins(1,4,5)P$_3$ accumulation stimulated by 300 μM 1S,3R-ACPD are shown in Figure 6. In agreement with previous work using cerebral cortex slices from adult rats (Baird & Nahorski, 1991), neither 10 nor 100 μM NMDA significantly affected basal Ins(1,4,5)P$_3$ accumulation (see Figure 7). At a concentration of 10 μM, NMDA caused a small (20%) enhancement of the initial increase in Ins(1,4,5)P$_3$ evoked by 1S,3R-ACPD at 15 s, and significantly enhanced agonist-stimulated Ins(1,4,5)P$_3$ accumulation at 1, 5 and 10 min ($P<0.01$ at each time-point). Thus at the 5 min time-point, the presence of 10 μM NMDA caused a 2.7 fold enhancement of the 1S,3R-ACPD-stimulated Ins(1,4,5)P$_3$ response (+ 1S, 3R-ACPD, 17.6 ± 2.7; + 1S,3R-ACPD/NMDA, 47.9 ± 3.9 pmol above basal mg$^{-1}$ protein; $P<0.01$). Compared to the

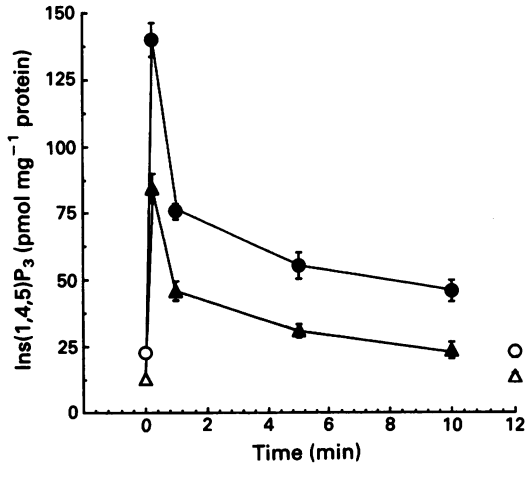


**Figure 5** Effect of extracellular calcium concentrations on the time-course of 1S,3R-ACPD-stimulated Ins(1,4,5)P$_3$ mass accumulation: cerebal cortex slices were incubated in normal KHB (1.3 mM $CaCl_2$) for 60 min. Slices were either dispensed into normal KHB (○, ●) or extensively washed (6 × 20 ml over a 15 min period) with $Ca^{2+}$-free KHB before dispensing slices into the same $Ca^{2+}$-free medium (△, ▲). 1S,3R-ACPD (300 μM) was added for the times indicated (filled symbols) and incubations terminated and processed for Ins(1,4,5)P$_3$ mass assay as described in the Methods section. Values are presented as means ± s.e.mean for four separate experiments performed in triplicate.

effect of 10 μM NMDA, 100 μM NMDA caused a greater (50%) enhancement of the initial increase in Ins(1,4,5)$P_3$ accumulation evoked by 1S,3R-ACPD (+ 1S,3R-ACPD, 145.6 ± 7.9; + 1S,3R-ACPD/NMDA, 202.5 ± 11.4 pmol $mg^{-1}$ protein at 15 s; $P<0.02$). Although the enhancement was still evident 1 min after 1S,3R-ACPD challenge, it decreased below that caused by 10 μM NMDA by 5 min. Furthermore, 10 min after 1S,3R-ACPD/100 μM NMDA addition, Ins(1,4,5)$P_3$ accumulation had returned to control values and was significantly lower than that evoked by 1S,3R-ACPD alone (Figure 6).

The concentration-dependency of the NMDA modulatory effects upon 1S,3R-ACPD-stimulated Ins(1,4,5)$P_3$ accumulation is presented in Figure 7. A time-point of 5 min after 1S,3R-ACPD ± NMDA was chosen for the study. Low concentrations of NMDA (3–30 μM) enhanced 1S,3R-ACPD-stimulated Ins(1,4,5)$P_3$ accumulation with a maximal effect being observed at 30 μM NMDA, whilst higher concentrations of

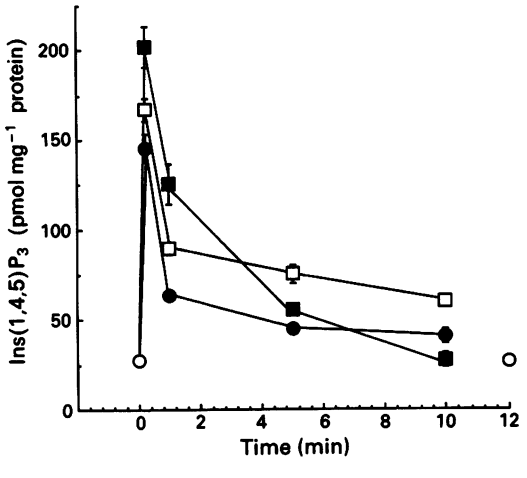


**Figure 6** Effect of NMDA on the time-course of 1S,3R-ACPD-stimulated Ins(1,4,5)$P_3$ mass accumulation: cerebral cortex slices were incubated for 60 min prior to addition of vehicle (○) or 300 μM 1S,3R-ACPD in the absence (●) or presence of 10 μM (□) or 100 μM (■) NMDA for the times indicated. Incubations were terminated and processed for measurement of Ins(1,4,5)$P_3$ mass as described in the Methods section. Values are shown as means ± s.e.mean for four separate experiments performed in duplicate or triplicate. Statistical comparisons (Student's $t$ test) to evaluate the effect of NMDA on the 1S,3R-ACPD-stimulated response demonstrated that at the earliest time points (0.25 and 1 min) both 10 and 100 μM NMDA significantly enhanced the control response ($P<0.05$). This significant enhancement was maintained at the 5 and 10 min time-points for 10 μM NMDA; however, in the presence of 100 μM NMDA, 1S,3R-ACPD-stimulated Ins(1,4,5)$P_3$ accumulation was significantly decreased by the 10 min time point ($P<0.01$).

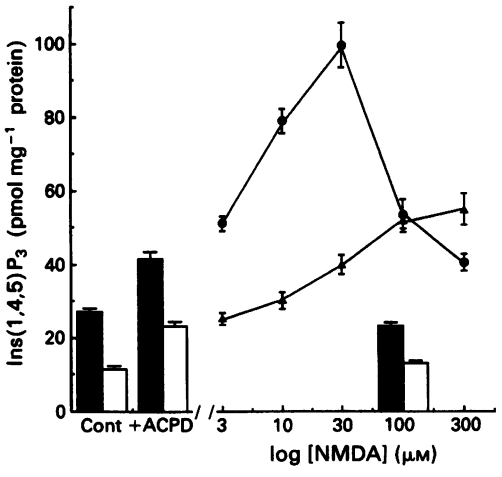


**Figure 7** Effect of extracellular calcium concentration on the modulation of 1S,3R-ACPD-stimulated Ins(1,4,5)$P_3$ mass accumulation by NMDA: cerebral cortex slices were incubated in normal KHB (1.3 mM $CaCl_2$) for 60 min. Slices were either dispensed into normal KHB (●) or extensively washed (6 × 20 ml over a 15 min period) with nominally $Ca^{2+}$-free KHB before dispensing slices into the same $Ca^{2+}$-free medium (▲). In all cases, the indicated concentrations of NMDA and/or 1S,3R-ACPD (300 μM) were added for 10 min before incubations were terminated and processed for Ins(1,4,5)$P_3$ mass as described in the Methods section. Columns depict basal accumulations (Cont) or those stimulated by 300 μM 1S,3R-ACPD (+ ACPD) or 100 μM NMDA in normal KHB (solid columns) or $Ca^{2+}$-free KHB (open columns). Values are presented as means ± s.e.mean for three separate experiments performed in triplicate.

**Table 1** Effects of pre-exposure to 100 μM N-methyl-D-aspartate (NMDA) for 15 min on subsequent inositol phospholipid labelling and agonist-stimulated [$^3$H]-Ins$P_x$ and Ins(1,4,5)$P_3$ accumulations

| | *Control* | *NMDA pre-exposure* |
|---|---|---|
| (i) [$^3$H]-PtdIns($P_x$) labelling (d.p.m. per 25 μl slice) | | |
| No addition | 66822 ± 1148 | 20775 ± 760 |
| + ACPD | 119987 ± 6896 | 29456 ± 1165 |
| + ACPD/NMDA (10 μM) | 128696 ± 8293 | 33461 ± 1374 |
| + ACPD/NMDA (100 μM) | 95163 ± 5620 | 29828 ± 300 |
| (ii) [$^3$H]-Ins$P_x$ accumulation (d.p.m. per 25 μl slice) | | |
| No addition | 3402 ± 424 | 2514 ± 268 |
| + ACPD | 27662 ± 895 | 12512 ± 1154 |
| + ACPD/NMDA (10 μM) | 63949 ± 671 | 16263 ± 611 |
| + ACPD/NMDA (100 μM) | 33288 ± 1788 | 13995 ± 751 |
| (iii) Ins(1,4,5)$P_3$ accumulation (pmol $mg^{-1}$ protein) | | |
| No addition | 21.8 ± 1.0 | 15.0 ± 0.8 |
| + ACPD | 42.7 ± 2.6 | 20.4 ± 1.5 |
| + ACPD/NMDA (10 μM) | 67.0 ± 3.6 | 24.1 ± 1.1 |
| + ACPD/NMDA (100 μM) | 53.7 ± 4.1 | 27.9 ± 1.8 |

Neonatal rat cerebral cortex slices were pre-incubated for 15 min in the absence (control) or presence (NMDA pre-exposure) of 100 μM NMDA. Slices were then washed extensively with multiple changes of KHB and 25 μl aliquots of packed slices dispensed into 250 μl KHB. For (i) and (ii), the KHB contained 0.5 μCi [$^3$H]-inositol and after the 60 min labelling period, LiCl (5 mM) was added. Incubations were terminated either 15 min (for (i) and (ii)) or 5 min (for (iii)) after addition of 300 μM 1S,3R-ACPD (ACPD) in the absence or presence of the indicated concentration of NMDA. Values are presented as means ± s.e.mean for a single triplicate experiment. Similar data were obtained on two further occasions.

NMDA (100, 300 μM) failed to cause any enhancement of this response. However, when experiments were performed in nominally $Ca^{2+}$-free KHB, NMDA concentration-dependently enhanced the 1S,3R-ACPD-stimulated responses ($EC_{50}$ 32.3 (25.4–41.2) μM), with the greatest effect being observed at 300 μM NMDA (Figure 7).

The data presented above (see Figures 2 and 6) suggest that whilst high concentrations of NMDA cause an initial enhancement of the agonist-stimulated phosphoinositide response, this is time-dependently superceded by an inhibitory effect, which is dependent upon the presence of physiological concentrations of $Ca^{2+}$ in the incubation medium (Figure 7). To explore this possibility further, the effect of pre-exposure to 100 μM NMDA on subsequent phosphoinositide labelling and 1S,3R-ACPD-stimulated [$^3$H]-$InsP_x$ and Ins(1,4,5)$P_3$ accumulations was studied (Table 1). Over three separate experiments, pretreatment with 100 μM NMDA attenuated subsequent [$^3$H]-inositol incorporation into phospholipids by 67.9 ± 3.4%. Although basal and 1S,3R-ACPD-stimulated [$^3$H]-$InsP_x$ accumulations were not as severely affected by NMDA pre-exposure compared to inositol phospholipid labelling, the magnitude of the stimulation-over-basal was attenuated by about 50% and the enhancement of the response to 1S,3R-ACPD in the presence of 10 μM NMDA was reduced from 2.3 fold potentiation in control slices compared to only a 30% enhancement in the NMDA pretreatment group (Table 1). The effect of NMDA pre-exposure was also observed with respect to Ins(1,4,5)$P_3$ accumulation evoked by 1S,3R-ACPD (± NMDA). Data are shown for Ins(1,4,5)$P_3$ accumulation at 5 min after agonist addition, however, similar attenuations caused by NMDA pre-exposure were seen throughout the time course (data not shown).

## Discussion

Since the original cloning and functional expression of a glutamate receptor (mGluR1) belonging to the guanine nucleotide binding protein-coupled superfamily (Masu *et al.*, 1991), a number of structurally-related receptors have been described (Abe *et al.*, 1992; Tanabe *et al.*, 1992; Pickering *et al.*, 1993). Of these, glutamate receptor agonists have been shown to stimulate phosphoinositide turnover in cells expressing mGluR1α/β and mGluR5 (Abe *et al.*, 1992; Tanabe *et al.*, 1992; Pickering *et al.*, 1993). The mGluRs linked to phosphoinositide turnover exhibit pharmacological differences (Abe *et al.*, 1992; Aramori & Nakanishi, 1992; Tanabe *et al.*, 1992; Pickering *et al.*, 1993), differential susceptibility to pertussis toxin (Abe *et al.*, 1992; Aramori & Nakanishi, 1992; Pickering *et al.*, 1993) and have been shown to be differentially expressed in rat brain (Abe *et al.*, 1992; Martin *et al.*, 1992; Fotuhi *et al.*, 1993).

In the present study, the mGluR agonist 1S,3R-ACPD has been shown to cause a sustained accumulation of [$^3$H]-$InsP_x$ in neonatal rat cerebral cortex slices pre-labelled with [$^3$H]-inositol and challenged with 1S,3R-ACPD in the presence of sufficient LiCl to block inositol monophosphate activity. The $EC_{50}$ for this response (16.6 μM) is in good agreement with values previously obtained in guinea-pig cerebral cortex (Jones & Roberts, 1993) and primary rat cerebro-cortical cultures (Birrell & Marcoux, 1993), although the magnitude of the response reported here is much greater. If it is assumed that the phosphoinositide-generating activity of *trans*-ACPD (a mixture of 1S,3R and 1R,3S enantiomers) resides in the 1S,3R enantiomer (Irving *et al.*, 1990; Jones & Roberts, 1993), then comparable $EC_{50}$ values for *trans*-ACPD-stimulated phosphoinositide turnover have been obtained for mGluR5 (50 μM; Abe *et al.*, 1992) and mGluR1α and MGluR1β (37 and 106 μM respectively; Pickering *et al.*, 1993) in cells transfected to express specific metabotropic receptor subtypes.

Further studies demonstrated that 1S,3R-ACPD (300 μM) also stimulated a dramatic initial increase in Ins(1,4,5)$P_3$ mass accumulation, which was maximal, some six fold above basal levels, within approximately 15 s. Although Ins(1,4,5)$P_3$ accumulation declined over the subsequent time-course, the accumulation of this second messenger was still significantly elevated above basal levels 10 min after addition of 1S,3R-ACPD. Whether this pattern of response reflects a rapid, but partial, desensitization of metabotropic receptor-mediated PI-PLC activity or an enhanced metabolism of Ins(1,4,5)$P_3$ via 3-kinase and/or 5-phosphatase routes is not known (see Wojcikiewicz *et al.*, 1993). Preliminary experiments using high performance liquid chromatography (h.p.l.c.) to investigate, in detail, changes in inositol (poly) phosphate isomer profiles following metabotropic receptor agonist challenge do not provide evidence for any shift in PI-PLC substrate selectivity from the initial fidelity for $PtdInsP_2$ to other inositol phospholipids (Gray, Challiss & Nahorski, unpublished results).

In the absence of extracellular $Ca^{2+}$, the 1S,3R-ACPD-induced increase in Ins(1,4,5)$P_3$ mass accumulation, was significantly reduced both during the initial peak and later sustained phases. However, in common with a previous study which investigated muscarinic cholinoceptor-stimulated Ins(1,4,5)$P_3$ mass accumulation in cerebral cortex prepared from adult rats (Challiss & Nahorski, 1991), the difference between responses in the presence or absence of physiological levels of extracellular $Ca^{2+}$ resided primarily in the changes in basal Ins(1,4,5)$P_3$ levels. Thus, removal of extracellular $Ca^{2+}$ caused a 40–50% decrease in basal Ins(1,4,5)$P_3$: if 1S,3R-ACPD-stimulated responses were expressed relative to the different basal values then almost identical 'fold' stimulations were observed in the absence and presence of $Ca^{2+}$. It should be noted that the nominal absence of $Ca^{2+}$ (extracellular [$Ca^{2+}$] approximately 2–5 μM) is sufficient to eliminate possible indirect mechanisms of 1S,3R-ACPD-stimulated phosphoinositide turnover (i.e. 1S,3R-ACPD-stimulated release of a neurotransmitter(s) which can in turn stimulate phosphoinositide turnover), strongly suggesting that the phosphoinositide responses observed in neonatal cerebral cortex slices, in common with adult cerebral cortex (Challiss & Nahorski, 1991) and foetally-derived cerebrocortical cultures (Birrell & Marcoux, 1993), are due to 1S,3R-ACPD interacting with a metabotropic receptor directly linked to phosphoinositide turnover.

Since the initial report by Baudry and colleagues (1986) that ionotropic glutamate receptor activation could influence signalling by a variety of agonists linked to phosphoinositide turnover, a considerable number of studies have investigated the mechanistic bases for such modulations, particularly with respect to the effects of NMDA on muscarinic cholinoceptor-stimulated phosphoinositide hydrolysis (Schmidt *et al.*, 1987; Godfrey *et al.*, 1988; Gonzales & Moerschbaecher, 1989; Morrisett *et al.*, 1990; Baird & Nahorski, 1991; Arias-Montana *et al.*, 1993). In contrast to the predominant inhibitory effect of NMDA on phosphoinositide hydrolysis elicited by muscarinic receptor agonists, our studies have demonstrated an enhancement of phosphoinositide hydrolysis in response to metabotropic glutamate receptor stimulation in the presence of NMDA. Thus, although addition of NMDA alone resulted in a significant increase in [$^3$H]-$InsP_x$ accumulation, co-addition of NMDA (10 μM) and 1S,3R-ACPD (300 μM) caused a much greater than additive increase in this response compared to that elicited by either agent added alone. The maximum facilitatory effect was observed at 10–30 μM NMDA and was evident at maximal and sub-maximal concentrations of 1S,3R-ACPD in the absence and presence of LiCl. Furthermore, the effect of NMDA was immediately apparent, with respect to the initial increases in both Ins(1,4,5)$P_3$ and [$^3$H]-$InsP_x$ accumulations.

The NMDA receptor responsible for modulation of the 1S,3R-ACPD-stimulated response was inhibited by the competitive antagonist D-AP5 (but not L-AP5) and the uncompetitive antagonist MK-801 with 50% inhibitory effects occurring at concentrations of each antagonist consistent with those previously reported for studies using similar experimental

paradigms or more direct indices of NMDA receptor/channel activity (Gonzales & Moerschbaecher, 1989; Morrisett *et al.*, 1990).

The most likely mechanism by which NMDA facilitates 1S,3R-ACPD-stimulated phosphoinositide turnover is through an increase in intracellular $Ca^{2+}$ concentration via activation of the NMDA receptor/ion channel to increase the influx of $Ca^{2+}$. A number of studies have demonstrated that manipulations which increase intracellular $Ca^{2+}$ can have variable, but generally small, stimulatory effects on basal PI-PLC activity, whilst synergistically increasing agonist-stimulated PI-PLC activity (Eberhard & Holz, 1988; Baird & Nahorski, 1990; Baird *et al.*, 1991; Challiss & Nahorski, 1991). Indeed, the effect of NMDA upon the concentration-response relationship for 1S,3R-ACPD-stimulated [$^3$H]-InsP$_x$ accumulation can be almost precisely mimicked, including the apparent shift to the left of the concentration-response curve, by increasing the extracellular $K^+$ concentration from 4.7 to 30 mM (data not shown). Thus, although mGluR coupling to PI-PLC is mediated, at least in part, via a pertussis toxin-sensitive G-protein (Abe *et al.*, 1992; Aramori & Nakanishi, 1992; Pickering *et al.*, 1993), the PI-PLC(s) activated by this transduction pathway appears to exhibit similar $Ca^{2+}$-sensitivity to PI-PLC(s) activated by receptors linked via pertussis toxin-insensitive G-proteins.

At concentrations of NMDA greater than 30 $\mu$M a less dramatic facilitation of 1S,3R-ACPD-stimulated [$^3$H]-InsP$_x$ was observed. Time-course studies of both [$^3$H]-InsP$_x$ and Ins(1,4,5)P$_3$ accumulations clearly demonstrated that NMDA (at 100 $\mu$M) initially caused a greater facilitation of 1S,3R-ACPD-stimulated responses than was seen in the presence of 10 $\mu$M NMDA; however, for both phosphoinositide responses the facilitation decreased with time. Thus, by 10 min after co-addition of 1S,3R-ACPD and 100 $\mu$M NMDA the rate of [$^3$H]-InsP$_x$ accumulation had decreased considerably (see Figure 2) and Ins(1,4,5)P$_3$ mass accumulation was lower than that observed in the presence of 1S,3R-ACPD only (see Figure 6). These data suggest that the initial stimulatory modulation of the 1S,3R-ACPD-stimulated phosphoinositide response is superceded by an inhibitory effect of NMDA which becomes predominant within about 10 min of addition to cerebral cortex slices. The time-dependency of the inhibitory effect of NMDA is consistent with a previous report by Palmer and colleagues (1988), who found that 100 $\mu$M NMDA severely inhibited quisqualate-stimulated [$^3$H]-InsP$_x$ accumulation in neonatal hippocampal slices when incubations were continued for 60 min.

To investigate further the temporal dependence of NMDA modulation of 1S,3R-ACPD-stimulated phosphoinositide responses, a different experimental paradigm was adopted. The effects of NMDA (100 $\mu$M) pretreatment upon inositol phospholipid-labelling with [$^3$H]-inositol, and [$^3$H]-InsP$_x$ and Ins(1,4,5)P$_3$ accumulations stimulated by 1S,3R-ACPD were investigated. NMDA pretreatment of neonatal cerebral cortex slices for 15 min resulted in a dramatic decrease in subsequent total inositol phospholipid labelling. Although 1S,3R-ACPD-stimulated [$^3$H]-InsP$_x$ accumulation was relatively well-maintained, co-addition of a maximally effective concentration of NMDA (10 $\mu$M) caused only a modest facilitation of the response stimulated by the metabotropic agonist compared to that elicited in slices not pre-exposed to NMDA (Table 1). Similar results have been reported recently in adult rat striatal discs, where pre-exposure to 100 $\mu$M NMDA markedly decreased subsequent [$^3$H]-inositol phospholipid-labelling and [$^3$H]-InsP$_x$ accumulations stimulated by carbachol (Arias-Montano *et al.*, 1993). Furthermore, in the present study we have also shown that the detrimental effect of pre-exposure to 100 $\mu$M NMDA is seen in experiments which are not dependent on interpretation of labelling data; thus, following NMDA pre-exposure the increase in Ins(1,4,5)P$_3$ accumulation caused by 1S,3R-ACPD and the facilitation caused by co-addition of 10 $\mu$M NMDA, were profoundly attenuated relative to the responses of slices not pretreated with NMDA.

In common with the conclusion reached by Arias-Montano and colleagues (1993), we feel that the simplest explanation for our own experimental data is that high concentrations of NMDA rapidly cause damage and destruction of a significant proportion of the cerebral cortical cells. How such a rapid neurotoxic effect may be brought about is unresolved; however, the independent or interactive roles of NMDA-receptor/ion channel-mediated $Ca^{2+}$ and $Na^+$ entry must be prime candidates. Thus, it is interesting to note that whilst NMDA (> 30 $\mu$M) appears to cause inhibition of 1S,3R-ACPD-stimulated Ins(1,4,5)P$_3$ accumulation (at 5 min) in normal medium ($[Ca^{2+}]_o$ = 1.3 mM), under nominally $Ca^{2+}$-free conditions a positive modulation is seen at all concentrations of NMDA, allowing an $EC_{50}$ of about 30 $\mu$M to be estimated for the facilitation of 1S,3R-ACPD-stimulated Ins(1,4,5)P$_3$ accumulation by NMDA. In addition, preliminary experiments have shown that pre-exposure to NMDA (100 $\mu$M) in nominally $Ca^{2+}$-free medium has little or no effect on the subsequent stimulation of Ins(1,4,5)P$_3$ accumulation by 1S,3R-ACPD, or the positive modulatory effect of 10 $\mu$M NMDA upon this response (Challis, Mistry & Nahorski, unpublished results). Taken together these data suggest that NMDA can 'gate' sufficient $Ca^{2+}$-entry, when extracellular $Ca^{2+}$ concentration is reduced to the low micromolar range, to facilitate agonist-stimulated PI-PLC activity, but $Ca^{2+}$ entering under these circumstances does not reach levels at which toxic effects begin to be seen (at least within the time courses of the experiments reported here).

Although these data, and those provided by others (Gonzales & Moeschbaecher, 1989; Baird & Nahorski, 1991; Arais-Montano *et al.*, 1993), suggest that acute $Ca^{2+}$ overload may be primarily responsible for the acute neurotoxic actions of NMDA at least on phosphoinositide signalling, the role of $Na^+$-influx via the NMDA-receptor/ion channel should not be discounted. Thus, other groups have found that inhibitory effects of NMDA on agonist-stimulated phosphoinositide responses are essentially unaffected by removal of extracellular $Ca^{2+}$, whilst removal of $Na^+$ (with suitable substitution to maintain osmolarity and [$Cl^-$]) profoundly affected the modulatory effect of NMDA (Baudry *et al.*, 1986; Morrisett *et al.*, 1990). Although it is difficult to reconcile these conflicting results, it is noteworthy that the latter studies were both performed in hippocampal slices, whereas the former, which support a pre-eminent role for $Ca^{2+}$ in the neurotoxic action of NMDA, were all conducted in cerebral cortical or striatal preparations. Perhaps this indicates that regional differences may provide a suitable explanation.

In conclusion, we have shown that NMDA can cause complex modulations of phosphoinositide responses stimulated by metabotropic glutamate-receptor activation in neonatal rat cerebral cortex slices. At low concentrations, NMDA potentiates 1S,3R-ACPD-stimulated Ins(1,4,5)P$_3$ accumulation, probably by increasing intracellular $Ca^{2+}$ concentration and facilitation of agonist-stimulated PI-PLC activity. These observations suggest that as well as NMDA-receptor/ion channel activity being affected by metabotropic receptor activation (Bleakman *et al.*, 1992; Courtney & Nicholls, 1992; Chen & Huang, 1992; Kelso *et al.*, 1992; Harvey & Collingridge, 1993), reciprocal modulations can also occur, providing additional evidence for the complexities of ionotropic/metabotropic 'cross-talk' between glutamate receptors which are considered to underlie phenomena such as synaptic plasticity (Madison *et al.*, 1991; Bashir *et al.*, 1993; Behnisch & Reymann, 1993; Bliss & Collingridge, 1993). In contrast, exposure of neonatal cerebral cortex slices to higher concentrations of NMDA causes a rapidly developing inhibition of agonist-stimulated phosphoinositide responses which are most likely to be due to an acute, $Ca^{2+}$-dependent neurotoxic action of NMDA in this brain preparation.

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# Cardiovascular and behavioural effects of centrally administered tachykinins in the rat: characterization of receptors with selective antagonists

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**1** The effects of intracerebroventricular (i.c.v.) injection of selective and potent $NK_1$ (RP 67580), $NK_2$ (SR 48968) and $NK_3$ (R 486, [Trp$^7$, β-Ala$^8$]NKA(4-10)) receptor antagonists were assessed on the cardiovascular and behavioural responses elicited by the i.c.v. injection of substance P (SP), neurokinin A (NKA) or [MePhe$^7$]neurokinin B ([MePhe$^7$]NKB) in the conscious freely moving rat.

**2** SP, NKA and [MePhe$^7$]NKB (5–650 pmol) evoked dose-dependent increases in mean arterial blood pressure (MAP) and heart rate (HR) with the rank order of potency SP > NKA > [MePhe$^7$]NKB. The cardiovascular responses were accompanied by excessive face washing, grooming and wet dog shakes.

**3** The cardiovascular effects and face washing behaviour induced by SP (25 pmol) were significantly reduced by the pre-injection (i.c.v., 5 min earlier) of RP 67580 (6.5 nmol). However, this antagonist failed to affect the central effects of 25 pmol NKA or [MePhe$^7$]NKB.

**4** The cardiovascular and behavioural responses (except for wet dog shakes) elicited by NKA (25 pmol) were significantly reduced by 6.5 nmol SR 48968. However, the latter antagonist had no effect on the SP or [MePhe$^7$]NKB-mediated responses.

**5** Both cardiovascular and behavioural effects produced by either SP or NKA (25 pmol) were completely abolished when rats were pretreated with a combination of RP 67580 (6.5 nmol) and SR 48968 (6.5 nmol), yet this combination of antagonists failed to modify the central effects of [MePhe$^7$]NKB.

**6** R 486 (6.5 nmol) inhibited the cardiovascular effects as well as wet dog shakes produced by [MePhe$^7$]NKB, but it was inactive against the responses induced by either SP or NKA.

**7** None of the tachykinin receptor antagonists or agonists caused motor impairment or respiratory distress. All antagonists blocked in a reversible manner and were devoid of intrinsic activity except R 486 (6.5 nmol) which produced a transient increase of MAP and HR.

**8** These results suggest that the central effects of SP, NKA and [MePhe$^7$]NKB are primarily mediated by central $NK_1$, $NK_2$ and $NK_3$ receptors, respectively. However, a minor activation of $NK_2$ receptors by SP and $NK_1$ receptors by NKA was seen during blockade of both receptors. This study therefore supports the existence of functional $NK_1$, $NK_2$ and $NK_3$ receptors in the adult rat brain.

**Keywords:** Substance P; neurokinin A; neurokinin B; tachykinin receptors; tachykinin antagonists; central effects; cardiovascular system; behaviour

## Introduction

The mammalian tachykinins, substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) are widely distributed in the central nervous system (CNS) and peripheral tissues. They are believed to play several neurotransmitter functions in central cardiovascular regulation, motor activity, and on sensory, autonomic and endocrine systems (for review see Otsuka & Yoshioka, 1993). So far, three tachykinin receptors termed $NK_1$, $NK_2$ and $NK_3$, have been cloned and pharmacologically characterized. The rank order of potency of tachykinins is SP > NKA > NKB for the $NK_1$ receptor, NKA > NKB > SP for the $NK_2$ receptor and NKB > NKA > SP for the $NK_3$ receptor (Guard & Watson, 1991; Maggi *et al.*, 1993b; Mussap *et al.*, 1993).

Intracerebroventricular (i.c.v.) injection of SP or NKA in the conscious freely moving rat leads to increase in mean arterial blood pressure (MAP), heart rate (HR), cardiac output, and to enhanced locomotor activity, awareness, scratching and face washing behaviour (Unger *et al.*, 1988; Itoi *et al.*, 1992; Tschöpe *et al.*, 1992). On the other hand, administration of NKB or the $NK_3$-selective agonist, senktide, into the lateral ventricle of the conscious rat also induces increases in blood pressure and HR, but evokes a unique behavioural pattern, the wet dog shake (Itoi *et al.*, 1992). Although all three tachykinins have been reported to act mainly on hypothalamic neurones (Itoi *et al.*, 1991; Massi *et al.*, 1991), the cardiovascular responses induced by either SP or NKA have been associated with an increased sympathoadrenal activity (Unger *et al.*, 1981; Takano *et al.*, 1990), while those induced by i.c.v. injection of $NK_3$ agonists would result mainly from the release of vasopressin from the hypothalamus and to a minor extent by activation of the sympathetic nervous system (Polidori *et al.*, 1989; Takano *et al.*, 1990; 1993).

Tachykinin antagonists may represent useful pharmacological tools to characterize central tachykinin receptors and to define better the role of these neuropeptides in central cardiovascular regulation. In a recent study, we provided evidence for the existence of distinct populations of functionally active $NK_1$ and $NK_2$ receptors in the adult rat brain with the use of selective tachykinin antagonists, namely (±)-CP 96345 to block the $NK_1$, MEN 10207, MEN 10376 and R 396 to inhibit the $NK_2$ receptor. It was concluded that the cardiovascular and behavioural effects of i.c.v. SP and NKA are mediated by $NK_1$ and $NK_2$ receptors, respectively (Tschöpe *et al.*, 1992). The role of $NK_1$ receptors in the central action of SP remains, however, to be confirmed as the quinuclidine antagonist (±)-CP 96345 may act as an

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antagonist of L-type calcium channels in rat cardiac and brain tissue (Schmidt *et al.*, 1992; Guard *et al.*, 1993) and as a blocker of voltage-dependent sodium currents in rat neocortical neurones (Caeser *et al.*, 1993). Hence, non-specific effects produced by (±)-CP 96345, probably not related to an interaction with tachykinin receptors, could not be excluded in our earlier study (Tschöpe *et al.*, 1992). In this respect, the perhydroisoindolone compound, RP 67580, is a new, potent and promising non-peptide antagonist selective for the $NK_1$ receptor showing higher potency in rat than in guinea-pig and man (Garret *et al.*, 1992; Carruette *et al.*, 1992; Rouissi *et al.*, 1993). Moreover, the new selective $NK_2$ receptor non-peptide antagonist, SR 48968, has higher affinity and stability than the former peptide antagonists (MEN 10207, MEN 10376, R 396, MDL 28564) (Advenier *et al.*, 1992; Emonds-Alt *et al.*, 1992). SR 48968 blocks in a dose-dependent and reversible manner the hyperalgesic response to NKA(4-10) (selective $NK_2$ agonist) but not that induced by [$Sar^9$, $Met(O_2)^{11}$]SP (selective $NK_1$ agonist) in the rat spinal cord (Picard *et al.*, 1993). RP 67580 and SR 48968 are potent antagonists which exert specific, competitive, reversible, yet non toxic antagonism and should therefore be suitable for studying the function of central $NK_1$ and $NK_2$ receptors (Advenier *et al.*, 1992; Rouissi *et al.*, 1993; Maggi *et al.*, 1993a; Picard *et al.*, 1993). Up to now, the lack of a potent and selective $NK_3$ receptor antagonist had limited our understanding of the role played by this receptor in central cardiovascular regulation. However, R 486, R 487 and GR 138676 belong to the first generation of peptide antagonists selective for the $NK_3$ receptor (Regoli *et al.*, 1991; Stables *et al.*, 1993). R 487 blocks in a specific manner the antinociceptive effect of intrathecally injected [$MePhe^7$]NKB (selective $NK_3$ agonist) in the rat tail-flick test (Couture *et al.*, 1993).

The purpose of the present study was twofold: firstly, to confirm the participation of $NK_1$ and $NK_2$ receptors in central cardiovascular and behavioural effects of SP and NKA, by using newly developed, non-peptide, receptor selective antagonists (RP 67580 and SR 48968). Secondly, to determine the participation of central $NK_3$ receptors in the cardiovascular and behavioural effects of tachykinins by using a selective agonist ([$MePhe^7$]NKB) and antagonist (R 486) of the $NK_3$ receptor. A preliminary account of this work has been presented elsewhere (Picard *et al.*, 1992).

## Methods

### *Animal preparation*

Male Wistar rats (Charles River, St. Constant, Québec, Canada) weighing 300–350 g were used. The animals were allowed free access to food and water and maintained on a 12 h light/dark cycle (lights on 06 h 00 min–18 h 00 min).

Rats were anaesthetized with an intraperitoneal (i.p.) injection of 65 mg $kg^{-1}$ sodium pentobarbitone (Somnotol; M.T.C. Pharmaceuticals, Cambridge, Ont. Canada) and an i.c.v. polyethylene cannula (PE-20; Intramedic, Clay Adams, NJ, U.S.A.) was implanted into the right brain ventricle by use of a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, U.S.A.) and fixed to the skull with dental cement (Reliance Dental MFG. Co., Worth, IL, U.S.A.). The co-ordinates were 1.3 mm lateral to the midline, 0.6 mm caudal to the bregma and 5.0 mm vertical from the skull surface. The angle of the head was adjusted according to the horizontal plan with respect to bregma and lambda reference points. The animals were then placed in individual plastic cages (40 cm × 23 cm × 20 cm). After a recovery period of 24 h, rats were anaesthetized again and a second polyethylene cannula (PE-50) was inserted through a femoral artery into the abdominal aorta for measurement of blood pressure and heart rate. The pre-siliconized intraarterial catheter was filled with physiological saline containing heparin sodium salt (100 i.u. $ml^{-1}$), passed through a subcutaneous tunnel and emerged at the back of the neck. Experiments were conducted 24 h after the intravascular surgery on conscious unrestrained rats kept in their resident cage.

Rats were injected i.c.v. with 25 pmol angiotensin II (AII) to verify the potency of the i.c.v. cannula. Only those animals which responded by an immediate sharp rise of blood pressure associated with an intense dipsogenic activity as reported earlier (Thunhorst & Johnson, 1993) were included in the study. The correct position of the i.c.v. cannula was verified histologically by post-mortem dissection.

### *Measurement of cardiovascular and behavioural responses*

The arterial pressure was monitored through the intra-arterial catheter with a Statham pressure transducer (P231D) while the heart rate was measured with a cardiac tachometer (model 7P4) and both variables were displayed on a Grass polygraph model 79D (Grass Instruments Co., Quincy, MA., U.S.A.). Experiments started when the animal was in a resting state and basal MAP and HR were stable.

The behavioural activity was recorded in their resident plastic cage over a 30 min period starting immediately with the i.c.v. injections. During the course of these experiments, the grid cage top was removed. The frequency of the individual behavioural responses: face washing and grooming, was determined according to the 15 s sampling procedure of Gispen *et al.* (1975). During every consecutive period of 15 s, a score 1 or 0 was given systematically depending on whether the animal showed the specific type of behaviour or not, whatever its frequency, intensity or duration during that period. Summation of scores for 30 min following the i.c.v. injection gave the behavioural scores for face washing and grooming in each experiment. The maximal theoretical score was 120 (15 s intervals × 30 min). The wet dog shake was measured according to the number of episodes (less than 1 s each) during the 30 min period, whatever the intensity.

### *Experimental protocols*

In the first series of experiments, the effects of three to four doses (5 pmol, 25 pmol, 65 pmol, 325 pmol or 650 pmol) of SP, NKA or [$MePhe^7$]NKB on MAP and HR were measured following i.c.v. administration. Only one peptide was administered to a rat at increasing doses; each dose was administered at intervals of 24 h to avoid tachyphylaxis (Itoi *et al.*, 1992), in a volume of 1 µl of artificial cerebrospinal fluid (CSF; composition in mM: NaCl 128.6, KCl 2.6, $MgCl_2$ 2.0 and $CaCl_2$ 1.4; pH adjusted to 7.2). The catheter was then flushed with 4 µl of CSF over a period of 20–30 s and the cardiovascular responses were measured for 30 min. Control animals were injected with 5 µl CSF only.

In the second series of experiments, the animals received randomly a single i.c.v. injection of either 25 pmol SP, NKA or [$MePhe^7$]NKB (1 µl of peptide solution flushed with 4 µl of CSF) and the cardiovascular and behavioural responses were measured over a period of 30 min. The vehicle CSF containing dimethylsulphoxide (DMSO), used to dissolve the tested antagonist, was injected i.c.v. 5 min prior to the agonist. On the second day, CSF or one of the three antagonists (RP 67580, SR 48968 and R 486) were randomly administered i.c.v., at 6.5 or 65 nmol, 5 min prior to SP, NKA or [$MePhe^7$]NKB. Only one antagonist was administered to each animal. On the third day, the tested agonist was injected alone to evaluate the reversibility of any inhibition produced by the antagonist. No tachyphylaxis to SP, NKA or [$MePhe^7$]NKB was seen on MAP, HR, face washing, grooming and wet dog shakes when each agonist (25 pmol) was injected i.c.v. on three consecutive days (Figure 1). The intrinsic activity of the antagonists was tested in separate experiments. Baseline MAP and HR values were calculated 1 min before the injection of 25 pmol SP, NKA or [$MePhe^7$]NKB.

### *Peptides and non-peptides*

The non-peptide $NK_1$ antagonist, RP 67580 (racemic form of 7,7-diphenyl-2[1-imino-2(2-methoxy-phenyl)-ethyl] perhydro-isoindol-4-one (3aR, 7aR); mol. wt: 475,0 for the hydrochloride salt) was a gift from Dr C. Garret, Rhône-Poulenc Rorer, Paris, France. The $NK_2$ antagonist SR 48968 ((S)-N-methyl-N[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)-butyl]benzamide; mol. wt: 570,0) was a gift from Dr J.-C. Brelière, Sanofi, Montpellier, France. R 486 (H-Asp-Ser-Phe-Trp-β-Ala-Leu-Met-$NH_2$; mol. wt.: 868.1) and [MePhe$^7$]-NKB (H-Asp-Met-His-Asp-Phe-Phe-MePhe-Gly-Leu-Met-$NH_2$) were synthesized in the laboratory of Dr D. Regoli at Sherbrooke University, Sherbrooke, Canada by conventional solid-phase methods. SP, NKA and AII were purchased from Hükabel Scientific Ltd, Montréal, Canada. Heparin sodium salt Grade II from porcine intestinal mucosa was purchased from Sigma chemicals (St-Louis, MO, U.S.A.). The antagonists and [MePhe$^7$]NKB were dissolved in DMSO (Fisher) and CSF was added to obtain the desired solution (the final solution contained a maximum of 30% of DMSO). SP, NKA and AII were dissolved directly in CSF. The stock solutions (1–10 mg ml$^{-1}$) of peptides and non-peptides were divided into 100 µl aliquots and stored at – 20°C until used. Daily dilutions were made in CSF before each experiment.

### *Statistical analysis of data*

The results are expressed as mean ± s.e.mean. Statistical differences were evaluated with Student's *t* test for paired samples or Wilcoxon–Mann–Whitney (U) test for unpaired samples on non parametric values (behaviour frequency). When more than one comparison was made, the significance of differences among groups was evaluated with a two-way analysis of variance (ANOVA) in conjunction with Bonferroni confidence intervals. Only probability values (*P*) smaller than 0.05 were considered to be statistically significant.

## Results

### *Central cardiovascular and behavioural effects induced by SP, NKA and [MePhe$^7$]NKB*

The i.c.v. injection of 5 pmol SP, NKA or [MePhe$^7$]NKB failed to cause significant cardiovascular changes when compared with CSF values (Figure 2). However, at 25 pmol all three agonists induced significant increases of MAP and HR ($P<0.001$) which reached a maximum at 3–5 min and returned gradually to pre-injection levels within 30 min (Figures 2 and 3). The doses of 65 pmol and 325 pmol SP produced further increases of MAP and HR. However, cardiovascular responses were maximal at 25 pmol NKA or [MePhe$^7$]NKB as higher doses (325 and 650 pmol) failed to cause further increases of MAP and HR (Figure 2). Thus, the agonists evoked maximal cardiovascular changes (3–5 min post-injection) with the rank order of potency SP>

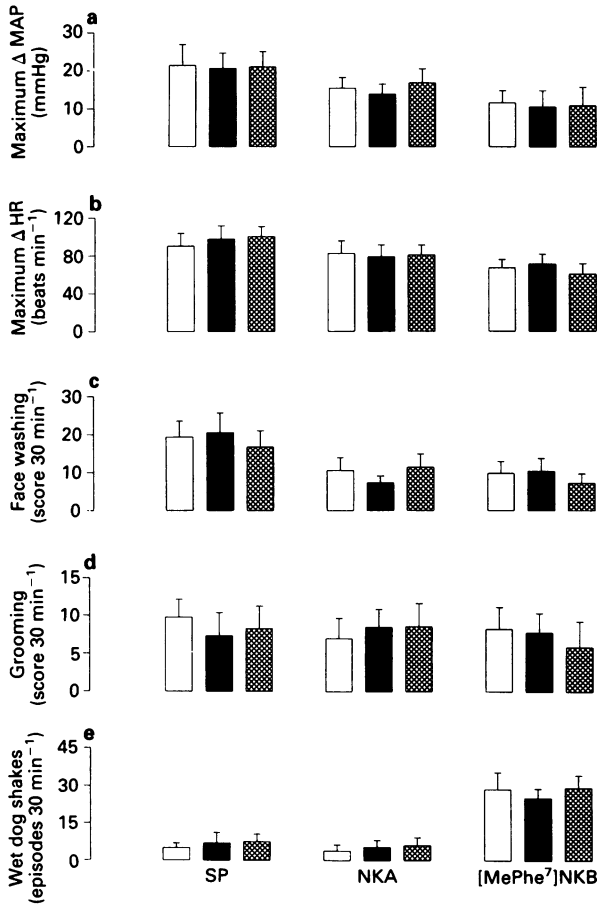


**Figure 1** Cardiovascular and behavioural effects of 25 pmol substance P (SP), neurokinin A (NKA) and [MePhe$^7$]NKB on three consecutive days in conscious rats. The agonist was injected i.c.v. on day 1 (open columns), day 2 (solid columns) and day 3 (cross-hatched columns). The increases in (a) mean arterial blood pressure (MAP) and (b) heart rate (HR) represent maximal values at 3–5 min post-injection. Individual behaviours were measured for a period of 30 min (c, d and e). Each value represents the mean ± s.e.mean of 8 rats for each agonist. There is no statistical difference between days 1, 2 and 3 for each agonist.

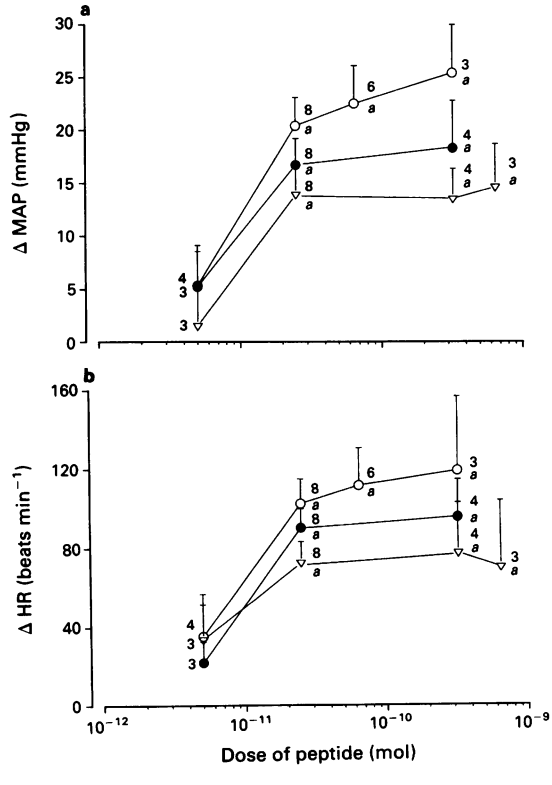


**Figure 2** Effects of several doses of substance P (○), neurokinin A (●) and [MePhe$^7$]NKB (▽) injected intracerebroventricularly in conscious rats. Maximal increases in (a) mean arterial blood pressure (MAP) and in (b) heart rate (HR) measured at 3–5 min post-injection are shown. Each point represents the mean ± s.e.mean of several rats indicated by numbers. Statistically significant difference compared with CSF values (2.1 ± 1.8 mmHg and 15.1 ± 7.4 beats min$^{-1}$) is indicated by $^aP<0.001$.

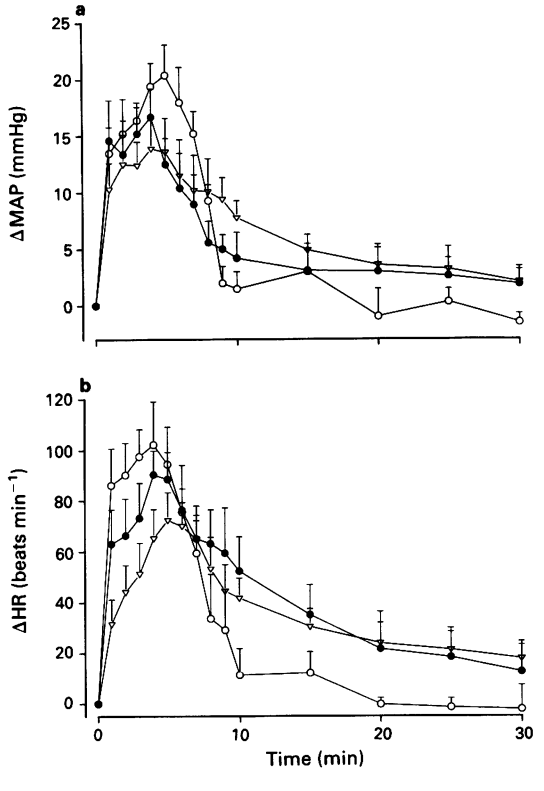


**Figure 3** Time course of (a) mean arterial blood pressure (MAP) and (b) heart rate (HR) changes evoked by the i.c.v. injection of 25 pmol substance P (○), neurokinin A (●) and [MePhe$^7$]NKB (▽) in conscious rats. Each point represents the mean ± s.e.mean of 8 rats.

NKA > [MePhe$^7$]NKB (Figure 2). The dose selected for each agonist in the further experiments was 25 pmol.

The cardiovascular responses elicited by SP and NKA were accompanied by excessive face washing and grooming/biting of hindlimbs (Table 3). These behavioural effects occurred simultaneously with the cardiovascular responses and presented a similar time course. On the other hand, i.c.v. injected [MePhe$^7$]NKB induced not exclusively but mainly uninterrupted wet dog shakes for the 30 min observation period; this behaviour was not parallel to the cardiovascular effects (Tables 1–3). When injected i.c.v., CSF (5 μl) produced no appreciable cardiovascular (Tables 1 and 2) or behavioural (Table 3) effects.

### *Effects of tachykinin receptor antagonists on the responses to SP, NKA and [MePhe$^7$]NKB*

Cardiovascular responses as well as face washing but not grooming and wet dog shake induced by 25 pmol SP were significantly reduced (by about 50–70%) when animals were pretreated with 6.5 nmol RP 67580 (Tables 1–3). The inhibitory effect of the antagonist was no longer observed when the agonist was reinjected 24 h later. The cardiovascular response to SP was not further inhibited by higher doses of RP 67580. Maximal ΔMAP and ΔHR induced by 25 pmol SP in the absence of RP 67580 were 17.3 ± 4.2 mmHg ($n = 8$) and 93.7 ± 13.8 beats min$^{-1}$ ($n = 8$) while in the presence of 65 nmol RP 67580, they were reduced to 4.0 ± 2.5 mmHg ($n = 5$) and 40.9 ± 10.3 beats min$^{-1}$ ($n = 5$), respectively. The residual responses to SP in the presence of 6.5 nmol (Tables 1 and 2) and 65 nmol RP 67580 were not statistically different from each other. In contrast, the $NK_1$ antagonist (6.5 nmol) was inactive against the central cardiovascular and behavioural effects induced by 25 pmol NKA or [MePhe$^7$]NKB (Tables 1–3).

At 6.5 nmol, SR 48968 significantly reduced by approximately 60% the central cardiovascular response and abolished the behavioural effects (but not wet dog shakes) induced by 25 pmol NKA. The inhibition was reversible 24 h later, yet SR 48968 had no effect on the responses to 25 pmol SP or [MePhe$^7$]NKB (Tables 1–3). The i.c.v. injection of 65 nmol SR 48968 did not reduce further the cardiovascular effects evoked by NKA. Maximal ΔMAP and ΔHR induced by 25 pmol NKA in the absence of SR 48968 were 13.4 ± 3.6 mmHg ($n = 8$) and 88.7 ± 10.5 beats min$^{-1}$ ($n = 8$) while in the presence of 65 nmol SR 48968, they were reduced to 3.1 ± 2.0 mmHg ($n = 5$) and 25.3 ± 10.1 beats min$^{-1}$ ($n = 5$), respectively. The residual responses to NKA in the presence of 6.5 nmol (Tables 1 and 2) and 65 nmol SR 48968 were not statistically different from each other.

RP 67580 (6.5 nmol) and SR 48968 (6.5 nmol) were co-injected i.c.v. and tested against the agonist-mediated effects. This combination of antagonists completely abolished the increases in MAP and HR as well as the face washing, grooming and wet dog shakes induced by SP or NKA (25 pmol), yet this antagonist mixture did not affect the cardiovascular and behavioural responses to [MePhe$^7$]NKB (Tables 1–3). The blockade of the SP or NKA mediated cardiovascular responses by RP 67580 plus SR 48968 was no longer observed when SP or NKA was re-administered alone 24 h later (Figure 4).

I.c.v. pretreatment with the selective $NK_3$ receptor antagonist, R 486 (6.5 nmol), inhibited the pressor and tachycardiac responses as well as wet dog shake induced by 25 pmol [MePhe$^7$]NKB (Tables 1–3). The same pretreatment with R 486 failed, however, to alter both cardiovascular and behavioural responses induced either by SP or NKA (Tables 1–3). The cardiovascular effects of [MePhe$^7$]NKB were entirely recovered 24 h after treatment with R 486 (Figure 4). There were no significant differences in MAP and HR basal values among the experimental groups (Tables 1–2). None of the tested antagonists or agonists showed any apparent toxic effects. Moreover, at 6.5 nmol, RP 67580 or SR 48968 had no significant effect on MAP or HR (Figure 5). On the other hand, the $NK_3$ antagonist, R 486 (6.5 nmol), caused a small transient increase of MAP and HR that lasted less than 5 min (Figure 5). Furthermore, the three antagonists had no direct effects on the individual behaviours namely face washing, grooming and wet dog shake (Table 4).

## Discussion

The intracerebroventricular injection of SP, NKA or [MePhe$^7$]NKB elicited dose-dependent increases in mean arterial blood pressure and heart rate accompanied by specific behavioural manifestations in conscious rats. SP and NKA prevailing responses were grooming and face washing, while activation of the $NK_3$ receptor induced mainly wet dog shake behaviour. Even though it is difficult to associate these behaviours with physiological correlates, the SP/NKA behaviours are typical behaviours observed during the defence reaction (Unger *et al.*, 1988; Itoi *et al.*, 1991).

The hypothalamus may be the site of action of tachykinins, since microinjections of SP into the anterior and ventromedial parts of hypothalamus evoked cardiovascular changes similar to those produced by i.c.v. injection of SP or NKA (Itoi *et al.*, 1991). Moreover, the magnocellular part of the rat hypothalamic paraventricular nucleus was identified as a site of action for the central effect of tachykinins ($NK_3$ agonists) on the release of vasopressin (Massi *et al.*, 1991; Takano *et al.*, 1993). However, it would be premature to reach any conclusions regarding the exact localization of the tachykinin receptors activated by i.c.v. SP, NKA or [MePhe$^7$]NKB. Nonetheless, the fast onset of the response to i.c.v. tachykinin agonists leads one to suggest that receptor sites must be localized in the circumventricular organs or in adjacent periventricular structures. A peripheral site of action is unlikely since i.v. injections of SP, NKA or [MePhe$^7$]NKB

**Table 1** Effects of selective tachykinin receptor antagonists on changes in mean arterial blood pressure (MAP) elicited by the i.c.v. injection of 25 pmol substance P (SP), neurokinin A (NKA) or [MePhe$^7$]NKB in the conscious rat

| | | | | ΔMAP (mmHg) Time (min) after agonist injection | | | | |
|---|---|---|---|---|---|---|---|---|
| *Antagonist* | *Agonist* | n | *Baseline MAP* (mmHg) | 3 | 5 | 10 | 20 | 30 |
| – | CSF | 10 | 97.6 ± 3.6 | 1.5 ± 0.6 | 1.0 ± 0.6 | 1.2 ± 0.7 | 0.5 ± 0.3 | 0.4 ± 0.5 |
| – | SP | 10 | 109.3 ± 7.7 | 18.5 ± 4.0*** | 19.2 ± 2.9*** | 3.3 ± 2.0 | 1.5 ± 1.6 | 1.1 ± 1.3 |
| RP 67580 | SP | 10 | 103.2 ± 6.0 | 5.8 ± 2.6*† | 8.3 ± 4.0*† | 2.1 ± 3.5 | 1.7 ± 1.3 | −0.5 ± 0.6 |
| – | NKA | 6 | 98.5 ± 5.6 | 13.9 ± 3.1** | 12.5 ± 2.4** | 8.4 ± 1.3** | 4.2 ± 1.3* | 2.6 ± 0.9* |
| RP 67580 | NKA | 6 | 105.6 ± 4.3 | 10.3 ± 2.5* | 9.3 ± 2.9** | 6.1 ± 2.1* | 3.6 ± 1.9* | 1.5 ± 1.8 |
| – | [MePhe$^7$]NKB | 7 | 93.6 ± 6.7 | 10.2 ± 1.9** | 7.3 ± 2.5* | 4.3 ± 2.0* | 4.6 ± 1.6* | 3.2 ± 1.2* |
| RP 67580 | [MePhe$^7$]NKB | 7 | 103.1 ± 7.2 | 12.1 ± 5.3** | 11.9 ± 3.4** | 8.2 ± 2.4** | 3.6 ± 2.7* | 1.9 ± 2.1 |
| – | SP | 8 | 106.5 ± 6.1 | 18.0 ± 4.3*** | 20.5 ± 3.0*** | 3.1 ± 1.9 | 0.5 ± 0.4 | −0.3 ± 1.1 |
| SR 48968 | SP | 8 | 105.2 ± 5.1 | 12.9 ± 3.2** | 17.1 ± 1.9*** | 2.1 ± 2.2 | −1.3 ± 1.6 | −2.1 ± 1.9 |
| – | NKA | 8 | 101.4 ± 4.1 | 12.5 ± 3.3** | 12.6 ± 3.9** | 9.0 ± 3.8* | 3.9 ± 2.3* | 2.2 ± 1.3 |
| SR 48968 | NKA | 8 | 97.1 ± 9.1 | 4.3 ± 1.8† | 5.8 ± 2.5*† | 4.4 ± 3.1 | 2.5 ± 1.3* | 2.4 ± 2.0 |
| – | [MePhe$^7$]NKB | 8 | 99.1 ± 5.6 | 9.8 ± 3.0** | 12.5 ± 1.7** | 5.4 ± 2.8* | 3.6 ± 2.0* | 2.8 ± 0.6* |
| SR 48968 | [MePhe$^7$]NKB | 8 | 102.5 ± 6.3 | 12.1 ± 4.1** | 13.5 ± 2.9** | 4.8 ± 1.9* | 2.2 ± 1.4 | 2.5 ± 1.7 |
| – | SP | 7 | 94.8 ± 5.6 | 15.9 ± 2.4*** | 19.7 ± 3.3*** | 3.6 ± 1.8* | 4.0 ± 1.9* | 1.6 ± 0.7 |
| RP 67580 + SR 48968 | SP | 7 | 97.5 ± 4.3 | 1.4 ± 2.5†† | 2.1 ± 0.9††† | 0.5 ± 1.9† | 1.3 ± 0.4† | 0.1 ± 0.9 |
| – | NKA | 8 | 95.1 ± 3.8 | 13.5 ± 2.0** | 10.9 ± 1.6** | 8.5 ± 1.0** | 3.2 ± 1.4* | 2.8 ± 0.6* |
| RP 67580 + SR 48968 | NKA | 8 | 100.8 ± 4.4 | −0.3 ± 1.8††† | 1.2 ± 1.3†† | 0.6 ± 0.8†† | 2.1 ± 0.5* | 2.2 ± 0.8* |
| – | [MePhe$^7$]NKB | 5 | 97.6 ± 6.8 | 9.9 ± 3.6** | 8.1 ± 2.4** | 5.3 ± 2.1* | 4.7 ± 2.2* | 4.8 ± 1.8* |
| RP 67580 + SR 48968 | [MePhe$^7$]NKB | 5 | 95.2 ± 4.5 | 8.5 ± 2.3** | 5.3 ± 2.9* | 4.1 ± 3.1 | 2.3 ± 1.9 | 1.6 ± 1.6 |
| – | SP | 7 | 102.6 ± 5.1 | 16.5 ± 2.3*** | 20.3 ± 3.1*** | 2.9 ± 0.5* | 0.2 ± 0.9 | −1.1 ± 0.3 |
| R 486 | SP | 7 | 107.1 ± 6.3 | 17.5 ± 3.6*** | 18.9 ± 5.1*** | 6.0 ± 3.2* | 1.5 ± 1.6 | 1.9 ± 1.0 |
| – | NKA | 6 | 106.3 ± 5.3 | 13.2 ± 3.5** | 11.0 ± 2.0** | 6.1 ± 1.2* | 2.1 ± 3.4 | 2.5 ± 1.6 |
| R 486 | NKA | 6 | 108.0 ± 9.1 | 13.5 ± 3.2** | 12.0 ± 2.2** | 3.9 ± 1.6* | 1.6 ± 1.8 | −0.2 ± 1.3 |
| – | [MePhe$^7$]NKB | 8 | 100.7 ± 7.0 | 11.0 ± 1.6** | 8.8 ± 1.5** | 6.0 ± 1.5* | 4.3 ± 3.9 | 3.0 ± 2.3 |
| R 486 | [MePhe$^7$]NKB | 8 | 98.3 ± 5.1 | 3.7 ± 3.1† | 1.2 ± 2.6† | −0.3 ± 2.9† | −0.9 ± 2.6 | 0.7 ± 2.8 |

Values represent the means ± s.e.mean of ($n$) rats. The antagonists were injected at the dose of 6.5 nmol, 5 min prior to the agonist. Statistical comparison to CSF (*) or to the agonist in the absence of antagonist (†) was calculated with a two-way ANOVA: *,†$P<0.05$; **,††$P<0.01$; ***,†††$P<0.001$.

**Table 2** Effects of selective tachykinin receptor antagonists on changes in heart rate (HR) elicited by the i.c.v. injection of 25 pmol substance P (SP), neurokinin A (NKA) or [MePhe$^7$]NKB in the conscious rat

| | | | | $\Delta HR$ (beats min$^{-1}$) Time (min) after agonist injection | | | | |
|---|---|---|---|---|---|---|---|---|
| *Antagonist* | *Agonist* | n | *Baseline HR* | 3 | 5 | 10 | 20 | 30 |
| – | CSF | 10 | 311.4 ± 19.5 | 7.3 ± 5.2 | 5.1 ± 9.3 | 8.2 ± 8.9 | 3.5 ± 6.1 | 4.8 ± 7.0 |
| – | SP | 10 | 305.6 ± 15.6 | 98.1 ± 12.1*** | 95.1 ± 9.4*** | 17.3 ± 6.9 | −2.0 ± 8.5 | −5.4 ± 7.1 |
| RP 67580 | SP | 10 | 318.0 ± 21.3 | 43.1 ± 9.5**† | 41.2 ± 12.5**† | 10.3 ± 8.2 | 12.5 ± 9.0 | 0.8 ± 7.2 |
| – | NKA | 6 | 293.1 ± 22.3 | 78.5 ± 9.3*** | 89.1 ± 11.6*** | 43.9 ± 8.3** | 28.1 ± 10.0* | 20.1 ± 5.2* |
| RP 67580 | NKA | 6 | 301.4 ± 17.6 | 82.5 ± 12.0*** | 87.3 ± 9.4*** | 50.1 ± 9.1** | 20.5 ± 5.2* | 12.3 ± 4.8 |
| – | [MePhe$^7$]NKB | 7 | 306.8 ± 16.3 | 60.1 ± 7.3** | 76.2 ± 10.1** | 38.4 ± 7.8** | 29.3 ± 6.1* | 25.4 ± 3.8* |
| RP 67580 | [MePhe$^7$]NKB | 7 | 319.5 ± 18.2 | 55.4 ± 8.0** | 81.2 ± 6.3*** | 40.3 ± 8.8** | 17.9 ± 7.3* | 25.1 ± 4.1* |
| – | SP | 8 | 311.9 ± 17.1 | 103.2 ± 11.4*** | 96.4 ± 10.3*** | 25.1 ± 12.0 | 7.8 ± 9.5 | −2.6 ± 5.7 |
| SR 48968 | SP | 8 | 298.1 ± 14.3 | 93.6 ± 8.6*** | 79.5 ± 9.4*** | 15.4 ± 7.1 | −1.9 ± 7.2 | −4.4 ± 6.0 |
| – | NKA | 8 | 305.0 ± 16.8 | 65.3 ± 8.1** | 93.1 ± 10.5*** | 51.1 ± 6.8** | 34.1 ± 7.4** | 12.5 ± 8.0 |
| SR 48968 | NKA | 8 | 303.8 ± 17.4 | 28.0 ± 9.3*† | 36.3 ± 7.0*† | 30.1 ± 4.2*† | 28.1 ± 4.1** | 17.3 ± 6.3* |
| – | [MePhe$^7$]NKB | 8 | 301.6 ± 18.3 | 63.1 ± 9.1** | 69.7 ± 7.8** | 51.3 ± 6.7** | 33.4 ± 9.0** | 10.1 ± 7.7 |
| SR 48968 | [MePhe$^7$]NKB | 8 | 289.1 ± 16.3 | 58.4 ± 7.5** | 59.2 ± 8.3** | 43.2 ± 5.4** | 27.1 ± 4.3** | 7.0 ± 8.1 |
| – | SP | 7 | 303.4 ± 22.1 | 87.5 ± 8.4*** | 80.6 ± 7.7*** | 12.3 ± 9.1 | 5.3 ± 3.8 | −5.6 ± 6.1 |
| RP 67580 + SR 48968 | SP | 7 | 305.1 ± 23.7 | 3.5 ± 6.8†† | 5.3 ± 8.1†† | −3.5 ± 6.7 | −0.8 ± 4.7 | −7.9 ± 5.6 |
| – | NKA | 8 | 287.1 ± 16.2 | 80.3 ± 13.5*** | 92.1 ± 9.1*** | 60.3 ± 7.2** | 31.4 ± 7.5** | 12.3 ± 8.1 |
| RP 67580 + SR 48968 | NKA | 8 | 296.1 ± 18.5 | 10.3 ± 5.3†† | 14.1 ± 6.0†† | 3.6 ± 8.0†† | 7.3 ± 5.1† | 4.3 ± 3.6 |
| – | [MePhe$^7$]NKB | 5 | 294.1 ± 16.0 | 55.1 ± 12.3** | 73.1 ± 9.9** | 40.6 ± 8.3** | 21.6 ± 8.0* | 18.5 ± 6.3* |
| RP 67580 + SR 48968 | [MePhe$^7$]NKB | 5 | 304.1 ± 14.2 | 48.3 ± 9.3** | 64.1 ± 8.3** | 34.2 ± 7.5** | 18.3 ± 5.1* | 7.3 ± 7.8 |
| – | SP | 7 | 309.1 ± 17.8 | 95.3 ± 12.7*** | 98.3 ± 10.9*** | 23.4 ± 7.1* | 8.6 ± 5.9 | −5.8 ± 4.9 |
| R 486 | SP | 7 | 301.2 ± 17.5 | 93.4 ± 13.0*** | 98.1 ± 8.3*** | 17.3 ± 10.8 | 9.1 ± 6.1 | 7.6 ± 4.8 |
| – | NKA | 6 | 297.0 ± 14.9 | 78.1 ± 9.6*** | 87.3 ± 10.7*** | 61.2 ± 8.5** | 34.1 ± 7.9** | 26.4 ± 5.8* |
| R 486 | NKA | 6 | 299.3 ± 18.0 | 62.9 ± 10.8** | 90.1 ± 9.3*** | 53.1 ± 8.0** | 27.1 ± 5.6** | 16.9 ± 8.1* |
| – | [MePhe$^7$]NKB | 8 | 316.2 ± 18.9 | 61.3 ± 9.7** | 89.1 ± 5.3*** | 43.8 ± 5.7** | 39.1 ± 7.3** | 21.5 ± 6.0* |
| R 486 | [MePhe$^7$]NKB | 8 | 320.1 ± 20.5 | 23.1 ± 8.2*† | 19.0 ± 7.0*†† | 14.7 ± 7.1† | 12.1 ± 9.0† | 8.3 ± 4.1† |

See footnote to Table 1.

**Table 3** Effects of selective tachykinin receptor antagonists on behavioural responses elicited by the i.c.v. injection of 25 pmol substance P (SP), neurokinin A (NKA) or [MePhe$^7$]NKB in the conscious rat

| *Antagonist* | *Agonist* | n | *Face washing score* (30 min$^{-1}$) | *Grooming score* (30 min$^{-1}$) | *Wet dog shake episodes* (30 min$^{-1}$) |
|---|---|---|---|---|---|
| – | CSF | 10 | 1.3 ± 0.4 | 1.2 ± 0.8 | 2.0 ± 1.3 |
| – | SP | 10 | 17.2 ± 3.1** | 9.3 ± 2.6* | 5.6 ± 2.8 |
| RP 67580 | SP | 10 | 5.3 ± 2.4*† | 8.3 ± 3.0* | 6.8 ± 3.7 |
| – | NKA | 6 | 9.2 ± 2.6* | 6.6 ± 3.0* | 4.0 ± 1.6 |
| RP 67580 | NKA | 6 | 7.5 ± 2.7* | 5.1 ± 1.2* | 7.4 ± 2.4* |
| – | [MePhe$^7$]NKB | 7 | 12.5 ± 3.6* | 9.3 ± 3.6* | 22.6 ± 7.1** |
| RP 67580 | [MePhe$^7$]NKB | 7 | 9.6 ± 3.7* | 8.2 ± 3.4* | 26.4 ± 8.3** |
| – | SP | 8 | 10.7 ± 3.6* | 8.5 ± 2.4* | 5.1 ± 3.2 |
| SR 48968 | SP | 8 | 12.5 ± 4.1* | 8.1 ± 2.0* | 5.9 ± 2.1 |
| – | NKA | 8 | 8.9 ± 2.9* | 7.0 ± 1.5* | 9.1 ± 4.3* |
| SR 48968 | NKA | 8 | 0.9 ± 0.2† | 1.2 ± 1.0† | 10.3 ± 2.9* |
| – | [MePhe$^7$]NKB | 8 | 9.7 ± 2.1* | 6.3 ± 1.2* | 25.6 ± 5.4** |
| SR 48968 | [MePhe$^7$]NKB | 8 | 10.1 ± 2.5* | 7.0 ± 2.5* | 21.6 ± 7.2** |
| – | SP | 7 | 15.7 ± 2.9** | 10.3 ± 2.6* | 8.0 ± 2.8* |
| RP 67580 + SR 48968 | SP | 7 | 2.0 ± 0.9†† | 3.2 ± 0.7† | 4.1 ± 2.0† |
| – | NKA | 8 | 13.2 ± 2.6* | 7.0 ± 3.6* | 5.1 ± 2.6 |
| RP 67580 + SR 48968 | NKA | 8 | 1.3 ± 0.8†† | 2.9 ± 1.1† | 2.6 ± 1.9 |
| – | [MePhe$^7$]NKB | 5 | 10.5 ± 1.9* | 6.5 ± 1.6* | 30.6 ± 7.1** |
| RP 67580 + SR 48968 | [MePhe$^7$]NKB | 5 | 7.8 ± 1.4* | 5.8 ± 2.0* | 28.5 ± 6.7** |
| – | SP | 7 | 15.4 ± 3.2* | 8.1 ± 1.2* | 12.5 ± 5.1* |
| R 486 | SP | 7 | 17.3 ± 2.7* | 7.2 ± 2.1* | 10.3 ± 3.1* |
| – | NKA | 6 | 9.3 ± 1.2* | 7.3 ± 2.4* | 8.5 ± 2.5* |
| R 486 | NKA | 6 | 7.3 ± 2.0* | 6.0 ± 2.0* | 9.1 ± 2.3* |
| – | [MePhe$^7$]NKB | 8 | 5.2 ± 1.7* | 5.3 ± 2.0* | 22.8 ± 8.5** |
| R 486 | [MePhe$^7$]NKB | 8 | 4.4 ± 1.4* | 3.4 ± 1.5 | 4.5 ± 1.5† |

Values represent the frequency of individual behaviour for 30 min and are indicated by the mean ± s.e.mean of ($n$) rats. The antagonists were injected at a dose of 6.5 nmol, 5 min prior to the agonist. Statistical comparison to CSF (*) was evaluated with a Wilcoxon–Mann–Whitney (U) test, while comparison to the agonist in the absence of antagonist (†) was calculated with Student's $t$ test for paired samples; *, †$P<0.05$; **, ††$P<0.01$.

cause decreases in blood pressure (Couture *et al.*, 1989). Furthermore, the possibility of a spinal activation can also be excluded since intrathecal injection of 65 nmol [MePhe$^7$]NKB produced no cardiovascular effect while 6.5 nmol of SP injected intrathecally was necessary to induce an increase of 10–15 mmHg (Hassésian *et al.*, 1988).

Immunocytochemistry, *in situ* hybridization and radioimmunoassay studies have shown discrete and abundant distribution of SP, NKA and NKB (and their preprotachykinin mRNAs) in all major subdivisions of the rat brain (Warden & Young, 1988; Harlan *et al.*, 1989; Marksteiner *et al.*, 1992; Merchenthaler *et al.*, 1992; Lucas *et al.*, 1992). Whereas SP is found throughout the rat brain, NKB is distributed more to forebrain than to brainstem structures (Cuello & Kanazawa, 1978; Lucas *et al.*, 1992; Merchenthaler *et al.*, 1992). A high density and widespread distribution of SP and NKA was reported in the rat hypothalamus (Larsen *et al.*, 1992). Also, the paraventricular and supraoptic nuclei of the hypothalamus and the substantia nigra have higher contents of NKB-like immunoreactivity than any other CNS areas (Tateishi *et al.*, 1989; Merchenthaler *et al.*, 1992). Both the $NK_1$ and $NK_3$ receptors have been found in moderate to high density in the paraventricular and supraoptic nuclei of the rat hypothalamus (Dam *et al.*, 1990a,b; Larsen *et al.*, 1992; Maeno *et al.*, 1993). However, the presence of $NK_2$ receptor binding sites in the rat brain remains controversial (Mantyh *et al.*, 1989; Quirion *et al.*, 1991; Takeda & Krause, 1991; Mussap *et al.*, 1993).

Our results support our earlier finding that i.c.v. SP acts primarily through the activation of $NK_1$ receptors to induce cardiovascular and behavioural changes; this conclusion was reached on the basis of results obtained with CP 96345 (Tschöpe *et al.*, 1992). These results needed to be confirmed with RP 67580, a novel $NK_1$ antagonist (Carruette *et al.*, 1992; Rouissi *et al.*, 1993) which does not have the non-specific action of CP 96345 on calcium and sodium channels (Schmidt *et al.*, 1992; Guard *et al.*, 1993; Caeser *et al.*, 1993). In addition, the present study provides an explanation for the failure of CP 96345 (Tschöpe *et al.*, 1992) or RP 67580 (even at high doses) to abolish the central effects of SP. The persisting residual cardiovascular and behavioural responses to SP measured after pretreatment with the $NK_1$ receptor antagonist (RP 67580) were blocked when RP 67580 was co-administered with the $NK_2$ antagonist, SR 48968. These results suggest that SP can activate $NK_2$ receptors during $NK_1$ receptor blockade, unmasking the non-selectivity of the natural peptide SP. Since individual pretreatments with SR 48968 and R 486 failed to modify the central effects of SP, it appears that SP acts preferentially on the $NK_1$ receptor when the latter is functional.

The central NKA effects were very similar to those mediated by SP. However, NKA seems to activate mainly $NK_2$ receptors since only the $NK_2$ receptor antagonist SR 48968 reduced the biological effects of NKA, while the $NK_1$ (RP 67580) or $NK_3$ (R 486) receptor antagonists were inactive against NKA. These data confirm a previous study in which we concluded that the cardiovascular and behavioural effects of i.c.v. NKA are mediated by a $NK_2$ receptor that was sensitive to R 396 ($NK_2$ selective antagonist) but not to CP 96345 ($NK_1$ selective antagonist) (Tschöpe *et al.*, 1992). However, a higher dose of SR 48968 (65 nmol) was unable to block entirely the NKA-mediated responses, and as was the case with SP administration, the residual effects of NKA were completely abolished when SR 48968 was co-injected with the $NK_1$ selective antagonist, RP 67580. These results

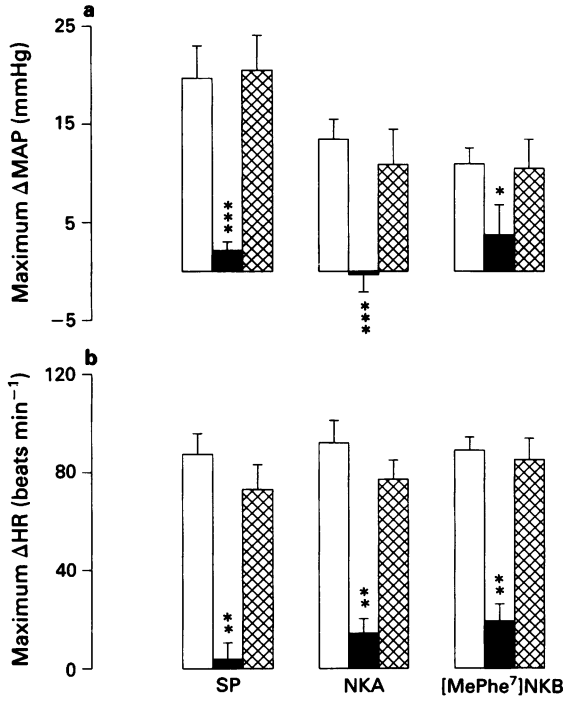


**Figure 4** Effects of selective tachykinin receptor antagonists on maximal changes in (a) mean arterial blood pressure (MAP) and (b) heart rate (HR) induced by the i.c.v. injection of 25 pmol substance P (SP), neurokinin A (NKA) or [MePhe$^7$]neurokinin B ([MePhe$^7$]NKB) in conscious rats. The agonist was injected alone on day 1 (open columns), 5 min after the antagonist on day 2 (solid columns) or alone on day 3 (cross-hatched columns). SP and NKA were tested in the presence of RP 67580 plus SR 48968 (6.5 nmol each) while [MePhe$^7$]NKB was tested in the presence of 6.5 nmol R 486. Values represent the means ± s.e.mean of 7–8 rats. Statistically significant difference compared to the agonist alone (open columns) is indicated by $*P<0.05$; $**P<0.01$; $***P<0.001$.

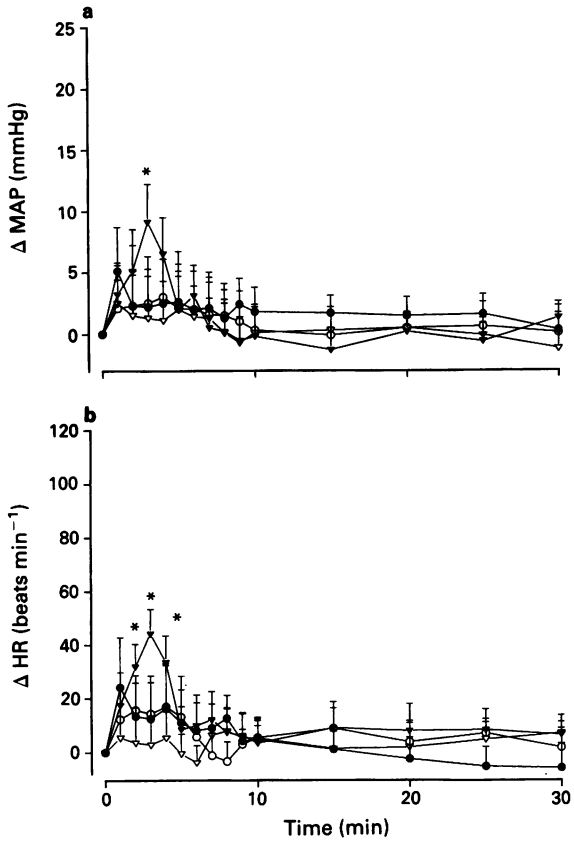


**Figure 5** Direct effects of 6.5 nmol RP 67580 (○), 6.5 nmol SR 48968 (●), 6.5 nmol R 486 (▼) or CSF (▽) on (a) mean arterial blood pressure (MAP) and (b) heart rate (HR) after i.c.v. injection in conscious rats. Values represent the means ± s.e.mean of 7–8 rats. Statistically significant difference compared to CSF values is indicated by $*P<0.05$.

**Table 4** Effects of selective tachykinin receptor antagonists on behavioural responses in the conscious rat

| *Treatment* | *Dose* (nmol) | *Face washing score* (30 min$^{-1}$) | *Grooming score* (30 min$^{-1}$) | *West dog shake episodes* (30 min$^{-1}$) |
|---|---|---|---|---|
| CSF | – | 1.5 ± 0.6 | 1.3 ± 1.0 | 1.9 ± 1.4 |
| RP 67580 | 6.5 | 2.1 ± 1.1 | 2.5 ± 1.3 | 1.4 ± 0.8 |
| SR 48968 | 6.5 | 2.3 ± 1.6 | 2.4 ± 1.5 | 2.9 ± 1.0 |
| RP 67580 + SR 48968 | 6.5 each | 2.6 ± 1.6 | 1.9 ± 1.2 | 2.0 ± 1.3 |
| R 486 | 6.5 | 1.5 ± 0.7 | 1.7 ± 0.9 | 3.1 ± 1.8 |

Values represent the frequency of individual behaviour for 30 min and are indicated by the mean ± s.e.mean of 8 rats in each group. No statistical difference was found when compared to CSF values.

can be interpreted in the same way as the SP-mediated responses, but in this case, NKA activates primarily $NK_2$ receptors. During $NK_2$ receptor blockade, a non selective activation of $NK_1$ receptors has been revealed with NKA.

The most prominent behavioural response (wet dog shakes) evoked by i.c.v. [MePhe$^7$]NKB injection is indicative of a different central neuronal pathway and confirms results obtained with senktide ($NK_3$ agonist), injected either subcutaneously or intracisternaly (Stoessl *et al.*, 1988) or i.c.v. (Itoi *et al.*, 1992) in the rat. Senktide also induced pressor responses through vasopressin release when injected either i.c.v. or directly into the hypothalamic paraventricular nucleus of the anaesthetized rat (Takano *et al.*, 1990; 1993). As expected, [MePhe$^7$]NKB induced cardiovascular and wet dog shake responses were selectively blocked by the $NK_3$ receptor antagonist, R 486 (Drapeau *et al.*, 1990; Regoli *et al.*, 1991) and were unaffected by RP 67580 or SR 48968 administered individually or in combination, thus assigning the $NK_3$ receptor as the sole functional tachykinin receptor site mediating [MePhe$^7$]NKB responses. Hence, our study confirms the presence of functionally active supraspinal $NK_3$ sites involved in the central cardiovascular and behavioural effects of tachykinins.

The inhibitory effect of the three antagonists was reversible and not related to motor deficits or to changes of baseline parameters. No residual agonists activity was shown with i.c.v. injection of SR 48968 or RP 67580 which is consistent with *in vitro* studies (Advenier *et al.*, 1992; Carruette *et al.*, 1992). On the other hand, the $NK_3$ antagonist, R 486, exhibited a direct central stimulatory effect which might be due to a residual agonistic activity on both $NK_1$ and $NK_2$ receptors (Regoli *et al.*, 1991). Since R 486 blocked selec-

tively the [MePhe$^7$]NKB-induced effects, this antagonist appears suitable for investigating the functional role of central $NK_3$ receptors. A similar $NK_3$ receptor antagonist, R 487 ([Phe$^7$, β-Ala$^8$]NKA(4-10), 6.5 nmol) was also found to block the central cardiovascular and behavioural effects of [MePhe$^7$]NKB in a selective and reversible manner (Picard *et al.*, 1992).

In summary, selective and potent antagonists of $NK_1$, $NK_2$ and $NK_3$ receptors have been used to characterize the receptor subtypes which are responsible for the central cardiovascular and behavioural effects of SP, NKA and [MePhe$^7$] NKB. SP activates mainly $NK_1$ but also $NK_2$ receptors whereas NKA-mediated effects are secondary to $NK_2$ and to a lesser extent $NK_1$ receptor activation. The interactions of SP and NKA with the $NK_1$ and $NK_2$ receptors were seen at doses as low as 25 pmol. The central effects of [MePhe$^7$]NKB are mediated by specific tachykinin receptors which are not identical with those activated by SP or by NKA and which belong to $NK_3$ receptor subclass. Hence, our data provide pharmacological evidence for the existence of distinct populations of functionally active $NK_1$, $NK_2$ and $NK_3$ receptors in the adult rat brain.

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# Cardiovascular and behavioural effects of centrally administered neuropeptide K in the rat: receptor characterization

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**1** The cardiovascular and behavioural responses to intracerebroventricularly (i.c.v.) administered neuropeptide K (NPK) were studied in conscious rats. The central effects of NPK were characterized by pretreatment (i.c.v.) with selective antagonists for the $NK_1$ ((±)-CP 96345 and RP 67580), $NK_2$ (SR 48968) and $NK_3$ (R 487) receptors.

**2** NPK (10–65 pmol) induced tachycardia and dose-dependent increases of mean arterial blood pressure. The cardiovascular responses reached a maximum within 3 min post-injection and lasted for more than 1 h. Concurrently, NPK produced dose-dependent increases of face washing, head scratching, grooming, walking and wet dog shakes.

**3** A desensitization of most of the behavioural responses (except head scratching) but not of the cardiovascular response was shown when two consecutive injections of 25 pmol NPK were given 24 h apart.

**4** Both the cardiovascular and behavioural responses (except the head scratching) to 25 pmol NPK were blocked by pre-administration (i.c.v.) of 6.5 nmol (±)-CP 96345 or RP 67580 given 5 min earlier. No inhibition of NPK responses was observed when 6.5 nmol SR 48968 or R 487 were used in a similar study. Additionally, NPK effects were significantly reduced 24 h after the prior injection of (±)-CP 96345 but not of RP 67580.

**5** These results support the involvement of $NK_1$ receptors in the cardiovascular and behavioural effects of i.c.v. NPK. Thus, this peptide may play a putative role in central cardiovascular regulation as it is the most potent endogenous tachykinin described centrally, to date.

**Keywords:** Neuropeptide K; $NK_1$ receptors; tachykinin antagonists; central cardiovascular regulation; behaviour

## Introduction

Six mammalian tachykinins namely substance P (SP), neurokinin A (NKA), NKA(3-10), neurokinin B (NKB), neuropeptide γ (NPγ) and neuropeptide K (NPK) have been identified in both the periphery and central nervous system (Helke *et al.*, 1990; Otsuka & Yoshioka, 1993). These endogenous neuropeptides bind to three receptor subtypes, termed neurokinin$_1$ ($NK_1$), $NK_2$ and $NK_3$; SP being the preferential ligand for the $NK_1$ receptor, NKA, NPK, NPγ and NKA(3-10) for the $NK_2$ receptor and NKB for the $NK_3$ receptor subtype (Regoli *et al.*, 1988; Guard & Watson, 1991; Maggi *et al.*, 1993).

NPK is a 36-amino acid peptide which was originally isolated from porcine brain extracts (Tatemoto *et al.*, 1985) and thought to be a precursor of NKA (Deacon *et al.*, 1987). It is postulated that β-preprotachykinin is processed to SP, NKA and NPK in neuronal cell bodies but that conversion of NPK to NKA takes place during packaging into storage vesicles for axonal transport (Deacon *et al.*, 1987). Moreover, NPK seems to be cleaved into NKA-LI in the plasma during intravenous (i.v.) infusion in the guinea-pig, suggesting as well the existence of an extraneuronal conversion of NPK to NKA (Martling *et al.*, 1987). Recent evidence suggests, however, that NPK is not only a precursor of NKA, but the most potent biologically active endogenous tachykinin *in vivo* whose effects appear to be mediated by $NK_1$ receptors (Takeda & Krause, 1989; Décarie & Couture, 1992; Pham & Couture, 1993). After i.v. injection in the anaesthetized rat, NPK produced sustained decreases in mean arterial blood pressure (MAP) and increases in heart rate (HR); the hypotensive effect was ascribed to a direct action of NPK on blood vessels, while the tachycardia derived from the non-reflex peripheral activation of the sympathoadrenal system (Décarie & Couture, 1992). In conscious rats, the intrathecal (i.t.) injection of NPK at the 9th thoracic level produced dose-dependent and prolonged increases in MAP and HR which were correlated with increases in plasma levels of catecholamines and neuropeptide Y. These NPK-mediated effects were greater than those evoked by similar doses of SP (Hasséssian *et al.*, 1990; Pham *et al.*, 1993). The cardiovascular responses to intrathecal NPK and SP were antagonized by prior i.t. or i.v. pretreatment with (±)-CP 96345 (Pham & Couture, 1993) a non-peptide, selective $NK_1$ receptor antagonist (Snider *et al.*, 1991). Moreover, NPK was found more potent and more efficacious than SP as a stimulator of phosphatidylinositol turnover in the adult rat spinal cord; the high potency of NPK did not appear to be entirely attributable to the metabolic stability of the peptide (Prat *et al.*, 1993a). NPK is also more potent than SP as sialagogue in the rat (Takeda & Krause, 1989) and as bronchoconstrictor in the guinea-pig *in vivo* (Martling *et al.*, 1987). Furthermore, NPK can be considered as a sensory neuropeptide since it is co-localized with NKA and SP in a subpopulation of guinea-pig primary sensory C-fibres (Hua *et al.*, 1985) and is highly concentrated in the dorsal horn of the rat spinal cord (Valentino *et al.*, 1986; Chambers *et al.*, 1988).

Tachykinins have been implicated in central cardiovascular regulation and in motor behaviour (Unger *et al.*, 1985; Itoi *et al.*, 1988). In conscious rats, SP and NKA injected intracerebroventricularly (i.c.v.) increase MAP and HR through the activation of $NK_1$ and $NK_2$ receptors, respectively (Tschöpe *et al.*, 1992). A role for NPK in central cardiovascular regulation remains a possibility as this neuropeptide is present in a high concentration in the brain (Tatemoto *et al.*, 1985; Arai & Emson, 1986; Valentino *et al.*, 1986).

The goals of this study were: (a) to examine the central

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(i.c.v.) action of NPK on the rat cardiovascular system and on behavioural activity and (b) to determine the tachykinin receptor subtype(s) involved in these effects. For that purpose, selective antagonists of the $NK_1$ ((±)-CP 96345 (Snider *et al.*, 1991) and RP 67580 (Garret *et al.*, 1992)), $NK_2$ (SR 48968) (Emonds-Alt *et al.*, 1992) and $NK_3$ (R 487) (Drapeau *et al.*, 1990) receptors were used. Some of these results have been described in a preliminary communication (Prat *et al.*, 1993b).

## Methods

### *Animal preparation*

Male Wistar rats (Charles River, St-Constant, Québec, Canada) weighing 300–350 g were anaesthetized with an intraperitoneal (i.p.) injection of 65 mg $kg^{-1}$ sodium pentobarbitone (Somnotol, MTC. Pharmaceuticals, Cambridge, Ont., Canada). By use of a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, U.S.A.), an i.c.v. polyethylene cannula (PE-20; Intramedic, Clay Adams, NJ, U.S.A.) was inserted into the right lateral ventricle (coordinates: 0.6 mm caudal to bregma, 1.3 mm lateral to midline and 5.0 mm vertical to the skull surface) and fixed to the skull with dental cement. The angle of the head was adjusted according to the horizontal plan with respect to bregma and lambda reference points. Five days later, the animals were anaesthetized again and a polyethylene cannula (PE-60) filled with physiological saline containing heparin (100 iu $ml^{-1}$) was inserted into one femoral artery, passed through a subcutaneous tunnel and emerged at the back of the neck. Following surgery, animals were housed individually in a plastic cage (43 × 23 × 20 cm) with free access to food and water and maintained in a room with a 12 h light/dark cycle.

### *Measurement of cardiovascular and behavioural parameters*

The experiments were conducted on freely moving rats 24 h after the intra-arterial surgery. The arterial blood pressure was monitored through the intra-femoral catheter with a Statham pressure transducer (P231D), while the HR was measured with a cardiac tachometer (model 7P4) and both parameters were displayed on a Grass Polygraph model 79D (Grass instruments Co., Quincy, MA, U.S.A.).

The behavioural activity was recorded in their resident plastic cage (43 × 23 × 20 cm) over a 30 min period at 15 s interval according to the procedure of Gispen *et al.* (1975). During the course of these experiments, the grid cage top was removed. During every consecutive period of 15 s, a score of 1 or 0 was given depending on the presence (1) or absence (0) of each behaviour (face washing, head scratching, grooming, walking and/or ipsilateral turning). The intensity, duration and frequency of each behaviour during the 15 s period were not taken into account. Summation of scores recorded over 30 min gave the behavioural score in each experiment. The maximal theoretical score was 120 in each behaviour (15 s intervals × 30 min). The wet dog shake was measured according to the number of episodes (duration of less than 1 s each) during the 30 min period whatever the intensity.

### *Experimental protocols*

In the first series of experiments, rats received a single i.c.v. injection of artificial cerebrospinal fluid (CSF) (composition in mM: NaCl 128.6, KCl 2.6, $MgCl_2$ 2.0 and $CaCl_2$ 1.4; pH adjusted to 7.2) in a volume of 1 µl. The catheter was flushed in 4 µl of CSF over a period of 10–15 s and the cardiovascular and behavioural responses were measured. One hour later, the animal received 1, 10, 25 or 65 pmol of NPK dissolved in 1 µl of CSF and flushed with 4 µl of CSF. Only one injection of NPK was given to a rat to construct the dose-response curve or to test the antagonists.

The second series of experiments was designed to evaluate possible tachyphylaxis after two consecutive injections of NPK to the same rats. On day 1, 8 rats received 1 µl of CSF and 8 rats received NPK (25 pmol, i.c.v.), then the cannula was flushed with 4 µl of CSF. The second day (24 h later), all animals received a single i.c.v. injection of 25 pmol NPK and the cardiovascular and behavioural effects were measured. The cardiovascular and behavioural responses obtained with the two groups were then compared.

In the third series of experiments, one of the four antagonists ((±)-CP 96345, RP 67580, SR 48968 or R 487) was given i.c.v. in a volume of 1 µl, 5 min or 24 h prior to the injection of 25 pmol NPK. An animal received only one antagonist at a single dose. The intrinsic activity of receptor antagonists was tested in separate experiments. The changes in MAP (ΔMAP) and in HR (ΔHR) at a given time were calculated from baseline values taken 1 min before injection.

At the end of each experiment, 25 pmol of angiotensin II was injected to ascertain the position of the i.c.v. cannula. Most rats (90–100%) displayed an immediate dipsogenic behaviour, characterized by a water intake of 10–15 ml during the first 15 min post-injection, as well as an increase in MAP. Only results from these rats which responded to this test were retained.

### *Peptides and non-peptides*

(±)-CP 93645 (racemic mixture of *cis*-3-(2-methoxybenzylamino)-2-benzhydryl-quinuclidine (2**R**,3**R**) and (2**S**,3**S**); mol. wt.: 412.6) was generously provided by Drs B. Gitter and J. Howbert at Eli Lilly, Indianapolis, U.S.A. while RP 67580 (racemic form of 7,7-diphenyl-2[1-imino-2(2-methoxy-phenyl)-ethyl]perhydroisoindol-4-one (3a**R**, 7a**R**); mol. wt.; 475,0 for the hydrochloride salt) was a gift from Dr C. Garret, Rhône-Poulenc Rorer, Paris, France. SR 48968 ((S)-N-methyl-N-[4-(4-acetylamino-4-phenyl piperidino)-2-(3,4-dichlorophenyl)-butyl]benzamide; mol. wt.: 570.0) was a kind gift from Dr J.C. Brelière, Sanofi, Montpellier, France. R 487 (Asp-Ser-Phe-Phe-β-Ala-Leu-Met-$NH_2$; mol. wt.: 829.1) was provided by Dr D. Regoli at Sherbrooke University, Sherbrooke, Canada. NPK (porcine) and angiotensin II were purchased from Hükabel Scientific Ltd. (Montréal, Québec, Canada) and prepared in CSF solution. Antagonists were made in dimethyl sulphoxide (DMSO; Fisher, Montreal, Canada) and CSF was added to obtain the desired dilution (the final concentration contained a maximum of 10% DMSO). Stock solutions (1–10 mg $ml^{-1}$) of peptides and non-peptides were divided in 100 µl aliquots and stored at −20°C until used.

### *Statistical analysis of data*

The results are expressed as mean ± s.e.mean. Statistical differences (time × effects) were evaluated with a two-way analysis of variance (ANOVA) and a Dunnett test *a posteriori*. Multiple comparisons with a single variable were evaluated with a one-way ANOVA followed by an unpaired Student's *t* test modified by Dunnett. Behavioural responses were evaluated by a one-way ANOVA and a U-Wilcoxon Mann Whitney test. Only probability values (*P*) less than 0.05 were considered to be statistically significant.

## Results

### *Effects of NPK on MAP and HR*

The time course of changes on MAP and HR elicited by the i.c.v. injection of NPK are illustrated in Figure 1. While 1 pmol NPK (data not shown) and CSF failed to cause cardiovascular changes, 10, 25 and 65 pmol of NPK elicited dose-dependent and significant increases in MAP. The pres-

sor response reached a maximum within 3 min and persisted for more than 1 h. However, the increase in HR was not dose-dependent since 25 pmol NPK elicited a greater increase in HR than 65 pmol.

### *Effect of NPK on behaviour*

The cardiovascular responses to i.c.v. NPK were simultaneously accompanied by a marked increase in behavioural activity. As shown in Table 1, face washing, head scratching, grooming, walking and/or ipsilateral turning as well as wet dog shakes increased dose-dependently following injection of 10, 25 and 65 pmol NPK. These changes in behaviour were significantly different from those elicited by CSF, which had little effect on behaviour.

### *Tachyphylaxis to NPK*

This series of experiments was undertaken to evaluate the possible desensitization of the cardiovascular and behavioural effects to two successive injections of 25 pmol NPK, given one day apart. The changes in MAP and HR produced by 25 pmol NPK were not significantly different between rats which received either an i.c.v. injection of CSF or NPK 24 h earlier. On the other hand, all behaviours, except head scratching, elicited by 25 pmol NPK were significantly reduced in

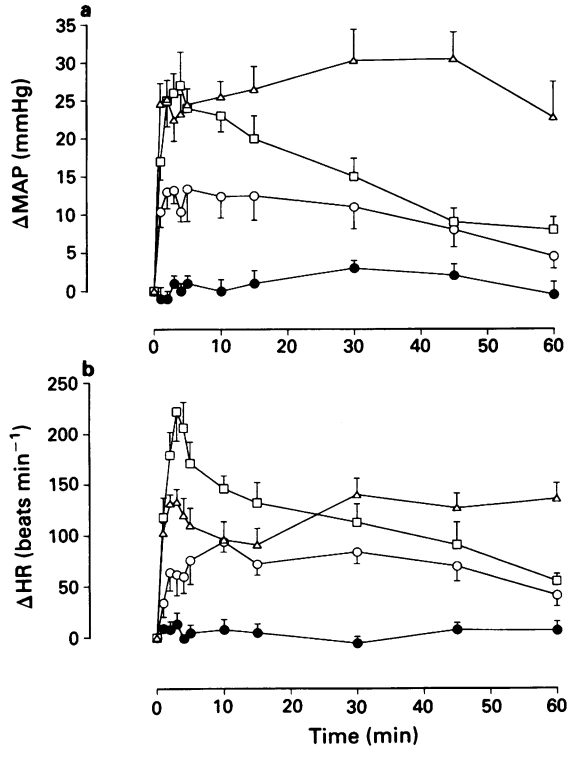


**Figure 1** Time course of cardiovascular responses to the intracerebroventricular injection of CSF (●, $n = 9$), or neuropeptide K (NPK) at the dose of 10 pmol (○, $n = 6$), 25 pmol (□, $n = 9$) and 65 pmol (△, $n = 6$) in conscious rats. Changes in (a) mean arterial pressure (MAP) and (b) heart rate (HR) are shown. Each point represents the mean ± s.e.mean of ($n$) rats in each group. The statistical comparison of CSF values (0–60 min) is given as follows: 10 pmol NPK [$F(1,14) = 16.4$; $P < 0.01$ for ΔMAP and $F(1,14) = 21.4$; $P < 0.01$ for ΔHR], 25 pmol NPK [$F(1,17) = 54.3$; $P < 0.001$ for ΔMAP and $F(1,17) = 47.8$; $P < 0.001$ for ΔHR] and 65 pmol NPK [$F(1,14) = 70.2$; $P < 0.001$ for ΔMAP and $F(1,14) = 44.5$; $P < 0.001$ for ΔHR]. The comparison between the three doses of NPK and CSF is highly significant: [$F(3,29) = 11.2$; $P < 0.001$ for ΔMAP and $F(3,29) = 13.8$; $P < 0.001$ for ΔHR].

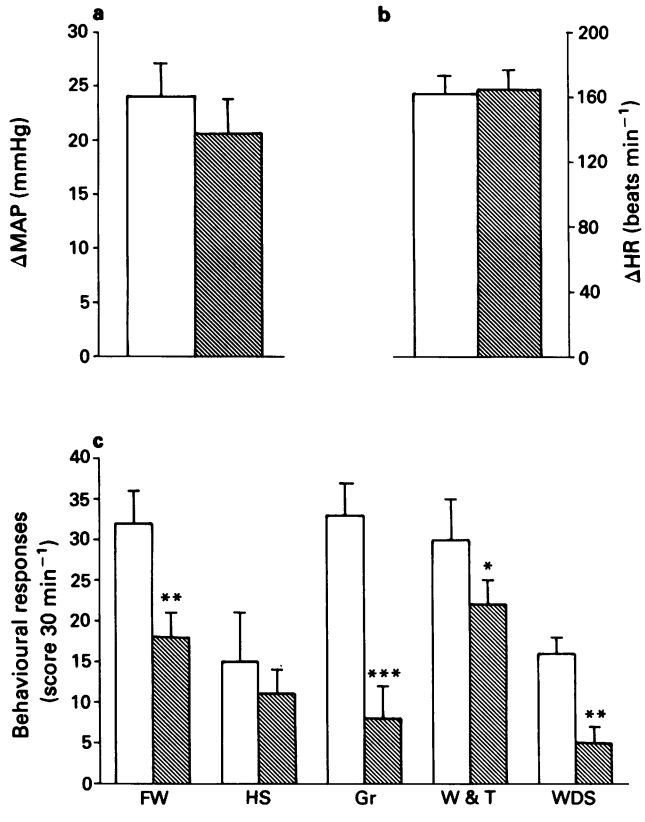


**Figure 2** Cardiovascular and behavioural effects induced by the intracerebroventricular injection of 25 pmol neuropeptide K (NPK) at 24 h pretreated with CSF at 0 h (open columns, $n = 8$) or 25 pmol NPK at 24 h pretreated with 25 pmol NPK at 0 h (hatched columns, $n = 8$). Maximal changes (a) in mean arterial pressure (MAP) and (b) heart rate (HR) or (c) behavioural activity for a 30 min period are shown. FW = face washing, HS = head scratching; Gr = grooming, W & T, walking and turning, WDS = wet dog shake. Each point represents the mean ± s.e.mean of ($n$) rats. Differences in MAP and HR between the two groups were non-significant. Differences in behaviour between the two groups are indicated by $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

**Table 1** Behavioural responses elicited by the i.c.v. injection of neuropeptide K (NPK) to conscious rats

| *Agonist* | n | *Face washing* (score 30 min$^{-1}$) | *Head scratching* (score 30 min$^{-1}$) | *Grooming* (score 30 min$^{-1}$) | *Walking and/or ipsilateral turning* (score 30 min$^{-1}$) | *Wet dog shakes* (episodes 30 min$^{-1}$) |
|---|---|---|---|---|---|---|
| CSF | 9 | 3.3 ± 1.6 | 0.2 ± 0.1 | 2.6 ± 1.3 | 3.3 ± 1.9 | 1.1 ± 0.7 |
| NPK 10 pmol | 6 | 6.5 ± 1.9 | 1.8 ± 1.4 | 12.2 ± 3.8* | 11.8 ± 5.1* | 7.2 ± 3.8* |
| NPK 25 pmol | 10 | 40.0 ± 4.5*** | 14.1 ± 3.4*** | 42.9 ± 4.5*** | 42.2 ± 3.4*** | 16.8 ± 2.7*** |
| NPK 65 pmol | 6 | 54.0 ± 11.7*** | 34.4 ± 8.7*** | 85.2 ± 18.3*** | 60.4 ± 18.0*** | 19.8 ± 4.7*** |

Values represent the mean ± s.e.mean of ($n$) rats for a period of 30 min. Statistical comparison to CSF was evaluated with a one-way ANOVA and a Wilcoxon-Mann-Whitney (U) test *a posteriori*: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

rats pre-injected, 24 h earlier, with the same dose of NPK when compared with rats pre-injected with CSF (Figure 2).

### *Effects of (±)-CP 96345 on NPK*

The i.c.v. injection of (±)-CP 96345 (6.5 nmol) 5 min before NPK, significantly blocked the pressor ($F(1,16) = 74.5$; $P<0.001$) and HR ($F(1,16) = 48.9$; $P<0.001$) responses induced by 25 pmol NPK (Figure 3). The cardiovascular response (0–60 min) to 25 pmol NPK in the presence of 6.5 nmol (±)-CP 96345 was not statistically different from CSF values ($F(1,16) = 1.5$; $P>0.05$ for ΔMAP and $F(1,16) = 1.4$; $P>0.05$ for ΔHR). Furthermore, (±)-CP 96345, reduced significantly all behaviours (except the head scratching) elicited by 25 pmol NPK (Table 2). Both the cardiovascular ($F(1,16) = 44.7$; $P<0.001$ for ΔMAP and $F(1,16) = 22.8$; $P<0.001$ for ΔHR) and behavioural responses to NPK were also significantly attenuated when the antagonist was given 24 h earlier (Figure 3 and Table 2). The residual cardiovascular response to 25 pmol NPK in rats pretreated 24 h earlier with (±)-CP 96345 was significantly different from CSF values and therefore was not completely abolished ($F(1,16) = 4.7$; $P<0.05$ for ΔMAP and $F(1,16) = 4.9$; $P<0.05$ for ΔHR). At 650 pmol, (±)-CP 96345 failed to alter the cardiovascular changes elicited by 25 pmol NPK (maximal ΔMAP and ΔHR were 24 ± 2 mmHg and 180 ± 17 beats $min^{-1}$ in the presence of antagonist ($n = 8$) versus 27 ± 3 mmHg and 215 ± 19 beats $min^{-1}$ in the absence of antagonist ($n = 8$): $P>0.05$).

### *Effects of RP 67580 on NPK*

The $NK_1$ receptor antagonist, RP 67580 (6.5 nmol, 5 min earlier) was found to reduce significantly ($F(1,16) = 45.8$; $P<0.001$ for ΔMAP and $F(1,16) = 49.5$; $P<0.001$ for ΔHR) both cardiovascular and behavioural responses (except the head scratching) to 25 pmol NPK (Figure 4 and Table 2). The cardiovascular response to 25 pmol NPK (0–60 min) in the presence of 6.5 nmol RP 67580 (5 min treatment) was not statistically different from CSF values ($F(1,16) = 1.5$; $P>0.05$ for ΔMAP and $F(1,16) = 3.9$; $P>0.05$ for ΔHR). However, RP 67580 injected i.c.v. 24 h before, had no significant effect on the cardiovascular ($F(1,16) = 1.9$; $P>0.05$ for ΔMAP and $F(1,16) = 2.8$; $P>0.05$ for ΔHR) and behavioural effects to 25 pmol NPK (Figure 4 and Table 2). Moreover, 650 pmol RP 67580 had no significant effect on the cardiovascular changes induced by 25 pmol NPK (maximal ΔMAP and ΔHR were 23 ± 4 mmHg and 200 ± 14 beats $min^{-1}$ in the

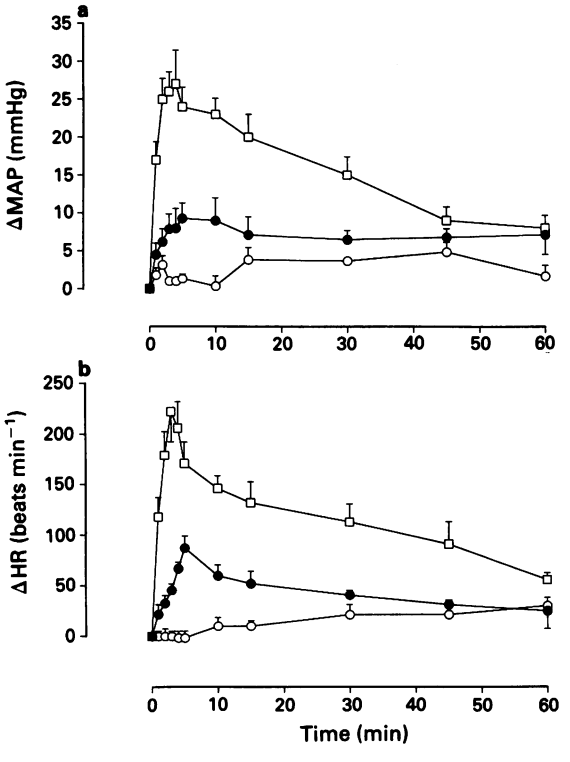


**Figure 3** Time course of cardiovascular effects to the intracerebroventricular injection of 25 pmol neuropeptide K (NPK) in the absence (□, $n = 9$) and presence of 6.5 nmol (±)-CP 96345 given 5 min (○, $n = 8$) or 24 h (●, $n = 8$) beforehand to conscious rats. Changes in (a) mean arterial pressure (MAP) and (b) heart rate (HR) are shown. Each point represents the mean ± s.e.mean of ($n$) rats in each group. When compared to CSF values (given in Table 3), the effects of NPK in the absence of antagonist were highly significant ($F(1,17) = 70.2$; $P<0.001$ for ΔMAP and $F(1,17) = 47.8$; $P<0.001$ for ΔHR).

**Table 2** Effects of tachykinin receptor antagonists on behavioural responses elicited by the i.c.v. injection of 25 pmol neuropeptide K (NPK) to conscious rats

| *Agonist* | *Antagonist* | n | *Face washing* (score 30 $min^{-1}$) | *Head scratching* (score 30 $min^{-1}$) | *Grooming* (score 30 $min^{-1}$) | *Walking and/or ipsilateral turning* (score 30 $min^{-1}$) | *Wet dog shakes* (episodes 30 $min^{-1}$) |
|---|---|---|---|---|---|---|---|
| CSF | – | 9 | 3.3 ± 1.6 | 0.2 ± 0.1 | 2.6 ± 1.3 | 3.3 ± 1.9 | 1.1 ± 0.7 |
| NPK | – | 10 | 40.0 ± 4.5*** | 14.1 ± 3.4*** | 42.9 ± 4.5*** | 42.2 ± 3.4*** | 16.8 ± 2.7*** |
| NPK | (±)-CP96345 6.5 nmol 5 min before | 8 | 18.4 ± 5.5††** | 5.4 ± 1.9** | 14.2 ± 3.8†††*** | 6.6 ± 3.1†††* | 2.2 ± 0.9††† |
| NPK | (±)-CP96345 6.5 nmol 24 h before | 8 | 19.0 ± 4.9†** | 9.6 ± 3.8** | 12.6 ± 3.3†††*** | 22.9 ± 4.8†*** | 4.3 ± 2.7††* |
| NPK | RP 67580 6.5 nmol 5 min before | 8 | 16.6 ± 4.2††** | 7.9 ± 0.9** | 20.1 ± 1.8††*** | 10.4 ± 5.1††* | 4.1 ± 2.1††* |
| NPK | RP 67580 6.5 nmol 24 h before | 8 | 34.5 ± 4.0*** | 19.0 ± 2.7*** | 45.8 ± 5.4*** | 38.0 ± 6.2*** | 14.8 ± 1.9*** |
| NPK | SR 48968 6.5 nmol 5 min before | 8 | 31.6 ± 6.7*** | 15.6 ± 5.3*** | 36.9 ± 10.8*** | 44.3 ± 9.4*** | 16.6 ± 1.6*** |
| NPK | R 487 6.5 nmol 5 min before | 6 | 45.5 ± 3.1*** | 46.3 ± 6.9†††*** | 45.6 ± 3.8*** | 55.1 ± 5.2*** | 18.2 ± 4.1*** |

Values represent the mean ± s.e.mean of ($n$) rats for a period of 30 min. Statistical comparison to NPK alone (†) or CSF (*) was evaluated with a one-way ANOVA and a Wilcoxon-Mann-Whitney (U) test *a posteriori*: *†$P<0.05$; **††$P<0.01$; ***†††$P<0.001$.

presence of antagonist ($n = 6$) versus 29 ± 5 mmHg and 220 ± 14 beats min$^{-1}$ in the absence of antagonist ($n = 6$); $P > 0.05$).

### *Effects of SR 48968 or R 487 on NPK*

Pre-administration (i.c.v., 5 min earlier) of 6.5 nmol of the selective $NK_2$ (SR 48968) or $NK_3$ (R 487) receptor antagonists did not significantly affect the MAP or HR increases and most behavioural activity elicited by 25 pmol NPK (Tables 2 and 3). However, 6.5 nmol R 487 enhanced head scratching, suggesting a possible agonist activity of the compound. Indeed, R 487 caused a transient increase in MAP at 1 min post-injection (Table 4). Otherwise, the three antagonists ((±)-CP 96345, RP 67580 and SR 48968) were devoid of direct effects on MAP and HR when given alone for a period of 15 min (Table 4). No apparent motor deficit nor respiratory distress were seen with any of the four antagonists.

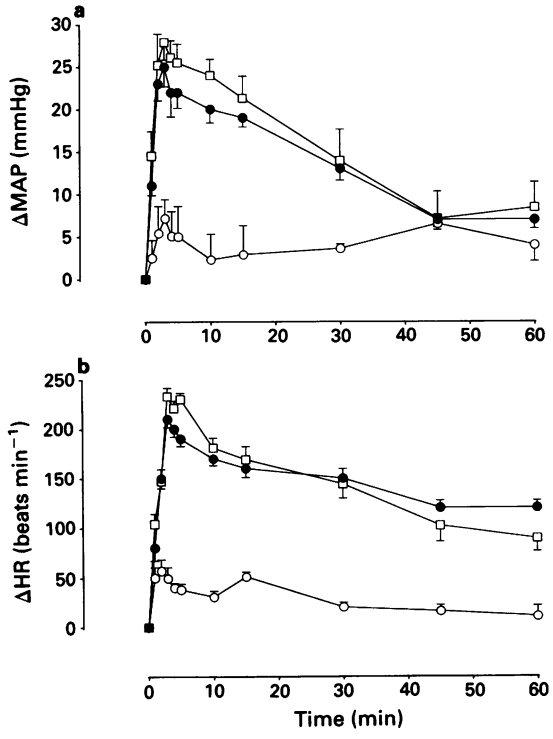


**Figure 4** Time course of cardiovascular effects to the intracerebroventricular injection of 25 pmol neuropeptide K (NPK) in the absence (□, $n = 9$) and presence of 6.5 nmol RP 67580 given 5 min (○, $n = 8$) or 24 h (●, $n = 8$) beforehand to conscious rats. Changes in (a) mean arterial pressure (MAP) and (b) heart rate (HR) are shown. Each point represents the mean ± s.e.mean of ($n$) rats in each group. When compared to CSF values (given in Table 3), the effects of NPK in the absence of antagonist were highly significant ($F(1,17) = 54.3$; $P < 0.001$ for ΔMAP and $F(1,17) = 47.8$; $P < 0.001$ for ΔHR).

## Discussion

The i.c.v. injection of NPK to the conscious rat produced rapid, prolonged and dose-dependent increases in MAP and behavioural activity. The increase in HR, however, did not appear to be dose-dependent. It is very unlikely that these central effects are produced by a leakage of NPK into the systemic circulation as intravenous injection of NPK caused a vasodepressor response (Décarie & Couture, 1992). A spinal site of action is also unlikely as the dose of NPK necessary to elicit pressor and tachycardiac responses after i.c.v. injection (10 pmol) was much lower than that required intrathecally (650 pmol) (Pham *et al.*, 1993). The higher efficacy of NPK in activating the cardiovascular system when injected i.c.v. is still unexplained, but it is consistent with a similar observation made with SP. While 25 pmol SP i.c.v. produced significant increases in MAP and HR (Tschöpe *et al.*, 1992), 6.5 nmol of SP was required to produce a significant pressor effect when injected i.t. in conscious rats (Hassessian *et al.*, 1988). The pressor and tachycardiac effects of i.c.v. or i.t. NPK are more prolonged and greater in intensity than those evoked by any other endogenous tachykinins namely SP, NKA and NKB (Hassessian *et al.*, 1988; Tschöpe *et al.*, 1992; Pham *et al.*, 1993).

This study shows that the central effects elicited by i.c.v. NPK are blocked by i.c.v. pretreatment with (±)-CP 96345 or RP 67580, two selective $NK_1$ receptor antagonists (Snider *et al.*, 1991; Emonds-Alt *et al.*, 1992; Garret *et al.*, 1992). In previous studies, (±)-CP 96345 (Tschöpe *et al.*, 1992) and RP 67580 (Picard *et al.*, 1994; preceding paper) were found to be good $NK_1$ antagonists that prevented by 40–50% the cardiovascular action of centrally administered SP. These antagonists did not affect the cardiovascular effect of i.c.v. NKA ($NK_2$ agonist) or [MePhe$^7$]NKB ($NK_3$ receptor agonist). Furthermore, the $NK_2$ (SR 48968) and $NK_3$ (R 487) receptor antagonists, did not affect the cardiovascular or the behavioural changes elicited by i.c.v. administered NPK, while they were found to block the central effects of NKA and [MePhe$^7$]NKB, respectively (Picard *et al.*, 1994). This pharmacological evidence suggests that NPK acts on $NK_1$

**Table 3** Changes in mean arterial pressure (MAP) and heart rate (HR) induced by the i.c.v. injection of 25 pmol neuropeptide (NPK) following the pre-injection of tachykinin receptor antagonists

| *Agonist* | *Antagonist* | n | | *Baseline MAP or HR* | *Time (min) after NPK injection (ΔMAP or ΔHR)* 1 | 5 | 10 | 30 | 60 |
|---|---|---|---|---|---|---|---|---|---|
| CSF | – | 8 | MAP | 99 ± 7 | 0 ± 1 | 1 ± 1 | 2 ± 1 | 3 ± 2 | 1 ± 1 |
| | | | HR | 350 ± 13 | 7 ± 8 | 5 ± 7 | 8 ± 5 | 3 ± 5 | 4 ± 4 |
| NPK | – | 9 | MAP | 110 ± 7 | 17 ± 2*** | 26 ± 2*** | 23 ± 2*** | 15 ± 2*** | 8 ± 1*** |
| | | | HR | 371 ± 12 | 118 ± 19*** | 222 ± 30*** | 146 ± 13*** | 113 ± 17*** | 55 ± 7*** |
| NPK | SR 48968 6.5 nmol 5 min before | 8 | MAP | 113 ± 7 | 16 ± 2*** | 23 ± 3*** | 24 ± 4*** | 23 ± 4*** | 12 ± 2*** |
| | | | HR | 391 ± 18 | 82 ± 8*** | 131 ± 13*** | 117 ± 14*** | 108 ± 17*** | 65 ± 15*** |
| NPK | R 487 6.5 nmol 5 min before | 6 | MAP | 117 ± 8 | 15 ± 2*** | 21 ± 3*** | 21 ± 3*** | 16 ± 4*** | 8 ± 2*** |
| | | | HR | 389 ± 11 | 80 ± 5*** | 140 ± 9*** | 124 ± 10*** | 121 ± 9*** | 81 ± 21*** |

Values represent the mean ± s.e.mean from ($n$) rats. Statistical comparison to CSF (***$P < 0.001$) or NPK without antagonist was evaluated with a two-way ANOVA. No significant differences were observed to NPK.

**Table 4** Changes in mean arterial pressure (MAP) and heart rate (HR) induced by the i.c.v. injection of tachykinin receptor antagonists

| *Antagonist* | n | | *Baseline MAP or HR* | *Time (min) after antagonist injection (ΔMAP or ΔHR) 1* | *5* | *10* | *15* |
|---|---|---|---|---|---|---|---|
| CSF | 8 | MAP | 113 ± 10 | 2 ± 2 | 4 ± 3 | 2 ± 2 | 0 ± 2 |
| | | HR | 323 ± 21 | 12 ± 7 | 6 ± 10 | 5 ± 7 | 9 ± 8 |
| (±)-CP 96345 | 6 | MAP | 115 ± 8 | −4 ± 3 | 0 ± 2 | −2 ± 5 | 3 ± 5 |
| (6.5 nmol) | | HR | 384 ± 16 | 25 ± 6 | 20 ± 4 | 21 ± 8 | 12 ± 10 |
| RP 67580 | 8 | MAP | 117 ± 7 | 5 ± 3 | 3 ± 4 | 2 ± 2 | 2 ± 1 |
| (6.5 nmol) | | HR | 321 ± 24 | 24 ± 18 | 11 ± 12 | 5 ± 5 | 1 ± 7 |
| SR 48968 | 6 | MAP | 120 ± 5 | 4 ± 5 | 2 ± 2 | −3 ± 6 | −3 ± 5 |
| (6.5 nmol) | | HR | 371 ± 13 | 10 ± 5 | 16 ± 7 | 9 ± 7 | 11 ± 8 |
| R 487 | 6 | MAP | 112 ± 9 | 15 ± 4* | 10 ± 3 | 3 ± 2 | 2 ± 4 |
| (6.5 nmol) | | HR | 379 ± 10 | 19 ± 10 | 13 ± 14 | 9 ± 5 | 9 ± 8 |

Values represent the mean ± s.e.mean from (*n*) rats. Statistical comparison to CSF was evaluated with a two-way ANOVA: $*P < 0.05$.

but not on $NK_2$ or $NK_3$ receptors to produce its effects in the rat brain.

It is worthwhile to note here that the central cardiovascular effect induced by NPK was entirely blocked by either (±)-CP 96345 or RP 67580, while the same antagonists reduced by only 40–50% the central cardiovascular changes induced by SP. The residual response to SP measured after treatment with RP 67580 was, however, blocked after the addition of SR 48968, indicating that SP activates both $NK_1$ and $NK_2$ receptors. It seems therefore that NPK is a better $NK_1$ receptor agonist than SP in the rat central nervous system.

Since the discovery of NPK by Tatemoto *et al.* (1985), results of numerous pharmacological experiments conducted *in vivo* suggest that NPK and SP may act on the same receptor which may be of the $NK_1$ subtype (Takeda & Krause, 1989; Décarie & Couture, 1992; Pham & Couture, 1993). Nevertheless, binding or radioligand assays identified NPK as a potent $NK_2$ receptor agonist in the rat duodenum (Beaujouan *et al.*, 1988) and hamster urinary bladder (Van Giersbergen *et al.*, 1992) with a high selectivity for $NK_2$ binding sites and very weak binding affinity for $NK_1$ and $NK_3$ binding sites on rat brain synaptosomes (Beaujouan *et al.*, 1988). In submandibular gland membranes, a tissue rich in $NK_1$ receptors, both NPK and NKA appear to interact with the $NK_1$ receptor with 100 times lower affinity than that of SP (Takeda & Krause, 1989). The reason for the discrepancy between binding assays and functional studies is still unknown. One major difference between functional and binding studies is the fact that no peptidase inhibitors have been used to prevent the metabolic transformation of NPK *in vivo*. Thus, one cannot exclude the possibility that a metabolite of NPK produced by enzymatic degradation *in vivo* has higher affinity for the $NK_1$ receptor, while the entire molecule may behave as an $NK_2$ receptor agonist in binding assays. The C-terminal part of NPK contains the sequence of NKA; however, the present results do not support the view that NKA is the biologically active metabolite of NPK as suggested earlier (Martling *et al.*, 1987) since NKA is much less potent (magnitude and duration) than NPK when given i.c.v., and the central effects of NKA are mediated primarily by $NK_2$ receptors (Tschöpe *et al.*, 1992; Picard *et al.*, 1994). A similar conclusion was reached on ingestive behaviour induced by i.c.v NPK in the rat; the spectrum of antidipsogenic activity of NPK was markedly different from that of NKA (Achapu *et al.*, 1992).

The two $NK_1$ receptor antagonists had dissimilar time course effects. While RP 67580 failed to block NPK when administered 24 h earlier, (±)-CP 96345 did significantly reduce the central effects of NPK. This finding is consistent with a previous study from our laboratory where it was shown that (±)-CP 96345 given either by the i.t. (6.5 nmol) or i.v. (0.13 mg $kg^{-1}$) route, blocks for several days the cardiovascular effects induced by i.t. injection of NPK (3.25 nmol) (Pham & Couture, 1993). Hence, RP 67580 shows a different pharmacological profile from CP 96345 on central $NK_1$ receptors.

The relation between tachykinins and behaviour has been studied previously (Itoi *et al.*, 1988; Stoessl *et al.*, 1988), but our paper is the first to show that NPK enhances several behaviours dose-dependently. The most prominent are face washing, grooming and locomotor activity (walking and/or ipsilateral turning) while head scratching and wet dog shakes are observed with lower frequency. The use of selective receptor antagonists showed that nearly all behaviours elicited by NPK, except for head scratching, are probably mediated by $NK_1$ receptors. We do not have any valid explanation to explain why head scratching behaviour remains resistant to $NK_1$, $NK_2$ and $NK_3$ receptor antagonists. Interestingly, head scratching was the only behaviour resistant to desensitization induced by two injections of NPK. Although the cardiovascular and behavioural responses to i.c.v. NPK occurred concurrently and via the $NK_1$ receptor, they appear to derive from different mechanisms. This assumption is based on the observation that the cardiovascular effects mediated by NPK were not altered when the behavioural responses were desensitized upon the second injection of NPK.

The increasing effect of R 487 on the head scratching behaviour induced by NPK may be related to a residual agonist activity of this compound on $NK_1$ and $NK_2$ receptors (Regoli *et al.*, 1991). The inhibitory effect of (±)-CP 96345 and RP 67580 was not related to motor deficits nor to changes of baseline parameters.

In summary, selective and potent antagonists of $NK_1$, $NK_2$ and $NK_3$ receptors have been used to characterize the receptor subtype which is responsible for the central cardiovascular and behavioural effects of NPK in the conscious rat. Our findings suggest that the response to NPK is mediated primarily by $NK_1$ receptors, unlike SP which has the ability to stimulate $NK_2$ receptors when $NK_1$ receptors are occupied by $NK_1$ antagonists. It is suggested that NPK, or one of its metabolites (that is not NKA), may play a role in central cardiovascular regulation.

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# Pharmacological relevance of peripheral type benzodiazepine receptors on motor nerve and skeletal muscle

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**1** Effects of agonists and antagonists of peripheral and central benzodiazepine receptors (pBZR and cBZR) on neuromuscular transmission were studied in mouse isolated phrenic nerve-diaphragm preparations.

**2** Ro5-4864, a pBZR agonist, had no effect on the neuromuscular transmission but increased muscle contractility and antagonized the tetanic fade induced by neostigmine.

**3** Ro5-4864 inhibited the regenerative tonic endplate depolarization caused by repetitive stimulation in the presence of neostigmine without affecting the amplitude and decay time of miniature and evoked single endplate potentials.

**4** All the effects of Ro5-4864 were shared by PK11195, a pBZR antagonist, but not by clonazepam and flumazenil, a cBZR agonist and antagonist, respectively.

**5** It is suggested that peripheral type benzodiazepine receptors modulate presynaptic function and muscle contraction.

**Keywords:** Peripheral benzodiazepine receptors; benzodiazepines; Ro5-4864; PK11195; neuromuscular transmission

## Introduction

Benzodiazepine receptors (BZR) have been classified into central and peripheral types according to their relative affinities for specific ligands (Braestrup & Squires, 1977; Rampe & Triggle, 1986). The central type (cBZR) displays high affinity for diazepam (nanomolar range) and is involved in the anxiolytic, muscle relaxing, sedative and hypnotic actions of benzodiazepines (Haefely *et al.*, 1979). Peripheral BZR (pBZR), having tens of nanomolar affinity for diazepam (Braestrup & Squires, 1977), were originally demonstrated in various peripheral nonneuronal tissues by a selective binding ligand Ro5-4864 (7-chloro-1, 3-dihydro-1-methyl-5-(p-chlorophenyl)-2*H*-1,4-benzodiazepine-2-one) and later also found in the central nervous system (Schoemaker *et al.*, 1981). The functional roles of pBZR in the peripheral nervous system are far from clear, although involvement in steroidogenesis, cell proliferation and immunoreactivity of non-neuronal tissues have been proposed (Ruff *et al.*, 1985; Anholt, 1986; Mukhin *et al.*, 1989; Papadopoulos *et al.*, 1990; Bruce *et al.*, 1991). In addition, a third type nonselective binding site for benzodiazepines with micromolar affinity has been demonstrated in the brain (Bowling & DeLorenzo, 1982) and leech neurones (Johansen *et al.*, 1985). This type of receptor providing no selectivity for either pBZR or cBZR ligands was suggested to be related to the inhibition of $Ca^{2+}$ channel in synaptosomes (Taft & DeLorenzo, 1984) and inhibition of electric shock-induced seizure by benzodiazepines (Bowling & DeLorenzo, 1982).

Since diazepam improves the peripheral neuromuscular transmission impaired by anticholinesterase agents and enhances the muscle contractility (Chiou & Chang, 1993), it seems possible that benzodiazepine receptors exist in the peripheral motor nerve and skeletal muscle and have modulatory function on synaptic transmission. Specific binding sites for [$^3$H]-flunitrazepam (Wilkinson *et al.*, 1982) and [$^3$H]-Ro5-4864 (Roeske & Yamamura, 1982) have been demonstrated in rat diaphragm and it has been proposed that they are the peripheral type. The present study was undertaken to compare the functional activities of both cBZR and pBZR agonists and antagonists and to elucidate the possible functional role(s) of pBZR in neuromuscular junction.

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## Methods

### *Muscle contractions*

Muscle contractions and electrophysiological experiments were performed in phrenic nerve-hemidiaphragm preparations isolated from ICR mice (20–25 g) of either sex. The organ bath contained 15 ml Tyrode solution (composition in mM: NaCl 137, KCl 2.8, $CaCl_2$ 1.8, $MgCl_2$ 1.2, $NaH_2PO_4$ 0.33, $NaHCO_3$ 11.9 and dextrose 11.2) oxygenated with 95% $O_2$ plus 5% $CO_2$ and kept at 36 ± 0.2°C. Tetanic contraction and single twitches were evoked at 50–100 Hz for 3 s and 0.1 Hz, respectively. Indirect muscle contractions were elicited by stimulation of the phrenic nerve with supramaximal rectangular pulses of 0.05 ms width. Direct muscle contractions were elicited by field stimulation of the diaphragm with 0.5 ms bipolar pulses in the presence of 4 μM tubocurarine to exclude any possible indirect component. Contractions were recorded isometrically via a transducer (BG25, Gould) coupled to a physiological recorder (Gould 3000). The tetanic performance was evaluated by measuring the area under the contractile curves (AUC).

### *Electrophysiological studies*

Electrophysiological experiments were performed by classical intracellular recording technique with 3 M KCl-filled microelectrodes (10–40 MΩ). Preparations were mounted horizontally in a perfusion chamber containing 4 ml oxygenated Tyrode solution and were perfused at a rate of 6–8 ml $min^{-1}$. Transmembrane potentials were recorded through a high impedance amplifier (Axoclamp-2A) and registered with a computer-aided digitizer of 100 kHz bandwidth (D6100, Analogic). Endplate potentials (e.p.ps) were evoked by nerve stimulation at 0.1 Hz or 75 Hz and recorded at endplate area of cut muscle preparations (Barstad & Lilleheil, 1968). The amplitude of e.p.p. was corrected for non-linear summation to − 40 mV, assuming a reversal potential of 0 mV (Chang *et al.*, 1986).

A regenerative tonic depolarization was provoked by stimulation with repetitive pulses at 75 Hz. This was determined by the upstroke depolarization and the associated shut-off of phasic e.p.ps (Chang & Hong, 1986). The duration of regenerative depolarization was measured from the upstroke to the resumption of phasic e.p.ps. The extent of depolarization was

determined by the maximal changes of the endplate membrane potential.

### *Chemicals*

Diazepam, clonazepam and flumazenil were generous gifts from Hoffmann-La Roche (Basle, Switzerland) and PK11195 (also coded as RP 52028) (1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide) was from Rhone-Poulenc Rorer (Cedex, France). Ro5-4864 was purchased from Fluka (Switzerland) and neostigmine bromide, tubocurarine chloride and γ-aminobutyric acid (GABA) from Sigma (USA). All benzodiazepines and PK11195 were dissolved in ethanol as stock solutions. Final vehicle concentrations added to the organ bath were less than 0.2% and had little effect on the neuromuscular transmission.

### *Statistics*

All data are expressed as mean ± s.e. Differences between means were analyzed by Student's *t* test.

## Results

### *Effect on muscle contractions in the absence of neostigmine*

Like diazepam, Ro5-4864, a pBZR agonist, dose-dependently increased the direct single twitch response at 5–50 μM in the presence of 4 μM tubocurarine (Figure 1). The indirect single twitch response was increased to the same extent by Ro5-4864 at 5–30 μM. At 50 μM, a stepwise decrease of the indirect twitch tension, implicating nerve conduction block, was observed in some preparations after the initial potentiation (Figure 2). The tetanic contractions evoked directly or indirectly at 50～100 Hz were also enhanced by Ro5-4864 in the same concentration-range. However, the higher the stimulation rate, the less was the enhancement. A slight tonic contracture was observed when Ro5-4864 was at concentrations higher than 30 μM.

The effects of PK11195, a pBZR ligand nominally regarded as an antagonist (Le Fur *et al.*, 1983a,b; Benavides *et al.*, 1984; Mizoule *et al.*, 1984; Ruff *et al.*, 1985; Slobodyansky *et al.*, 1989; Bruce *et al.*, 1991), were compared. Interestingly, this compound also increased the muscle contractility evoked directly or indirectly by either single or repetitive pulses, at the same concentration-range. Muscle contracture and axonal conduction block were also observed at 100 μM. Ro5-4864 and PK11195, though classified as agonist and antagonist, respectively, appeared to act additively with no sign of antagonism. Their effects were analogous to diazepam (Chiou & Chang, 1993) but were more potent.

In contrast, clonazepam and flumazenil, the cBZR agonist and antagonist (Braestrup & Squires, 1977; Laurent *et al.*, 1981), respectively, did not affect the direct or indirect twitch responses at concentrations up to 50 μM (Figure 1). Moreover, the effects of Ro5-4864, PK11195 or diazepam were not antagonized after pretreatment with flumazenil.

### *Effects on muscle contractions in the presence of neostigmine*

Three characteristic actions of anticholinesterase agents on isolated nerve-skeletal muscle preparations, i.e., spontaneous muscle fasciculation, twitch potentiation and tetanic fade, an inability to maintain a tetanus during high frequency stimulation of motor nerve (Hobbiger, 1976), were reproduced by pretreating the diaphragm with 0.3 μM neostigmine. Ro5-4864, PK11195 and diazepam at 1～20 μM dose-dependently inhibited the twitch potentiation (Figure 3). All three ligands appeared equipotent. Clonazepam was effective only at concentrations higher than 20 μM while flumazenil was ineffective at concentrations up to 50 μM.

The tetanic fade was antagonized by Ro5-4864 and PK 11195 (Figure 4) in a manner analogous to diazepam (Chiou & Chang, 1993) with a bell-shape dose-response relation (Figure 5). The potencies were in the order of PK11195 ≥ Ro5-4864 > diazepam. By contrast, both cBZR ligands were ineffective in restoring tetanic fade and did not affect the restoration by Ro5-4864, PK11195 and diazepam.

Similar to the effects on twitch potentiation and tetanic fade, the spontaneous muscle fasciculation was abolished by Ro5-4864 and PK11195 at 10 μM and by clonazepam at 20 μM but not by flumazenil at concentrations up to 50 μM.

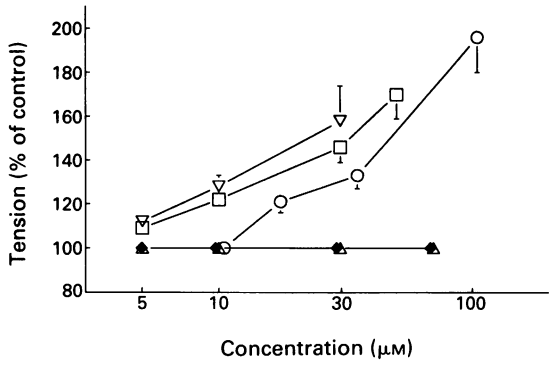


**Figure 1** Dose-response curves of benzodiazepine receptor ligands on the direct twitch responses. Twitch responses were evoked at 0.1 Hz directly in the presence of 4 μM tubocurarine; diazepam (○); Ro5-4864 (□); PK11195 (▽); clonazepam (◆); flumazenil (△). $n = 3-6$.

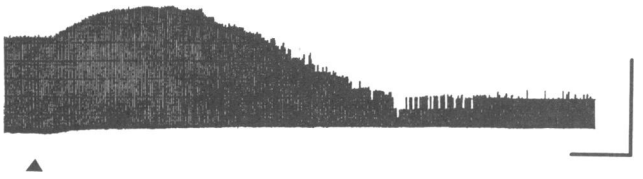

**Figure 2** Effect of Ro5-4864 on the indirect twitch response of mouse isolated phrenic nerve-diaphragm preparation. The indirect twitch response was evoked at 0.1 Hz. Ro5-4864, 50 μM was added at ▲. Calibration: 5 min, 1 g.

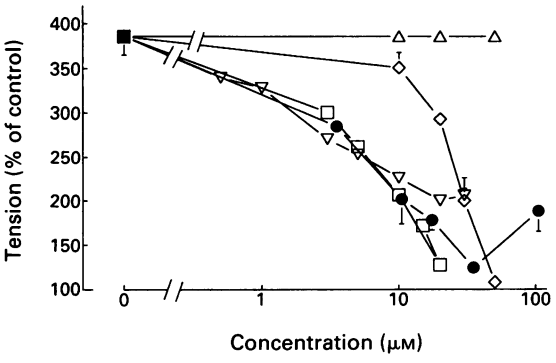


**Figure 3** Effect of benzodiazepine receptor ligands on the indirect twitch response in the presence of neostigmine. Indirect twitch response evoked at 0.1 Hz in the presence of 0.3 μM neostigmine is expressed as % of control in the absence of drugs. Concentration zero represents the average responses produced by neostigmine alone: diazepam (●); Ro5-4864 (□); PK11195 (▽); clonazepam (◇); flumazenil (△). $n = 3-8$.

*Effect on the endplate responses*

Ro5-4864, at concentrations up to 30 μM, did not affect the amplitude and decay time of e.p.p. or m.e.p.p. In the presence of neostigmine, single e.p.ps as well as m.e.p.ps that were enlarged and prolonged were also unaffected by Ro5-4864 (Table 1). The regenerative depolarization, a unique phenomenon provoked by high frequency stimulation in anticholinesterase-treated endplates (Chang & Hong, 1986; Hong & Chang, 1989) was inhibited by Ro5-4864 and PK 11195. The incidence of tonic depolarization, as characterized by the upstroke depolarization followed by complete shut-off of phasic e.p.ps was decreased from about 95% to two-thirds of junctions after treatment with 10 μM Ro5-4864, and the duration, if it occurred, was shortened (Table 1). The extent of depolarization was decreased resulting in a restoration of train e.p.ps (Figure 6). PK11195, at 5 μM, produced the same degree of inhibition as 10 μM Ro5-4864. The effects of both Ro5-4864 and PK11195 were again similar to that of diazepam (Chiou & Chang, 1993) but the potency of diazepam was lower. Again, in contrast to pBZR ligands, both clonazepam and flumazenil did not affect the neostigmine-induced regenerative depolarization.

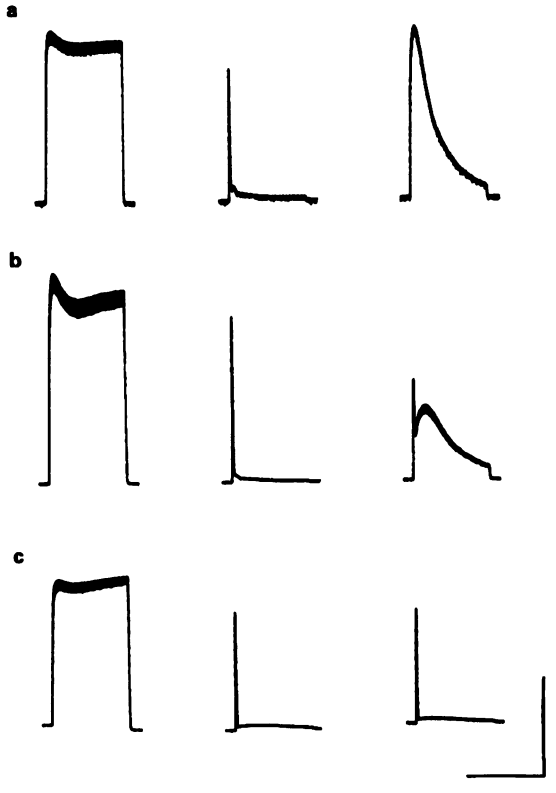


**Figure 4** Effect of Ro5-4864, PK11195 and flumazenil on the tetanic fade in the presence of neostigmine. Phrenic nerve was stimulated at 75 Hz for 3 s in preparations before (left column) and after treatment with 0.3 μM neostigmine (middle column) and after further treatment (right column) with 10 μM Ro5-4864 (a), 5 μM PK 11195 (b), or 10 μM flumazenil (c). Calibration: 3 s, 5 g.

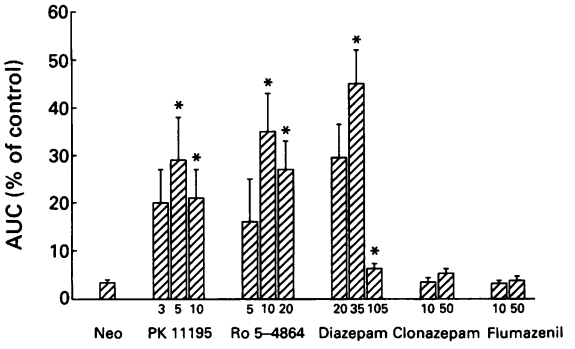


**Figure 5** Effect of benzodiazepine receptor ligands on tetanic contraction in the presence of neostigmine. Tetanic contractions induced by 75 Hz nerve stimulation for 3 s in the presence of 0.3 μM neostigmine (Neo) were measured from the area under the curve (AUC) of contraction and expressed as % of control before drug treatment. Figures below each column are the concentrations of BZR ligands in μM. $n = 4-7$. $^*P < 0.05$ vs. the next smaller concentration in paired $t$ test.

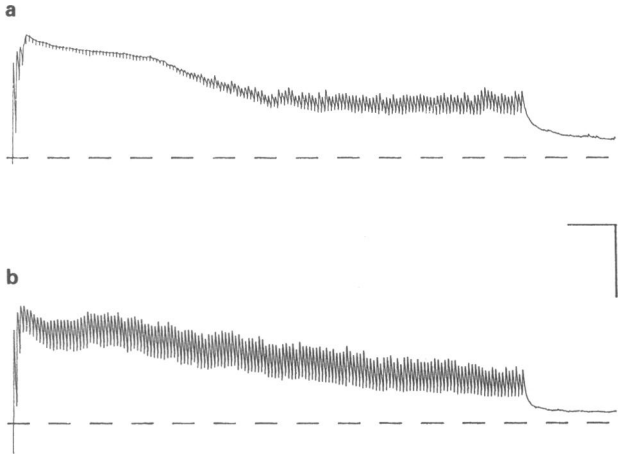


**Figure 6** Effect of Ro5-4864 on the endplate responses during repetitive stimulation in the presence of neostigmine. The preparation was treated with 0.3 μM neostigmine and stimulated at 75 Hz. The responses at the endplate were recorded from the same junction before (a) and after (b) treatment with 10 μM Ro5-4864. Resting membrane potential denoted by the broken lines was about − 46 mV. Calibration: 200 ms, 10 mV.

**Table 1** Effect of Ro5-4864 on the endplate responses in the presence of neostigmine

| | *m.e.p.p.*[a] | | *e.p.p.*[b] | | *Regenerative depolarization*[c] | | |
|---|---|---|---|---|---|---|---|
| *Treatment* | *Amplitude* (mV) | $t_{\frac{1}{2}}$[d] (ms) | *Amplitude* (mV) | $t_{\frac{1}{2}}$ (ms) | *Incidence* | *Duration* (ms) | *Extent* (mV) |
| Control | 2.9 ± 0.2 | 3.1 ± 0.1 | 16.0 ± 0.2 | 6.1 ± 0.1 | 21/22 | 887 ± 134 | 19.6 ± 1.3 |
| Ro5-4864 (10 μM) | 3.0 ± 0.2 | 3.0 ± 0.2 | 15.8 ± 0.2 | 6.4 ± 0.1 | 12/18* | 451 ± 73* | 14.0 ± 1.4* |

Neostigmine (0.3 μM) was present throughout.
[a]Recorded in uncut muscle preparations (resting membrane potentials: − 82 ± 2 mV) in the presence of 1 μM tetrodotoxin.
[b]Evoked at 0.6 Hz and recorded in cut muscle preparations (resting membrane potentials: − 41 ± 1 mV).
[c]Provoked by stimulating nerve at 75 Hz for 2.5 s and calculated as described in Methods.
[d]Half decay time.
*$P < 0.05$.

### *Effect of GABA on the actions of pBZR ligands*

GABA, at 100 μM, did not affect the neuromuscular transmission in the presence or absence of neostigmine nor did it affect the enhancement of muscle contraction and the restoration of neostigmine-impaired tetanic contraction caused by diazepam, Ro5-4864 or PK11195.

## Disccusion

BZR of the peripheral type have been demonstrated in skeletal muscle by binding studies (Roeske & Yamamura, 1982; Wilkinson *et al.*, 1982). Clonazepam, a cBZR ligand, was 3～4 order less potent than Ro5-4864 in displacing the specific binding of benzodiazepine ligands. In this study, we found that there was a clear-cut distinction between the pBZR and cBZR ligands in their effects on neuromuscular transmission. The pBZR ligands Ro5-4864 and PK11195, irrespective of agonist or antagonist activity, shared with diazepam the same effects on mouse phrenic nerve-diaphragm (Chiou & Chang, 1993), i.e., an enhancement of muscle contractility and a restoration from anticholinesterase-induced neuromuscular transmission disorders. The cBZR ligands, clonazepam and flumazenil, were much less effective or ineffective. This pattern of ligands selectively suggested that the receptor involved was pBZR but not cBZR or the third type. The effects of the pBZR ligands were unlikely to be due to a nonspecific membrane action of benzodiazepine derivatives since clonazepam and flumazenil are congeners of benzodiazepines but were ineffective while PK11195, a non benzodiazepine, was effective. It is noteworthy that these pBZR ligands have no appreciable effect on the process of neuromuscular transmission in the absence of neostigmine, as judged from the unchanged single or train e.p.ps. Accordingly, it may be inferred that pBZR exist in motor nerve-skeletal muscle system and have functional relevance at least in opposing pathophysiological or pharmacological distresses. These effects of pBZR ligands seem unrelated to GABA receptors since GABA showed neither action of its own nor interaction with pBZR ligands.

PK11195, though classified as a pBZR antagonist in cardiac and other tissues (Le Fur *et al.*, 1983a,b; Benavides *et al.*, 1984; Mizoule *et al.*, 1985; Ruff *et al.*, 1985; Slobodyansky *et al.*, 1989; Bruce *et al.*, 1991), probably acts as an agonist in motor nerve and skeletal muscle as in PC12 cells (Ohara-Imaizumi *et al.*, 1991), coronary blood vessel (Grupp *et al.*, 1987), bronchiole (Marao *et al.*, 1990), duodenum (Escubedo *et al.*, 1992) and steroidogenic cells (Mukhin *et al.*, 1989; Papadopoulos *et al.*, 1990).

The effective concentrations of diazepam and Ro5-4864 for neuromuscular actions were two orders of magnitude higher than their $K_D$ value. In general, however, the effective concentrations for pharmacological action on intact organ preparations were much higher than their binding affinity constant measured in homogenized membranes (cf. Hullihan *et al.*, 1983). The difference may be due to penetration barrier and/or to higher receptor occupancy required to generate the functional responses. Compared with brain or other peripheral tissues such as liver, kidney or heart, the specific binding sites for [$^3$H]-diazepam or [$^3$H]-Ro5-4864 in skeletal muscle are rather sparse (Braestrup & Squires, 1977; Roeske & Yamamura, 1982; Anholt, 1986). Therefore, higher concentrations may be required to produce pharmacological effects. Another reason may be the decreased binding of diazepam or Ro5-4864 when the temperature was elevated from 0～4°C to 37°C (Braestrup & Squires, 1977; Le Fur *et al.*, 1984b; Raeburn *et al.*, 1988; Mihara & Fujimoto, 1989).

The restoration of neostigmine-induced tetanic fade by Ro5-4864 and PK11195 can be explained by their inhibition of the train pulse-induced tonic depolarization. This depolarization, a novel phenomenon produced by anticholinesterase agents and resulting in tetanic fade, is attributed to a regenerative ACh release triggered by the accumulated ACh through activation of presynaptic nicotinic ACh receptor and $Ca^{2+}$ channels (Chang & Hong, 1986; Hong & Chang, 1989). How the pBZR ligands inhibit this regenerative depolarization is not known. Since the ligands did not affect the postsynaptic expression of e.p.ps, a presynaptic interaction seems more likely. Whether a $Ca^{2+}$ channel inhibition is involved remains to be elucidated. Indeed, various pharmacological effects of benzodiazepines have been related to an inhibition of $Ca^{2+}$ channels, e.g. inhibition of dopamine release in phaeochromocytoma cells (Nakazawa *et al.*, 1991; Ohara-Imaizumi *et al.*, 1991), ileal longitudinal muscle contraction (Hullihan *et al.*, 1983; Rampe & Triggle, 1987), vas deferens constriction (Escubedo *et al.*, 1992), action potential duration and contractility of cardiac muscle (Mestre *et al.*, 1984; Holck & Osterrieder, 1985) and β-endorphin release from At T20 cells (Bisserbe *et al.*, 1986). In addition, Ro5-4864 competitively inhibited the specific binding of dihydropyridine $Ca^{2+}$ channel blockers on $Ca^{2+}$ channels (Bender & Hertz, 1985; Holck & Ostereider, 1985; Rampe & Triggle, 1987) and *vice versa* (Cantor *et al.*, 1984). However, opposite results were also reported (cf. Rampe & Triggle, 1986; Raeburn *et al.*, 1988).

The enhancement of muscle contractility by pBZR ligands could be attributed to an effect on dihydropyridine receptors located on the T-tubular membrane of skeletal muscle, which probably function mostly as a voltage sensor in the process of excitation-contraction coupling (Rios & Pizarro, 1991). Several L-type $Ca^{2+}$ channel antagonists have been shown to enhance the contractility of skeletal muscle (Chang *et al.*, 1988; Dulhunty & Gage, 1988; Neuhaus *et al.*, 1990) and shift the activation curve of excitation-contraction coupling (Dulhunty & Gage, 1988; Neuhaus *et al.*, 1990). The possibility remains that the pBZR ligands modulate the excitation-contraction coupling and enhance muscle contractility by acting on this voltage sensor.

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# The influence of the trigeminal ganglion on carotid blood flow in anaesthetized guinea-pigs

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**1** The influence of the trigeminal ganglion on the carotid circulation has been investigated by measuring electrical stimulation-induced alterations in carotid arterial blood flow and resistance in anaesthetized guinea-pigs. The effects of several receptor antagonists were assessed to determine which neurotransmitters are involved in regulating carotid blood flow.

**2** Arterial blood pressure and carotid vascular resistance were reduced by electrical stimulation (0.5 mA, 1 ms, 5 Hz, 60 s) of the trigeminal ganglion ipsilateral to the carotid artery from which flow was measured. No consistent effect of electrical stimulation on carotid blood flow was observed. However, when guinea-pigs were pretreated with guanethidine (30 mg $kg^{-1}$, s.c., 24 h prior to experiments), stimulation produced little change in blood pressure, while carotid blood flow was increased and vascular resistance decreased, consistent with vasodilatation in the cranial circulation. Stimulation of the trigeminal ganglion contralateral to the carotid artery from which blood flow was measured, had little effect on either carotid blood flow or vascular resistance.

**3** In animals pretreated with guanethidine, intravenous administration of the vasoactive intestinal polypeptide (VIP) receptor antagonist, [p-Cl-D-Phe$^6$,Leu$^{17}$]-VIP (50 $\mu$g $kg^{-1}$) significantly attenuated the increase in carotid blood flow and decrease in carotid vascular resistance evoked by trigeminal ganglion stimulation. Responses evoked by trigeminal ganglion stimulation were, however, unaffected by intravenous injection of the tachykinin $NK_1$ receptor antagonists, GR82334 (0.3 mg $kg^{-1}$) and CP-99,994 (0.4 mg $kg^{-1}$), calcitonin gene-related peptide (CGRP) receptor antagonist, $CGRP_{8-37}$ (0.9 mg $kg^{-1}$) and the ganglion blocking agent, hexamethonium (10 mg $kg^{-1}$).

**4** It is concluded that in the guanethidine-pretreated guinea-pig, electrical stimulation of the trigeminal ganglion increases carotid blood flow and produces an accompanying decrease in carotid vascular resistance, consistent with the dilatation of carotid blood vessels. The transmitter mediating this effect is most likely to be VIP.

**Keywords:** Trigeminal ganglion; carotid blood flow; electrical stimulation; VIP; $NK_1$; CGRP; hexamethonium; neuropeptide receptor antagonists

## Introduction

The trigeminal nerve may be involved in the regulation of cranial blood flow (McCulloch *et al.*, 1986; Suzuki *et al.*, 1990). Stimulation of either the trigeminal ganglion or the nasociliary branch of its ophthalmic division increases blood flow in both cerebral and dural blood vessels of anaesthetized cats and rats (Hardebo *et al.*, 1991; Goadsby, 1993). The increase in blood flow arises from dilatation of cranial blood vessels, particularly those of the extracerebral circulation (Goadsby *et al.*, 1986). The vasodilatation has been shown in cats to be mediated in part by a parasympathetic reflex involving the seventh cranial nerve and the sphenopalatine and otic ganglia (Lambert *et al.*, 1984). Consistent with this mechanism, sectioning the seventh cranial nerve or administration of the ganglion blocking agent, hexamethonium, prevents the increases in carotid arterial blood flow and decreases in carotid vascular resistance which are evoked by trigeminal ganglion stimulation in cats and which reflect vasodilatation in the cranial vasculature (Lambert *et al.*, 1984; Goadsby & Macdonald, 1985). The neurotransmitter mediating this reflex vasodilatation and subsequent increase in cranial blood flow is likely to be vasoactive intestinal polypeptide (VIP) (Goadsby & Shelley, 1990). Cranial blood vessels receive a dense distribution of VIP-containing nerve fibres and stimulation of the efferent parasympathetic nerve releases VIP-like immunoreactivity. In addition, pretreatment with VIP antiserum blocks the increase in flow caused by trigeminal ganglion stimulation in anaesthetized cats (Larsson *et al.*, 1976; Goadsby & Macdonald, 1985; Goadsby & Shelley, 1990).

The neuropeptides, substance P and calcitonin gene-related peptide (CGRP) may also be important in the regulation of cranial blood flow (Suzuki *et al.*, 1990; Goadsby, 1993). Cranial blood vessels possess a dense distribution of substance P and CGRP-like immunoreactive nerve fibres originating in the trigeminal ganglion and stimulation of these fibres results in antidromic release of substance P and CGRP from perivascular nerve terminals (Edvinsson *et al.*, 1983; Liu-Chen *et al.*, 1983; Skofitsch & Jacobowitz, 1985). Both neuropeptides are potent dilators of cranial blood vessels. Moreover, recently it was demonstrated that increases in cerebral blood flow evoked by stimulation of the nasociliary nerve in cats are inhibited by human $\alpha$ $CGRP_{8-37}$, the putative $CGRP_1$ receptor antagonist (Jansen, 1992), indicating that CGRP may have a role in mediating this effect (Goadsby, 1993).

The relative importance of these neurotransmitters in the regulation of cranial blood flow remains to be clearly defined; previous studies have been hindered by the lack of selective and potent receptor antagonists for the transmitters implicated. The use of the CGRP receptor antagonist, human $\alpha$ $CGRP_{8-37}$, and the VIP and tachykinin receptor antagonists now available, such as [*p*-Cl-D-Phe$^6$,Leu$^{17}$]-VIP (Pandol *et al.*, 1986) and GR82334 (Hagan *et al.*, 1991) respectively, should allow the roles of these neuropeptides in the trigeminal nerve-mediated increase in carotid blood flow to be clarified.

The objectives of the present study were two fold. Firstly, it was intended to determine the effects of trigeminal ganglion stimulation on the carotid circulation, as measured by alterations in carotid arterial blood flow and vascular resistance (Lambert *et al.*, 1984; Spokes & Middlefell, 1993) and

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secondly, to characterize pharmacologically the influence of selective VIP, CGRP and $NK_1$ receptor antagonists on responses evoked by trigeminal ganglion stimulation to determine which transmitters were implicated. The use of these antagonists may also identify the neurotransmitters which regulate, under resting conditions, carotid vascular tone and blood flow. Guinea-pigs were used in the present study as in this species, the $NK_1$ and $CGRP_1$ receptor subtypes have been identified as those responsible for the cranial vasodilatation mediated by substance P or CGRP respectively (Jansen *et al.*, 1991; Nilsson *et al.*, 1992; Beattie *et al.*, 1993).

## Methods

Adult male Dunkin-Hartley guinea-pigs (300–450 g) were anaesthetized (i.p.) with ketamine (50 mg $kg^{-1}$) and pentobarbitone (25 mg $kg^{-1}$). The trachea was cannulated and animals artifically respired (60 strokes $min^{-1}$, 12 ml $kg^{-1}$) with room air supplemented by oxygen. The right carotid artery and jugular vein were cannulated to permit, respectively, the continuous measurement of arterial blood pressure and administration of drugs. Arterial blood flow was recorded by a Doppler flow probe (Bioengineering Department, University of Iowa, model 545C-4), placed around the left common carotid artery. Flow was recorded as a d.c. voltage (0.5 V per kHz Doppler shift). Carotid vascular resistance, equivalent to the mean arterial blood pressure divided by the carotid blood flow was monitored continuously by passing the pressure and flow signals to a peripheral resistance meter (Bioengineering Department, Glaxo Research and Development Ltd). The peak changes in blood pressure, carotid blood flow and vascular resistance were recorded. Anaesthesia was maintained by the infusion of pentobarbitone (15 mg $kg^{-1}$ $h^{-1}$, i.p.). Rectal temperature was monitored with a thermistor (CFP 8185) and maintained at 37–38°C with a heated blanket.

Guinea-pigs were placed in a stereotaxic frame (David Kopf) and a longitudinal incision made in the scalp. Two burr holes were drilled in the skull and a bipolar stimulating electrode (Rhodes NE-200) was lowered by a micromanipulator into each trigeminal ganglion, 0.37 cm dorsal to bregma, ± 0.45 cm lateral from the midline and 1.05 cm below the dural surface. Electrode placements in the trigeminal ganglia were confirmed visually at the end of each experiment; the results described below are only those from animals in which the electrodes were located in the trigeminal ganglia. After lowering the electrodes into the trigeminal ganglia, 30 min were allowed to lapse to achieve stable resting levels in blood pressure, carotid arterial flow and vascular resistance before stimulation. In most experiments, the trigeminal ganglion ipsilateral to the carotid artery from which blood flow measurements were made, was subjected to two periods of electrical stimulation (0.5–1.5 mA, 1 ms, 5 Hz, 60 s), separated by 1 h. The first stimulation period served as a control for the second. In one group of experiments, the contralateral trigeminal ganglion was stimulated (1.0 mA, 1 ms, 5 Hz, 60 s). Drugs were dissolved in saline (0.9% w:v) immediately before use and administered (0.5 ml $kg^{-1}$, i.v.) approximately 5 min prior to the second stimulation period. Drug vehicle was injected as control, in a separate group of animals.

### *Drugs and solutions*

The following drugs were used in this study: human α calcitonin gene-related peptide (CGRP), human α $CGRP_{8-37}$, [*p*-Cl-D-Phe$^6$,Leu$^{17}$]-porcine VIP (Bachem UK Ltd), guanethidine monosulphate (Sigma), hexamethonium bromide (Sigma) and substance P methyl ester (SPOMe; Cambridge Research Biochemicals Ltd). GR82334 ([D-Pro$^9$[Spiro-γ-Lactam]Leu$^{10}$,Trp$^{11}$]physalaemin(1–11) and CP-99,994 (2S,3S)-3-(2-methoxybenzylamino)-2-phenylpiperidine) were synthesized in the Medicinal Chemistry Department, Glaxo Research and Development Ltd, Greenford. Peptides were dissolved in acetic acid (0.1 mM) and stock solutions (1 or 10 mM) stored in aliquots at − 20°C until needed. Solutions were diluted to the final concentrations in saline (0.9% w:v).

### *Statistical analysis*

The effects of trigeminal ganglion stimulation on mean arterial blood pressure, carotid arterial blood flow and carotid vascular resistance were expressed as a percentage change from pre-stimulation levels. Values shown are means ± s.e.mean and *n* values quoted refer to the number of animals used. Comparisons between drug treatments were made by Student's *t* test for paired or unpaired samples, as appropriate, and a '*P*' value of less than 0.05 taken to indicate a significant difference between treatments.

## Results

Guinea-pigs had a mean resting arterial blood pressure of 49.6 ± 2.1 mmHg ($n = 23$) and left carotid arterial blood flow of 12.0 ± 1.3 ml $min^{-1}$ ($n = 20$). Electrical stimulation (0.5 mA, 1.0 ms, 5 Hz, 60 s) of the left trigeminal ganglion produced reductions in arterial blood pressure and left carotid vascular resistance with no consistent alteration in left carotid blood flow (changes from resting levels of − 29.8 ± 3.5, − 13.4 ± 3.9 and 3.2 ± 14.1 (each $n = 7$) respectively). A second period of stimulation, 1 h after the first, produced similar effects, both qualitatively and quantitatively, on blood pressure, carotid arterial blood flow and vascular resistance (changes from resting levels of − 34.4 ± 4.2, − 4.6 ± 11.6 and − 10.1 ± 4.5 (each $n = 7$) respectively). Stimulation, however, failed to evoke any response when the electrodes were placed outside the trigeminal ganglion.

Resting blood pressure and carotid blood flow were not significantly different in guinea-pigs pretreated with guanethidine (30 mg $kg^{-1}$, s.c.) compared to untreated animals. However, trigeminal ganglion stimulation (0.5–1.5 mA, 1.0 ms, 5 Hz, 60 s) in the pretreated animals produced little effect on blood pressure, but caused a consistent increase in carotid arterial blood flow and decrease in carotid vascular resistance (Figure 1). These effects were again reproducible when 1 h was left between stimulation periods (Table 1). The magnitude of the changes in carotid blood flow and vascular resistance evoked by trigeminal ganglion stimulation increased with increasing stimulation amplitude (Figure 1). Stimula-

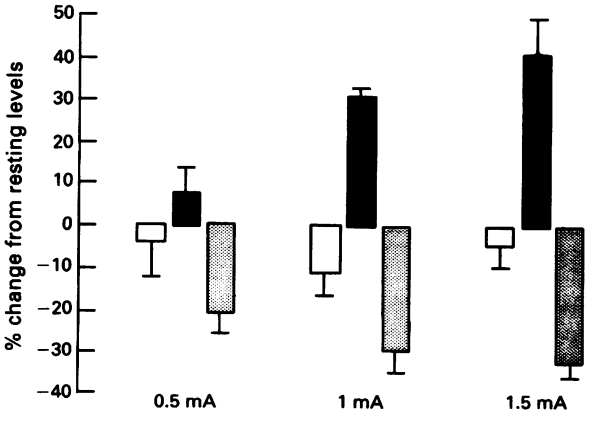


**Figure 1** The effect of trigeminal ganglion stimulation (0.5, 1.0 and 1.5 mA, 1 ms, 5 Hz, 60 s) on mean arterial blood pressure (open columns), carotid blood flow (solid columns) and vascular resistance (cross-hatched columns) in guinea-pigs pretreated with guanethidine (30 mg $kg^{-1}$, s.c.). Results are expressed as a percentage change from resting levels ($n = 5$).

**Table 1** Effects of two periods of trigeminal ganglion stimulation, separated by 1 h, on blood pressure and carotid blood flow and vascular resistance in guanethidine-pretreated guinea-pigs

| | *1st Stimulation* (1.0 ms, 5 Hz, 60 s) | | | *2nd Stimulation* (1.0 ms, 5 Hz, 60 s) | | |
|---|---|---|---|---|---|---|
| | *0.5 mA* | *1.0 mA* | *1.5 mA* | *0.5 mA* | *1.0 mA* | *1.5 mA* |
| % change in blood pressure | − 3.7 ± 8.2 | − 10.9 ± 4.8 | − 4.4 ± 5.0 | − 3.0 ± 8.8 | − 10.9 ± 4.1 | − 10.5 ± 4.8 |
| % change in carotid blood flow | 7.3 ± 6.8 | 30.6 ± 1.5 | 40.9 ± 8.4 | 7.1 ± 5.8 | 24.3 ± 9.9 | 34.8 ± 6.7 |
| % change in carotid vascular resistance | − 20.4 ± 5.1 | − 29.5 ± 5.1 | − 31.9 ± 3.5 | − 17.2 ± 8.6 | − 26.6 ± 4.2 | − 33.4 ± 5.1 |

$n = 4-6$.

**Table 2** Changes in blood pressure, carotid blood flow and vascular resistance evoked by trigeminal ganglion stimulation before (I) and following (II) administration of peptide receptor antagonists.

| *Drug treatment* | *% change in blood pressure* | | *% change in carotid blood flow* | | *% change in carotid vascular resistance* | |
|---|---|---|---|---|---|---|
| | I | II | I | II | I | II |
| Saline vehicle (0.5 mg kg$^{-1}$, i.v.) | − 1.7 ± 7.9 | − 7.9 ± 5.3 | 32.2 ± 11.6 | 26.9 ± 8.4 | − 27.8 ± 3.4 | − 26.6 ± 4.2 |
| Hexamethonium (10 mg kg$^{-1}$, i.v.) | 3.2 ± 4.4 | − 2.1 ± 0.7 | 25.4 ± 2.3 | 26.2 ± 5.2 | − 18.9 ± 3.8 | − 22.0 ± 3.6 |
| [*p*-Cl-D-Phe$^6$,Leu$^{17}$]-VIP (50 μg kg$^{-1}$) | − 3.2 ± 3.1 | − 6.2 ± 2.2 | 30.0 ± 9.3 | 9.7 ± 6.5* | − 24.8 ± 4.6 | − 11.0 ± 3.7* |
| $CGRP_{8-37}$ (0.9 mg kg$^{-1}$) | − 5.6 ± 7.4 | − 12.6 ± 3.9 | 26.9 ± 8.5 | 19.7 ± 10.5 | − 20.8 ± 2.4 | − 20.1 ± 4.8 |
| GR82334 (0.3 mg kg$^{-1}$) | 3.7 ± 5.8 | − 3.0 ± 6.6 | 37.6 ± 9.8 | 34.7 ± 17.3 | − 23.6 ± 3.7 | − 23.2 ± 9.7 |
| CP-99,994 (0.4 mg kg$^{-1}$) | − 2.2 ± 3.1 | − 6.4 ± 0.5 | 29.3 ± 9.4 | 29.8 ± 1.0 | − 23.9 ± 3.1 | − 27.0 ± 0.9 |

$n = 4-6$.
*$P < 0.05$ compared to the first stimulation.

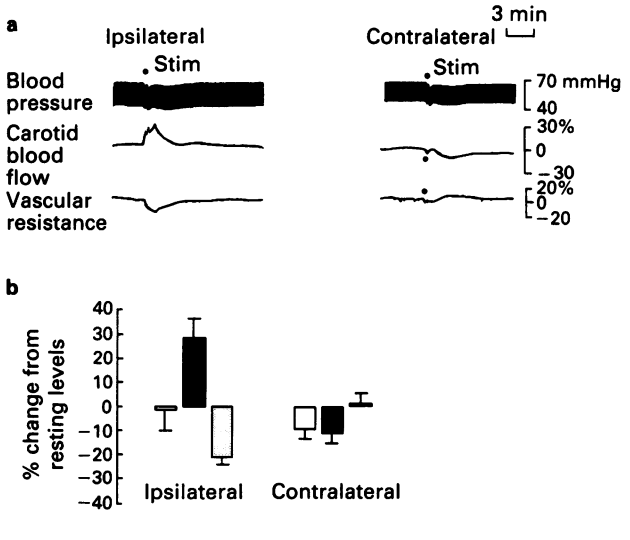


**Figure 2** (a) Responses to stimulation (1 mA, 1 ms, 5 Hz, 60 s) of the trigeminal ganglion, ipsilateral and contralateral to the carotid artery from which flow was measured in guinea-pigs pretreated with guanethidine (30 mg kg$^{-1}$, s.c.). (b) A comparison of the effect of electrical stimulation (1 mA) of the ipsilateral and contralateral trigeminal ganglia on blood pressure (open columns), carotid blood flow (solid columns) and vascular resistance (cross-hatched columns), alterations expressed as a percentage change from resting levels ($n = 5$).

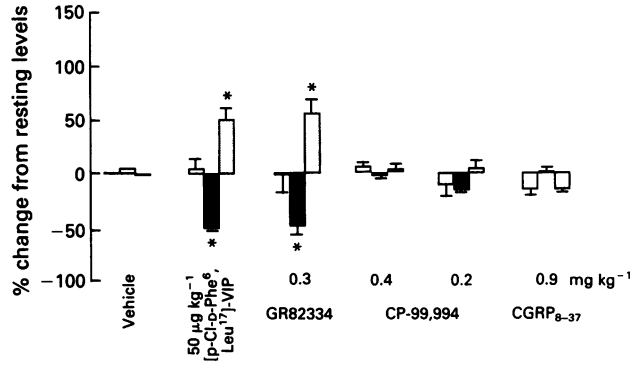


**Figure 3** The effects of vehicle (0.5 ml kg$^{-1}$), [*p*-Cl-D-Phe$^6$,Leu$^{17}$]-VIP (50 μg kg$^{-1}$), the $NK_1$ receptor antagonists, GR82334 (0.3 mg kg$^{-1}$) and CP-99,994 (0.4 and 2.2 mg kg$^{-1}$), and $CGRP_{8-37}$ (0.9 mg kg$^{-1}$) following i.v. administration, on blood pressure (open columns), carotid blood flow (solid columns) and carotid vascular resistance (cross-hatched columns) in anaesthetized guinea-pigs. [*p*-Cl-D-Phe$^6$,Leu$^{17}$]-VIP and GR82334, but not CP-99,994 significantly (*$P < 0.05$ compared to vehicle) increased vascular resistance and decreased flow ($n = 3-6$).

tion (1 mA), however, of the trigeminal ganglion contralateral to the carotid artery from which blood flow was measured, failed either to increase carotid blood flow or decrease vascular resistance in guanethidine-pretreated guinea-pigs (Figure 2). In all further studies, animals were pretreated with guanethidine (30 mg kg$^{-1}$, s.c.) 24 h prior to experiments.

Injection of saline vehicle (0.5 ml kg$^{-1}$, i.v.) 5 min before the second period of stimulation had no effect on mean arterial blood pressure, carotid blood flow or carotid vascular resistance in its own right (Figure 3), nor on respones evoked by trigeminal ganglion stimulation (Table 2). The ganglion blocking agent, hexamethonium (10 mg kg$^{-1}$, i.v.) on its own produced transient reductions, of 23.9 ± 0.8 and 33.5 ± 17% ($n = 4$), in blood pressure and carotid blood flow respectively. Carotid vascular resistance was little affected (an alteration of 3.6 ± 13.0% ($n = 4$) from the pre-injection level).

Responses to trigeminal ganglion stimulation (1 mA), 5 min later, when blood pressure and carotid blood flow had returned to basal levels, were not however significantly affected by hexamethonium (Table 2). The VIP receptor antagonist, [*p*-Cl-D-Phe$^6$,Leu$^{17}$]-VIP (50 $\mu$g kg$^{-1}$, i.v.) had no significant effect on basal blood pressure (an alteration of 3.9 ± 9.3% ($n$ = 6) from the resting level). However, carotid arterial blood flow was decreased and vascular resistance increased in all animals by [*p*-Cl-D-Phe$^6$,Leu$^{17}$]-VIP (50 $\mu$g kg$^{-1}$) (peak changes from pre-injection levels of − 47.5 ± 4.5 and 48.7 ± 13.1% respectively ($n$ = 6); Figure 3). When these effects had plateaued (approximately 5 min), the trigeminal ganglion was stimulated. The stimulation-induced alterations in carotid flow and vascular resistance, following [*p*-Cl-D-Phe$^6$,Leu$^{17}$]-VIP (50 $\mu$g kg$^{-1}$, i.v.) administration, were significantly ($P <$ 0.05) attenuated (Table 2).

The CGRP receptor antagonist, CGRP$_{8-37}$ (0.9 mg kg$^{-1}$, i.v.) reduced transiently (duration <2 min) blood pressure and carotid vascular resistance, but had no effect on carotid blood flow; the peak changes from resting levels were − 14.5 ± 5.8, − 12.9 ± 4.7 and 1.7 ± 3.6% respectively ($n$ = 6; Figure 3). Responses evoked by trigeminal ganglion stimulation, however, when blood pressure and vascular resistance had returned to basal levels, were not significantly affected by CGRP$_{8-37}$ (Table 2). The NK$_1$ receptor antagonist, GR82334 (0.3 mg kg$^{-1}$, i.v.), but not CP-99,994 (0.4 and 2.2 mg kg$^{-1}$, i.v.), produced a marked and longlasting (in excess of 30 min) decrease in carotid blood flow and an accompanying increase in vascular resistance (Figure 3). Despite the alterations in carotid blood flow and vascular resistance, responses to trigeminal ganglion stimulation were unaffected by either GR82334 (0.3 mg kg$^{-1}$) or CP-99,994 (0.4 mg kg$^{-1}$) (Table 2).

## Discussion

The trigeminal ganglion provides the principal sensory innervation of cranial blood vessels and is implicated in the regulation of blood flow in both cerebral and dural blood vessels (McCulloch *et al.*, 1986; Suzuki *et al.*, 1990; Hardebo *et al.*, 1991). The neurotransmitter(s) which may be responsible include substance P and CGRP, released antidromically from trigeminal nerve terminals, and VIP, released via a centrally-mediated parasympathetic reflex (Goadsby & Macdonald, 1985; Suzuki *et al.*, 1990). Each peptide is localized in nerve terminals innervating cranial blood vessels and their release and vasodilator activity have been demonstrated following trigeminal nerve activation (Edvinsson *et al.*, 1983; Goadsby & Shelley, 1990; Skofitsch & Jacobowitz, 1985; Buzzi *et al.*, 1991). The present study investigated the influence of the trigeminal ganglion on carotid blood flow in anaesthetized guinea-pigs by monitoring stimulation-induced changes in carotid arterial blood flow and vascular resistance and attempted to identify the neurotransmitters involved in this response by the use of selective antagonists.

Trigeminal ganglion stimulation reduced arterial blood pressure in anaesthetized guinea-pigs, a response similar to that observed in the cat (Lambert *et al.*, 1984) and monkey (Goadsby *et al.*, 1986), but contrasting with the pressor response reported in the rat (Spokes & Middlefell, 1993). Pretreatment of guinea-pigs with guanethidine significantly attenuated the hypotensive response suggesting that trigeminal ganglion stimulation in untreated animals may inhibit a sympathetic vascular tone. Moreover, only in guanethidine-pretreated guinea-pigs was trigeminal ganglion stimulation observed to increase carotid arterial blood flow. This enhancement of carotid blood flow occurred only following stimulation of the trigeminal ganglion ipsilateral to the carotid artery from which flow was measured, and is consistent with a localized dilatation of the cranial vasculature; stimulation of the contralateral ganglion resulted in small reductions in carotid arterial flow and blood pressure, but had no effect on carotid vascular resistance.

An aim of the study was to determine which neurotransmitter was responsible for the increase in carotid arterial blood flow and decrease in carotid vascular resistance evoked by trigeminal ganglion stimulation. The VIP receptor antagonist, [p-Cl-D-Phe$^6$,Leu$^{17}$]-VIP (Pandol *et al.*, 1986), significantly inhibited responses evoked by trigeminal nerve activation at a dose similar to that which inhibits VIP-induced coronary vasodilatation in the dog (Quebbemann *et al.*, 1991). The inhibition of the evoked responses in this study is unlikely to be due to physiological antagonism as the NK$_1$ receptor antagonist, GR82334, had no inhibitory action, despite having a similar effect to [*p*-Cl-D-Phe$^6$,Leu$^{17}$]-VIP on basal carotid blood flow and vascular resistance. The ganglion blocking agent, hexamethonium, at a dose (10 mg kg$^{-1}$, i.v.) which prevents the effects of trigeminal ganglion stimulation on carotid blood flow in cats (Lambert *et al.*, 1984), failed to influence the electrically-evoked responses implying that in the guinea-pig, ganglionic transmission is not involved in this process. The results suggest, therefore, that while VIP is responsible for the increased carotid blood flow produced by trigeminal ganglion stimulation in the guinea-pig, as in the cat (Goadsby & Macdonald, 1985), the nerve pathways may differ in the two species. It is possible that in the guinea-pig, VIP is released directly from sensory afferent trigeminal nerve terminals in the cranial vasculature, although to our knowledge there is no immunohistochemical evidence to suggest this; VIP-like immunoreactivity appears to be localised exclusively in parasympathetic efferent nerve fibres (Hara *et al.*, 1985; Uemura *et al.*, 1988). The precise mechanism underlying the VIP-induced increase in carotid blood flow requires further investigation.

The inability of CGRP$_{8-37}$ (0.9 mg kg$^{-1}$, i.v.), GR82334 (0.3 mg kg$^{-1}$, i.v.) and CP-99,994 (0.4 mg kg$^{-1}$, i.v.) (Chiba *et al.*, 1989; Hagan *et al.*, 1991; Desai *et al.*, 1992) to inhibit the changes in carotid blood flow and vascular resistance evoked by trigeminal ganglion stimulation suggests that neither CGRP$_1$ nor tachykinin NK$_1$ receptors mediate these responses. CGRP$_{8-37}$, at similar doses to that used in the present study (0.9 mg kg$^{-1}$), blocks cardiovascular effects of CGRP in conscious rats (Gardiner *et al.*, 1991) and increases in rat hindlimb blood flow evoked by saphenous nerve stimulation (Delay-Goyet *et al.*, 1992). Moreover, in the present study, in a different group of guinea-pigs, vasodepressor responses to CGRP (0.4 $\mu$g kg$^{-1}$, i.v.) were abolished by CGRP$_{8-37}$ (0.9 mg kg$^{-1}$, i.v.). Responses to trigeminal ganglion stimulation were also unaffected by doses of GR82334 (0.3 mg kg$^{-1}$) and CP-99,994 (0.4 mg kg$^{-1}$) which antagonized the vasodepressor activity of the tachykinin NK$_1$ receptor agonist, SPOMe (40 ng kg$^{-1}$, i.v.). While it is unlikely that either CGRP$_1$ or NK$_1$ receptors mediated the observed effects on carotid blood flow and vascular resistance in this study, the method used may not have been sensitive enough to detect any involvement of the peptides in discrete vascular beds, such as the cerebral or dural vasculature. Indeed, CGRP$_{8-37}$ has been shown to attenuate the increase in cerebral blood flow in cats following nasociliary nerve stimulation (Goadsby, 1993).

While the putative CGRP$_1$ receptor antagonist, CGRP$_{8-37}$, had only small effects on blood pressure, carotid blood flow or vascular resistance in its own right, the NK$_1$ antagonist, GR82334, and the VIP receptor antagonist, [*p*-Cl-D-Phe$^6$, Leu$^{17}$]-VIP, had profound effects on these parameters. Carotid blood flow was reduced and vascular resistance increased by both agents, effects consistent with antagonism of a resting NK$_1$ and VIP-regulated tone in the cranial circulation. While there may indeed be a regulatory role for VIP, the absence of any effect with CP-99,994 argues against involvement of NK$_1$ receptors. Although the NK$_1$ receptor antagonist, GR82334, has little affinity for other peptide receptors, such as those activated by neurokinins A and B, cholecystokinin, bradykinin or bombesin (Hagan, personal communication), another, as yet unidentified, mechanism would seem to

be responsible for its effects on carotid blood flow. This merits further investigation.

The results from this study provide evidence that the trigeminal ganglion is involved in the regulation of cranial blood flow in guinea-pigs, as in other species. The principal neurotransmitter mediating this process is likely to be VIP, rather than CGRP or substance P. It remains possible, however, that CGRP or substance P regulate blood flow in discrete regions of the carotid vascular bed, but that the changes in flow which they produce, represent only a small fraction of the blood flowing in the carotid artery.

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# The involvement of endothelial dysfunction, nitric oxide and prostanoids in the rat gastric microcirculatory responses to endothelin-1

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**1** The role of endothelial dysfunction in the gastric microcirculatory responses during local endothelin-1 (ET-1) infusion has been investigated in the pentobarbitone-anaesthetized rat. Furthermore, the involvement of prostanoids or nitric oxide (NO) in these actions has been investigated by the use of indomethacin to inhibit cyclo-oxygenase and $N^G$-nitro-L-arginine methyl ester (L-NAME) to inhibit NO synthase.

**2** Close-arterial infusion of ET-1 (1–10 pmol $kg^{-1}$ $min^{-1}$ for 10 min) induced a dose-dependent increase in the gastric leakage of radiolabelled albumin, used as an index of endothelial cell dysfunction.

**3** Close-arterial infusion of a submaximal dose of ET-1 (5 pmol $kg^{-1}$ $min^{-1}$ for 10 min) significantly increased gastric albumin leakage after 2 min infusion, which reached maximal levels after 10 min, and only slowly declined during the 30 min observation period.

**4** By contrast, gastric blood flow, as assessed by laser Doppler flowmetry, did not significantly increase until after 5 min of infusion of ET-1 (5 pmol $kg^{-1}$ $min^{-1}$ for 10 min), reaching a maximum after 17 min, and was sustained for the 30 min observation period.

**5** Pretreatment with L-NAME (2 mg $kg^{-1}$, i.v.) or indomethacin (5 mg $kg^{-1}$, i.v.) significantly reduced both the hyperaemic response to ET-1 and the increase in gastric albumin leakage, and in combination abolished these responses.

**6** These results suggest that locally released NO and prostanoids mediate the gastric vasodilator response to close arterial infusion of ET-1. This hyperaemia is preceded by changes in gastric albumin extravasation and hence may be initiated as a response to direct endothelial injury by ET-1.

**Keywords:** Vascular permeability; gastric blood flow; endothelin-1; nitric oxide; prostaglandins

## Introduction

Close-arterial infusion of picomole quantities of endothelin-1 (ET-1, Yanagisawa *et al.*, 1988a,b; Inoue *et al.*, 1989) induces extensive haemorrhagic injury to the gastric mucosa of the anaesthetized rat (Whittle & Esplugues, 1988; Whittle & Lopez-Belmonte, 1991). Furthermore, intravenous infusion of ET-1 can significantly enhance the gastric mucosal injury induced by topical application of ethanol or acid in the rat (Wallace *et al.*, 1989; MacNaughton *et al.*, 1989). These effects of ET-1 were thought to be the result of its potent vasoconstrictor activity, since other vasoconstrictor agents are also known to cause gastric injury (see Whittle, 1993). Indeed, ET-1 can induce vasoconstriction in the rat stomach both *in vitro* and *in vivo* (Wallace *et al.*, 1989; Lopez-Belmonte & Whittle, 1993).

However, ET-1 can also cause regional vasodilator responses following intravenous administration, (Wright & Fozard, 1988; Hoffman *et al.*, 1989; Gardiner *et al.*, 1990). Furthermore, close-arterial infusion of low doses of ET-1, which induce extensive gastric mucosal damage, only produce a significant and prolonged gastric mucosal hyperaemia (Lopez-Belmonte & Whittle, 1993).

In the present study, the possible role of endothelial cell dysfunction and injury, as assessed by changes in microvascular permeability to radiolabelled albumin, in the gastric microcirculatory responses to local infusion of ET-1 has now been investigated. Thus, temporal changes in albumin leakage have been correlated with changes in gastric blood flow, as determined by laser Doppler flowmetry in the anaesthetized rat. Furthermore, the involvement of endogenous prostanoids and nitric oxide in these actions has been assessed by the use of selective inhibitors of their synthesis, indomethacin and $N^G$-nitro-L-arginine methyl ester (L-NAME), respectively.

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## Methods

### *Measurement of gastric blood flow*

Male Wistar rats (230–250 g body weight) were deprived of food but not water for 18–20 h prior to the experiment. The animals were anaesthetized with sodium pentobarbitone (60 mg $kg^{-1}$, i.p.) and the stomach exposed by a midline incision. The left gastric artery was isolated and cannulated with a 24 g teflon cannula under a stereomicroscope (Esplugues *et al.*, 1989). A plastic cannula (8.5 mm diameter) was inserted in the forestomach and tied in place to allow free access to the gastric lumen for the measurement of blood flow by laser Doppler flowmetry (LDF), as described previously (Tepperman & Whittle, 1992; Lopez-Belmonte & Whittle, 1993). A teflon-coated laser optic probe (Periflux PF308, standard probe, 0.25 mm fibre separation) was inserted into the gastric lumen via the forestomach cannula and allowed to rest on the corpus mucosa. A resting period of 20–30 min was allowed after surgery to allow gastric blood flow to reach stable values. Gastric blood flow was then recorded continuously with a laser Doppler flow monitor (Perimed PF3, helium-neon laser of wavelength 632.8 nm).

### *Time-course of ET-1 induced gastric hyperaemia*

Endothelin-1 (5 pmol $kg^{-1}$ $min^{-1}$) or its vehicle (0.1% solution of bovine serum albumin (BSA) in saline) were infused

close-arterially (13 μl $min^{-1}$) for a period of 10 min. Average LDF values were determined for a 3 min period prior to ET-1 infusion which was used as the control resting value (measured in arbitrary perfusion units). LDF was then determined at different time-points during and after ET-1 infusion (2, 5, 10 and 30 min), and expressed as changes in LDF expressed as % of the control resting value.

### *Measurement of gastric vascular leakage*

Gastric vascular leakage was determined as the extravasation of $^{125}$I-labelled human serum albumin [$^{125}$I]-HSA, 2 μCi $kg^{-1}$, 1 ml $kg^{-1}$, i.v.) injected 10 min prior to ET-1 infusion (1–10 pmol $kg^{-1}$ $min^{-1}$). The [$^{125}$I]-HSA content in the gastric tissue and in plasma prepared from blood withdrawn from the abdominal aorta (1 ml, centrifuged for 10 min at 12,000 r.p.m. 4°C), was measured in a gamma spectrometer (Nuclear Enterprises, NE 1600). The stomachs were cut open along the greater curvature, blotted dry and weighed.

Gastric blood volume was measured in a separate group of animals ($n = 4$ in each group) for each dose and time, by the administration of the [$^{125}$I]-HSA, 2 min before removing the tissue. The radioactive content of the stomachs was then assessed as described above and the difference between the total albumin and the blood volume content was expressed as plasma leakage, μl $g^{-1}$ tissue. Increases in vascular leakage following infusion of ET-1 were calculated as the change from the corresponding control value following local intra-arterial infusion of the vehicle, and expressed as Δμl plasma $g^{-1}$ tissue.

### *Time-course of ET-1-induced albumin leakage*

Endothelin-1 (5 pmol $kg^{-1}$ $min^{-1}$) or its vehicle (0.1% BSA in saline) were infused close-arterially for 2, 5 and 10 min in separate groups of animals and vascular albumin leakage measured. In a further group, albumin leakage was determined for a further 20 min following termination of a 10 min infusion of ET-1.

### *Effect of L-NAME and indomethacin pretreatment*

In these experiments the effects of inhibitors of NO synthase and cyclo-oxygenase on the gastric hyperaemia and albumin leakage induced by ET-1 (5 pmol $kg^{-1}$ $min^{-1}$; 10 min) were investigated. L-NAME (2 mg $kg^{-1}$, i.v.) or indomethacin (5 mg $kg^{-1}$, i.v.) were administered 10 and 15 min prior to ET-1 infusion respectively, in doses taken from previous studies (Whittle *et al.*, 1990; 1992). In a further set of experiments, L-NAME (2 mg $kg^{-1}$) and indomethacin (5 mg $kg^{-1}$) were administered concurrently, 15 min prior to ET-1 infusion.

The effects of higher doses of L-NAME on the ET-1 responses were not investigated since these would reduce resting gastric blood flow (Tepperman & Whittle, 1992; Whittle *et al.*, 1992) and hence make interpretation of the findings more difficult. The dose of indomethacin selected was sufficient to inhibit gastric prostanoid biosynthesis by over 90% (Whittle *et al.*, 1990).

### *Materials*

Endothelin-1 (Human-porcine, Peninsula Laboratories, St. Helens, Merseyside) was dissolved in isotonic saline and kept frozen (– 20°C) in aliquots. Samples were freshly diluted when required in a 0.1% solution of bovine serum albumin in saline. Indomethacin (Sigma Chemical Company, Poole, Dorset) was dissolved in 5% sodium bicarbonate solution and diluted with distilled water prior to injection. L-NAME (Sigma Chemical Co.) was made up freshly in isotonic saline.

$^{125}$I-labelled human serum albumin (92.5 kBq $mg^{-1}$ Amersham international) was prepared freshly each day from a stock in isotonic saline.

### *Statistical analysis*

All data are expressed as mean ± s.e.mean, where ($n$) is the number of animals. Comparisons between groups were made by Student's $t$ test for unpaired data or by One-Way Analysis of Variance (ANOVA) and Bonferroni test for multiple comparisons where appropriate. $P$ values of less than 0.05 were taken as significant.

## Results

### *Effect of ET-1 on gastric vascular leakage*

Resting plasma leakage following the local 10 min infusion of the vehicle (0.1% BSA in saline) was 19 ± 3, 21 ± 4, 15 ± 2 and 24 ± 4 μl $g^{-1}$ tissue ($n = 6$) at 2, 5, 10 and 30 min after the start of the infusion. Local intra-arterial infusion of ET-1 (1–10 pmol $kg^{-1}$ $min^{-1}$) for 10 min induced a dose-dependent increase in the gastric extravasation of plasma, when determined immediately after termination of ET-1 infusion (Figure 1). A near-maximal effect was observed at a dose of ET-1 of 5 pmol $kg^{-1}$ $min^{-1}$ (Δ26 ± 4 μl plasma $g^{-1}$ tissue from control values) which was therefore used for all subsequent experiments.

Local intra-arterial infusion of ET-1 (5 pmol $kg^{-1}$ $min^{-1}$) for 10 min induced a time-dependent increase in gastric vascular leakage (Figure 2). Thus, a significant increase in albumin leakage from the level observed with vehicle alone could be detected after 2 min of ET-1 infusion. This effect reached a plateau at 5 min of infusion and then increased again after 10 min of the infusion. This increase in vascular leakage showed only a slow decline over the next 20 min after termination of ET-1 infusion, and the value at this time was not significantly different from the value after 10 min of infusion (Figure 2).

### *Time-course of ET-1 induced gastric hyperaemia*

Resting values for LDF did not significantly change from the initial control value throughout the experimental period, during or following the 10 min local intra-arterial infusion of the vehicle, being 1 ± 1%, 3 ± 2%, 3 ± 2% and 1 ± 3% of basal ($n = 8$), at 2, 5, 10 and 30 min from the start of the infusion. Local intra-arterial infusion of ET-1 (5 pmol $kg^{-1}$ $min^{-1}$) for 10 min, however, induced a time-dependent increase in gastric LDF (Figure 2). LDF did not increase until 5 min of the ET-1 infusion, after which a significant hyperaemic response was observed, reaching a maximal value after 17 min (93 ± 8% increase from basal; $n = 13$ $P < 0.01$). Furthermore, this hyperaemic response was well maintained for the remainder

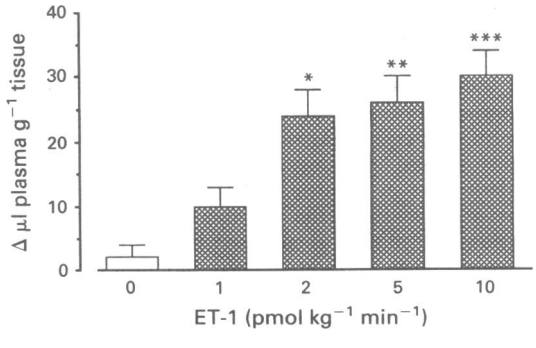


**Figure 1** Effect of close-arterial infusion of endothelin-1 (ET-1; 1–10 pmol $kg^{-1}$ $min^{-1}$; 10 min) on gastric vascular permeability to radiolabelled albumin in the anaesthetized rat. Results, expressed as plasma leakage, Δμl $g^{-1}$ tissue compared with control values (vehicle infusion), are shown as the mean ± s.e.mean of 5–8 experiments in each group. Significant difference from vehicle infusion is shown as $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

of the 30 min experimental period (Figure 2). Systemic arterial blood pressure (125 ± 9 mmHg, $n = 6$) was not significantly altered by close-arterial infusion of ET-1 (5 or 10 pmol $kg^{-1}$ $min^{-1}$; Δ13 ± 4 and Δ13 ± 6 mmHg, $n = 6$, respectively).

### *Effect of L-NAME and indomethacin on ET-1-induced hyperaemia*

Pretreatment with L-NAME (2 mg $kg^{-1}$, i.v.) significantly ($P < 0.05$) reduced the gastric hyperaemia observed after close-arterial ET-1 infusion (5 pmol $kg^{-1}$ $min^{-1}$) as shown in Figure 3. Similarly, pretreatment with indomethacin (5 mg $kg^{-1}$, i.v.) also attenuated the increase in LDF induced by ET-1 (43 ± 11% inhibition; $P < 0.01$; $n = 12$). Furthermore, the combination of both inhibitors abolished the ET-1 induced hyperaemia (Figure 3).

Neither L-NAME nor indomethacin had any significant effect on resting basal gastric LDF at the low doses used (− 10 ± 3%, $n = 7$ and − 10 ± 7% of basal, $n = 12$, respectively). Furthermore, the combination of L-NAME and indomethacin in these doses did not significantly affect resting LDF (− 10 ± 4%, $n = 7$).

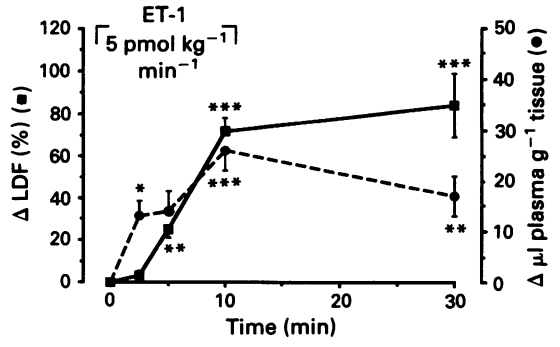


**Figure 2** Time-dependent effect of close-arterial infusion of endothelin-1 (ET-1; 5 pmol $kg^{-1}$ $min^{-1}$; 10 min) on gastric blood flow (as assessed by laser Doppler flowmetry, LDF) and vascular permeability to albumin. Changes in LDF are expressed as the % change from resting values (■), while changes in vascular permeability are shown as plasma leakage Δµl $g^{-1}$ tissue (●), compared with vehicle infusion values at corresponding times. Results are the mean ± s.e.mean of 6–8 experiments in each group. Significant difference from vehicle infusion at each time point is given as $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

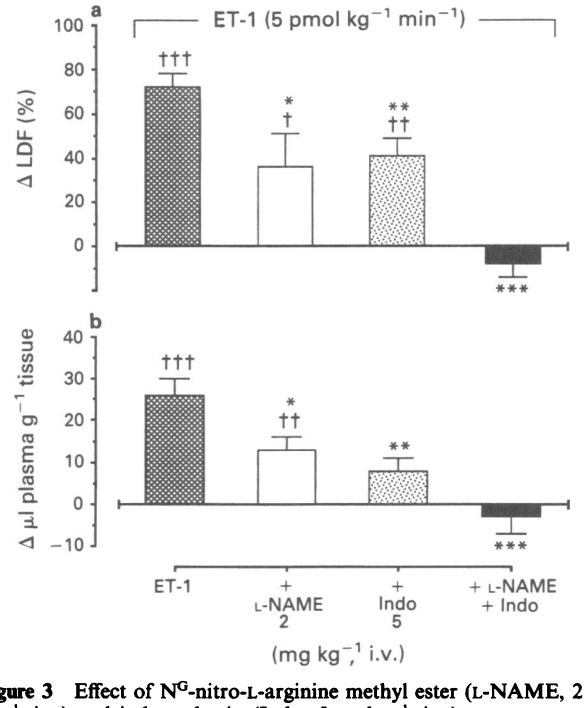


**Figure 3** Effect of $N^{G}$-nitro-L-arginine methyl ester (L-NAME, 2 mg $kg^{-1}$, i.v.) and indomethacin (Indo, 5 mg $kg^{-1}$, i.v.) pretreatment on the (a) gastric hyperaemic response (as assessed by laser Doppler flowmetry, LDF) and (b) extravasation of albumin induced by close-arterial infusion of endothelin-1 (ET-1; 5 pmol $kg^{-1}$ $min^{-1}$; 10 min). Results, shown as the change in LDF (% of resting values) or plasma leakage (Δµl $g^{-1}$ tissue) are the mean ± s.e.mean of 6–13 experiments in each group. Significant difference from vehicle infusion is given as: $^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$; $^{\dagger\dagger\dagger}P < 0.001$. Significant difference from ET-1 is given as: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

### *Effect of L-NAME and indomethacin on ET-1 induced albumin leakage*

Pretreatment with L-NAME (2 mg $kg^{-1}$, i.v.) significantly reduced the increase in gastric albumin leakage observed after a 10 min close-arterial infusion of ET-1 (5 pmol $kg^{-1}$ $min^{-1}$; 51 ± 10% inhibition, $P < 0.05$; $n = 7$). Similarly, pretreatment with indomethacin (5 mg $kg^{-1}$, i.v.) also significantly ($P < 0.01$) reduced the ET-1-induced increase in albumin leakage. Moreover, the combination of both inhibitors abolished the albumin leakage following ET-1 infusion (Figure 4).

In control experiments, administration of either L-NAME or indomethacin alone augmented gastric plasma leakage when administered either separately (by 11 ± 5 and by 10 ± 1 µl $g^{-1}$ tissue; $P < 0.05$; $n = 4$ respectively), or in combination (by 13 ± 3 µl $g^{-1}$ tissue; $P < 0.01$, $n = 4$).

## Discussion

Close intra-arterial infusion of ET-1 induced a dose-dependent increase in gastric blood flow and in gastric vascular leakage of radiolabelled albumin. Inhibition of the synthesis of prostanoids or nitric oxide attenuated both the hyperaemia and the increased vascular leakage induced by ET-1, suggesting the local release of these mediators play an important role in these microvascular responses.

Although endothelin-1 has been shown to be a potent vasoconstrictor agent, both *in vitro* and *in vivo* (Yanagisawa *et al.*, 1988a,b), ET-1 has also been observed to cause regional vasodilator responses. Thus in the rat, ET-1 has been demonstrated to reduce systemic arterial blood pressure and to induce vasodilatation in the vascular beds supplied by the carotid artery and the lower abdominal aorta (Wright & Fozard, 1988; De Nucci *et al.*, 1988; Hoffman *et al.*, 1989; Whittle *et al.*, 1989; Gardiner *et al.*, 1990). Likewise, following intracarotid administration of ET-1, an increase in cortical perfusion and a reduction in cerebrovascular resistance was observed at low doses of ET-1, although at higher doses, a reduction in cortical perfusion and an increase in cerebrovascular resistance was seen (Willette *et al.*, 1990). Similarly, in the intact pulmonary circulation, ET-1 at subnanomolar concentrations caused a transient vasodilatation, whereas higher concentrations induced vasoconstriction (Hasunuma *et al.*, 1990; Lippton *et al.*, 1991). In the gastric microvasculature of the anaesthetized rat, close-arterial infusion of increasing doses of ET-1 induced a biphasic response, consisting of a transient vasodilator response followed by a pronounced and sustained vasoconstriction. However, at lower doses of ET-1, only a vasodilator response could be observed (Lopez-Belmonte & Whittle, 1993), as confirmed in the present study.

ET-1 can induce the release of prostacyclin and NO (De Nucci *et al.*, 1988; Rakugi *et al.*, 1989; Warner *et al.*, 1989) which contribute to the vasodilator activity observed. Moreover, inhibition of NO can enhance the vasoconstrictor effect of ET-1 in the skin microvasculature (Lawrence & Brain, 1992) and rabbit kidney (Rogerson *et al.*, 1993). In the present study, pretreatment with either L-NAME or indome-

thacin significantly attenuated both the hyperaemia and the increase in vascular permeability observed after a 10 min close-arterial infusion of ET-1. Furthermore, when given in combination, L-NAME and indomethacin abolished both these vascular changes induced by ET-1. These results suggest that the synthesis and release of both NO and a prostanoid could account for the hyperaemic response induced by ET-1 in the gastric microvasculature.

Intradermal administration of ET-1 inhibits oedema formation induced by chemotactic agents in rabbit skin (Brain *et al.*, 1989; Chander *et al.*, 1989), an effect which was attributed to its potent constrictor activity in the microvasculature (Brain *et al.*, 1988; 1989). By contrast, intravenous administration of ET-1 has been shown to enhance protein extravasation in various organs of the rat including the stomach, changes that were independent of mean arterial blood pressure and were abolished by the $ET_A$ receptor antagonist, BQ-123 (Filep *et al.*, 1991; 1993). Similarly, in the present study close-arterial administration of picomole quantities of ET-1 induced a dose-dependent increase in gastric vascular leakage of radiolabelled albumin. This was not a consequence of systemic haemodynamic changes as ET-1 did not affect systemic arterial blood pressure and the observed increase in gastric blood flow during ET-1 infusion cannot account for the initial increase in albumin leakage, since these permeability changes occurred at an earlier time. Increased vascular permeability to protein is considered to reflect disruption of the endothelial barrier as a consequence of gap formation between these cells (Grega *et al.*, 1986) and hence reflect endothelial dysfunction and injury. However, the sustained increase in blood flow may subsequently enhance and maintain this increase in vascular albumin flux, and indeed a plateau increase in albumin leakage during ET-1 infusion was followed by a secondary increase which coincided with the initiation of the increase in blood flow. Therefore, it is feasible that there is an initial increase in vascular permeability which takes place in the first 2 min of ET-1 infusion and is the result of endothelial injury, followed by a secondary increase which is due to a prolonged increase in gastric blood flow promoting the leakage of albumin through an already damaged tissue.

ET-1 did not affect transendothelial [$^{125}$I]-HSA flux in endothelial cell monolayers (Horgan *et al.*, 1991; Rodman *et al.*, 1992), although it did induce a dose-dependent release of $^{51}$Cr from human cultured brain microvascular endothelium (Stanimirovic *et al.*, 1993). However, following its administration or release *in vivo*, ET-1 may induce vascular dysfunction by the release of secondary mediators, such as PAF, prostaglandins or thromboxanes. Indeed, it has been shown that PAF-antagonists, indomethacin, a thromboxane synthase inhibitor or a thromboxane/endoperoxide antagonist, can substantially inhibit protein extravasation induced by ET-1 in rats (Filep *et al.*, 1991; Sirois *et al.*, 1992). In addition, the gastric mucosal injury following bolus intra-arterial administration of ET-1 can be attenuated by a PAF-receptor antagonist (Kurose *et al.*, 1991), but an additional direct injurious action of ET-1 on the microvasculature cannot yet be excluded.

The reduction in albumin leakage following administration of the inhibitors of NO and prostanoid biosynthesis could occur, in part, indirectly as a consequence of reduction of the gastric hyperaemia by these agents. However, neither L-NAME nor indomethacin had any significant effect on resting blood flow at the low doses used. L-NAME itself significantly increased albumin leakage under control conditions which supports the suggestion that NO plays a role in the maintenance of gastro-intestinal microvascular integrity under these experimental conditions (Hutcheson *et al.*, 1990; Kubes & Granger, 1991). Furthermore, the comparable action of indomethacin supports the suggestion that both NO and a prostanoid can influence the integrity of gastric microvasculature (Whittle *et al.*, 1990), although no synergistic interaction could be observed in the present study.

The present findings indicate that changes in vascular permeability to albumin precede the increase in gastric blood flow observed in the rat gastric microcirculation induced by local intra-arterial infusion of ET-1. The local release of the vasodilators, NO and a prostanoid such as prostacyclin, which appear to mediate this hyperaemia, may thus be initiated as a response to endothelial injury induced by ET-1 in the gastric microvasculature.

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# Comparative effects of anti-platelet agents as adjuncts to tissue plasminogen activator in a dog model of occlusive coronary thrombosis

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**1** This study compares a cyclo-oxygenase inhibitor (aspirin), a 5-$HT_2$ antagonist (ZM170809) and a combined thromboxane synthase inhibitor/receptor antagonist (ZD1542) as adjuncts to tissue plasminogen activator (rt-PA).

**2** Application of an anodal current (332 ± 4.1 μA) to the stenosed left circumflex coronary artery of 20 anaesthetized dogs produced a stable platelet-rich occlusive thrombus.

**3** After initial i.v. administration of recombinant human tissue type plasminogen activator (rt-PA, 3 mg bolus + 2 mg $kg^{-1}$ $h^{-1}$ for 30 min) thrombolysis occurred in 15 out of 20 dogs. All 15 dogs reoccluded.

**4** The second i.v. administration of rt-PA in the presence of either aspirin, ZM170809, ZD1542 or saline resulted in thrombolysis in all 20 dogs.

**5** Both the combined thromboxane synthase inhibitor/receptor antagonist (ZD1542) and 5-$HT_2$ antagonist (ZM170809) significantly ($P<0.05$) reduced the time taken to lyse the thrombus compared with the saline group. The times were 14.4 ± 2.7 min, 18.0 ± 3.9 min and 36.8 ± 6.2 min for ZD1542, ZM170809 and saline respectively.

**6** Aspirin did not offer any additional benefit to using rt-PA alone. The times to thrombolysis were 36.8 ± 8.4 min for aspirin and 36.8 ± 6.2 min for the saline group.

**7** The number of dogs in which the circumflex coronary artery reoccluded within 60 min of terminating the second infusion of rt-PA were five for saline, four for aspirin, two for ZD1542 and two for ZM170809.

**8** These results indicate that both ZD1542 and ZM170809 are more effective adjuncts than aspirin in thombolysis and may provide an improvement in current clinical practice.

**Keywords:** Thrombolysis; platelet aggregation; cyclo-oxygenase; thromboxane; 5-$HT_2$; rt-PA

## Introduction

Treatment of coronary artery thrombosis with recombinant human tissue-type plasminogen activator (rt-PA) results in recanalization in between 50–80% of patients (van de Werf *et al.*, 1984; TIMI-1 trial, 1985). The limitations of coronary thrombolysis are the number of patients who fail to reperfuse (20–50%), and the high incidence of re-occlusion (20–30%) after the termination of rt-PA infusion. Early recanalization of the thrombosed vessel is important in the prevention of myocardial necrosis and patient survival (TIMI-1 Trial, 1985).

Jang *et al.* (1989) demonstrated that arterial thrombi consist of a high proportion of activated platelets; these platelet-rich thrombi are particularly resistant to thrombolytic therapy. Fitzgerald *et al.* (1989) have also shown that platelets play a pivotal role in the mechanism of failed or delayed reperfusion, an effect that is partly thromboxane $A_2$ ($TXA_2$) dependent. $TXA_2$ is the major cyclo-oxygenase metabolite of arachidonic acid produced by the platelet. It is a potent vasoconstrictor and platelet activator and also enhances platelet aggregation in response to various platelet agonists, including ADP, adrenaline, collagen and thrombin.

5-Hydroxytryptamine (5-HT) may also be involved in re-occlusion (Golino *et al.*, 1989). 5-HT is both plentiful in platelets and released upon platelet activation. Although 5-HT is a weak agonist for platelet activation, it enhances the activity of other platelet aggregating agents (De Clerck *et al.*, 1984).

Despite simultaneous administration of conventional anti-thrombotic agents, e.g. heparin and aspirin, early reocclusion following thrombolysis still occurs (ISIS-2, 1988). Consequently, there is a need for adjunctive therapy, to enhance the rate of thrombolysis and prevent reocclusion.

In the present study, we examined the effects of ZD1542 a combined $TXA_2$ synthetase inhibitor/TP-receptor antagonist (Brownrigg *et al.*, 1992), a cyclo-oxygenase inhibitor (aspirin) and ZM170809 a selective 5-$HT_2$ antagonist (Blackburn *et al.*, 1988; 1990) on the thrombolytic activity of rt-PA, in a dog model of acute coronary thrombosis.

## Methods

Male beagle dogs weighing between 15–18 kg were pretreated with morphine sulphate (10 mg, s.c.) and 30 min later were anaesthetized with pentobarbitone (induction 30 mg $kg^{-1}$, i.v.; maintenance 3 mg $kg^{-1}$, i.v. every 30 min) through a cannula inserted into the long saphenous vein under local anaesthesia. The animals were ventilated through a tracheotomy with a mixture of 40% oxygen in room air supplied by a Harvard animal ventilator. When the chest was opened a resistance to expiration was produced by placing the expiratory output of the pump under 3 cm of water. Samples of arterial blood were withdrawn at intervals throughout the experiment and pH, $PCO_2$, $PO_2$ were measured with a Ciba Corning blood gas analyzer (Halstead, Essex). End tidal $PCO_2$ was continuously monitored by aspirating expired air from the trachea into an infra-red $CO_2$ analyser (P.K. Mor-

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gan Ltd., Rainham, Kent). The arterial $P_{CO_2}$ was kept within the limits of 35–42 mmHg and the arterial pH between 7.35–7.41, either by adjustment of respiration or the intravenous injection of 1.0 M $NaHCO_3$ solution. Oesophageal temperature was recorded and maintained at 37°C by heating lamps above and a thermostatically controlled heating blanket below the animal. The ECG lead II was recorded and the signal used to drive a cardiotachometer. A catheter was inserted via the femoral vein into the inferior vena cava for the intravenous injection and infusion of drugs. Systemic arterial pressure was recorded through a cannula inserted into the femoral artery. All pressures were measured using strain guage manometers (Lectromed, Letchworth, Herts.) attached to d.c. bridge amplifiers. Blood flow to the left circumflex artery was measured with an ultra-sound flowmeter (Transonic Inc., Ithaca, New York, U.S.A.). The ECG, heart rate (HR), systemic arterial pressure (BP) and the mean and pulsatile coronary blood flow were recorded using a thermal array recorder (Gould TA4000, Valley View, Ohio, U.S.A.).

### *Surgical procedures*

The chest was opened in the fifth intercostal space, the left phrenic nerve divided and the pericardium opened to expose the left circumflex artery and vein. About 2 cm of the left circumflex artery was dissected free of surrounding tissue. The electrode was inserted into the lumen of the circumflex artery so that approximately 2–5 mm of the silver wire tip was within the vessel lumen and in contact with the intimal lining. The stimulating electrode was then fixed in place by the use of tissue adhesive. A stiff plastic cuff was placed over the tip of the wire to produce a fixed stenosis on the coronary artery of about 60%. A flow probe was placed proximal to the point of entry of the wire. An anodal current of approximately 350 μA was delivered to the stimulating electrode via a 9V nickel-cadium battery with the anode connected in series to a 250,000 ohm potentiometer. The cathode was placed subcutaneously completing the circuit (Figure 1). The current was discontinued 30 min after the formation of a persistent stable thrombus as indicated by zero coronary flow.

### *Morphological assessment*

In a separate series of experiments, the morphology of the occlusive thrombi formed after: (1) anodal stimulation (1st thrombus), (2) anodal stimulation and lytic failure (1st thrombus), (3) rt-PA infusion (2nd thrombus) was performed. At termination of the experiment, the left circumflex coronary artery was ligated proximal and distal to the occlusive thrombus. The wire and occluder were left *in situ* and the artery was excised and fixed by immersion in a solution of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2–7.4) for 48 h. After 48 h the occluder was removed from the section of the fixed artery, and the thrombus exposed by a longitudinal incision through the artery wall. A sample of thrombus was taken at the electrode tip. This tissue was then fixed for a further 2 h in 1% osmium tetroxide in the same buffer, dehydrated in an ascending acetone series and finally infiltrated with and embedded in Araldite resin. Semi-thin sections (1 μm thick) were stained with a solution of toluidine blue in borax and examined by light microscopy.

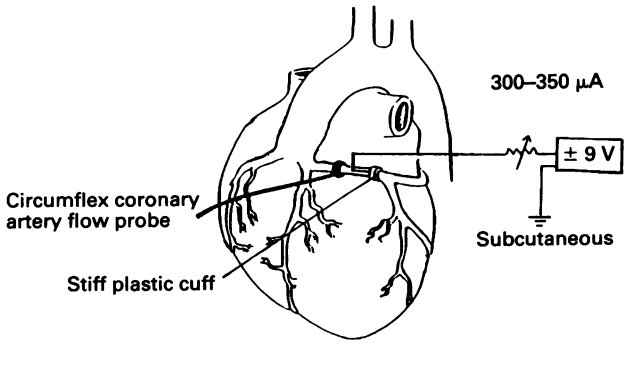


**Figure 1** Dog model of coronary artery thrombosis with a fixed stenosis. Application of anodal current (332 ± 4.1 μA) to the intimal lining leads to the production of a persistent occlusive thrombus. The cathode was placed subcutaneously completing the circuit.

### *Experimental protocol*

One hour after the occurrence of complete coronary occlusion, rt-PA was administered as a 3 mg bolus (i.v.) immediately followed by an i.v. infusion of 2 mg $kg^{-1}$ $h^{-1}$ for 30 min. Successful thrombolysis was defined as a return of mean coronary blood flow to at least 50% of the initial baseline value. Infusion of rt-PA was then terminated and reocclusion allowed to occur. This second occlusive thrombus was left for 1 h. Twenty dogs were then assigned radomly to one of four groups, after which adjunctive therapy was given as shown in Table 1.

Aspirin, ZM170809 and ZD1542 were administered at doses that we have previously shown to prevent thrombus formation in a dog model of coronary stenosis with damaged endothelium (McAuliffe *et al.*, 1992; 1993a,b). The effects of ZD1542 (1 mg $kg^{-1}$, i.v.) and aspirin (5 mg $kg^{-1}$, i.v.) on arachidonic acid metabolism were also measured *ex-vivo* in collagen-activated platelets. Aspirin and ZD1542 both caused a >95% inhibition of $TXB_2$, a measure of thromboxane production. In the case of ZD1542, there were corresponding increases in the production of prostaglandin $E_2$ ($PGE_2$), $PGF_{2\alpha}$ and $PGD_2$ (McAuliffe *et al.*, 1993a).

To determine whether adjunctive treatment could accelerate thrombolysis and prevent reocclusion, each agent was given as a bolus intravenous injection (10 ml fluid volume) 10 min prior to readministration of rt-PA, protocol as before (Figure 2). After the rt-PA infusion was terminated, the occurrence of cyclic flow reductions (CFR), reocclusion and ECG changes were recorded for a period of 60 min.

### *Drugs*

Actilyse (Boehringer Ingelheim, Bracknell, Berkshire), acetylsalicyclic acid (Sigma, U.K.), ZD1542 (4(Z)-6-[2S,4S,5R)-2-[1methyl-1-(2-nitro-4-tolyloxy)ethyl]-4-(3-pyridyl)-1,3-dioxan-5-yl]hex-4-enoic acid) (Zeneca Pharmaceuticals, U.K.) and ZM170809 (2,2[dimethylamino-2-methylamino-2-methylpropylthio]-3-phenylquinoline hydrochloride) (Zeneca Pharmaceuticals, U.K.) were used. All drugs were dissolved in sterile pyrogen free 0.85% sodium chloride.

### *Statistical analysis*

Results are expressed as the mean ± s.e.mean. Analysis of variance was used to compare the saline control group with the treated groups (Snedecor & Cochrane, 1989). A probability value of $P<0.05$ was considered statistically significant.

## Results

Within 20 min (19.6 ± 2.5 min) of applying an anodal current (332 ± 4.1 μA), a stable occlusive thrombus formed within the lumen of the left circumflex coronary artery in all 20 dogs. When recording began about 1 h after the completion of the surgical procedures, the pH, $P_{CO_2}$ and $P_{O_2}$ of the arterial blood were respectively: 7.396 ± 0.007, 37.6 ± 0.82 mmHg and 219 ± 9.2 mmHg. Mean systemic blood pressure and heart rate remained unchanged, at

**Table 1** Adjunctive treatment protocols

| *Group* | n | *Compound* | *Mode of action* | *Dose* (i.v.) |
|---|---|---|---|---|
| 1 | 5 | Saline | | 10 ml |
| 2 | 5 | ZM170809 | 5-$HT_2$ antagonist | 1 mg $kg^{-1}$ |
| 3 | 5 | Acetylsalicylic acid (ASA) | Cyclo-oxygenase inhibitor | 5 mg $kg^{-1}$ |
| 4 | 5 | ZD1542 | $TXA_2$ synthetase inhibitor/ TP-receptor antagonist | 1 mg $kg^{-1}$ |

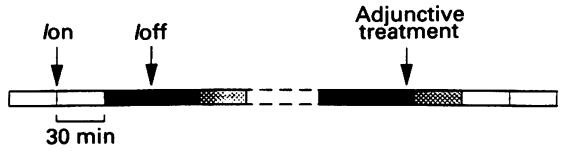


**Figure 2** A schematic diagram of the protocol indicating the periods of occlusion (solid bar) of the left circumflex coronary artery and the times of the rt-PA infusions (cross-hatched bar); $I$ = current.

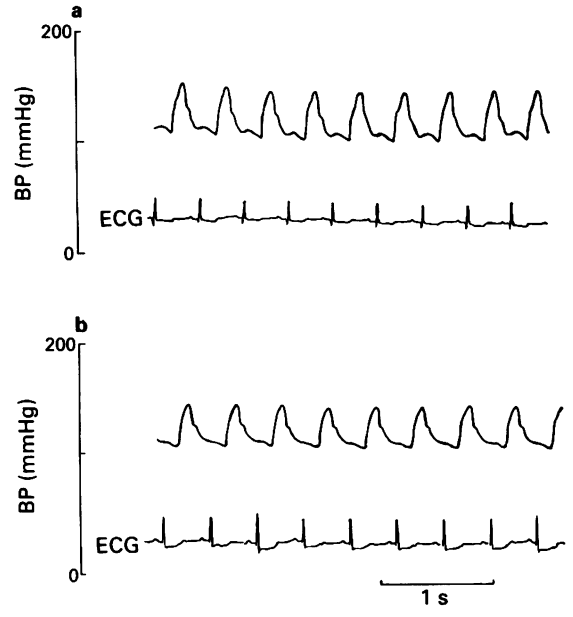


**Figure 3** The effect of an occlusive thrombus in the left circumflex coronary artery of a dog, on BP and ECG: (a) is in the absence and (b) is in the presence of the thrombus. Mean BP remains unchanged. The ECG ST segment is depressed in (b), indicative of ischaemia.

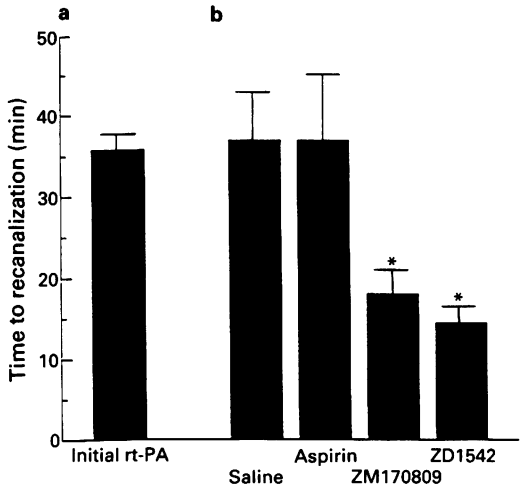


**Figure 4** (a) Time to recanalization after initial rt-PA infusion. (b) The effect of adjunctive anti-platelet agents on time to recanalization after the second infusion of rt-PA. $*P<0.05$ vs saline.

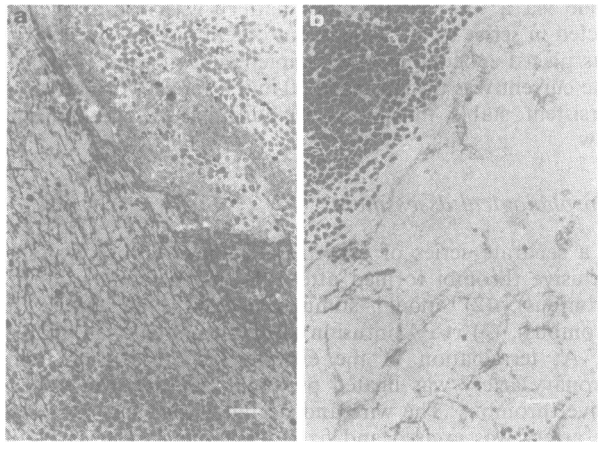

**Figure 5** Photomicrographs of platelet-rich thrombi after application of anodal current showing (a) a heterogeneous tissue structure of platelet-rich areas separated from erythrocyte-rich zones by abundant strands of fibrin and (b) following rt-PA infusion indicating reduced fibrin content and showing an adjacent area of blood, indicative perhaps of the site of reperfusion. Toluidine blue-stained plastic sections. Calibration for bar: $\mu$ (a) and (b), 10 mm = 1.81 mm.

121.3 ± 2.5 mmHg and 152.1 ± 3.4 beats $min^{-1}$, after either the application of anodal current or on the formation of a stable occlusive thrombus. During the period of occlusion there was ST segment depression in all dogs, indicative of ischaemia (Figure 3). None developed ventricular fibrillation.

Infusion of rt-PA caused an attenuation in the amplitude of the pulsatile coronary blood flow suggesting further activation of platelets.

In 15 dogs, thrombolysis occurred during or within 60 min after the first infusion of rt-PA. The time taken to lyse the thrombus ranged from 20 to 56 min (35.7 ± 2.3 min). The mean time to lyse the thrombi was similar in all four groups: saline 40.1 ± 5.3 min, ZM170809 34.4 ± 2.5 min, aspirin 36.7 ± 5.2 min, ZD1542 35.3 ± 4.9 min. After the infusion of rt-PA was terminated, reperfusion was followed by cyclical flow reductions and eventually complete coronary re-occlusion occurred in all 15 dogs. In the five remaining animals the initial thrombus was not lysed.

The second 30 min infusion of rt-PA, in the presence of either saline or adjunctive therapy resulted in thrombolysis in all 20 dogs. Adjunctive treatment with either ZM170809 or ZD1542 significantly reduced the time taken to thrombolysis compared with the saline group ($P<0.05$) (see Figure 4). Aspirin did not offer any additional benefit when compared with rt-PA alone. The number of dogs in each treatment group in which the coronary artery reoccluded within 60 min of terminating the infusion of rt-PA was:- saline 5, aspirin 4, ZM170809 2 and ZD1542 2.

Both the 5-$HT_2$ antagonist (ZM170809) and a combined thromboxane synthase inhibitor/TP-receptor antagonist (ZD

1542) significantly reduced the time taken to thrombolysis, and decreased the number of dogs in which reocclusion occurred.

### *Morphology*

The semi-thin sections of thrombi were examined by light microscopy. The initial thrombi formed after application of anodal current contained abundant platelets and fibrin which had enmeshed relatively few erythrocytes and leucocytes (Figure 5a). Examination of the thrombi formed after termination of the initial rt-PA infusion revealed platelet-rich areas which were associated with significantly less fibrin than the initial thrombi (Figure 5b). Areas of erythrocyte-rich and fibrin-poor tissue were sometimes seen which may represent a portion of re-canalised thrombus.

## Discussion

Recent evidence from both experimental models of coronary thrombosis and the clinic suggest that thrombolytic therapy is limited by platelet activation (Fitzgerald *et al.*, 1989; Coller, 1990). The method used in this study was specifically selected to produce a platelet-rich arterial thrombus similar to that which occurs in human coronary arteries (Sandritter & Thomas, 1979; Falk, 1985), so that the efficacy of anti-platelet agents as adjuncts to rt-PA could be assessed.

Application of anodal current to the endothelium, imparts a positive charge to the surrounding area, which attracts negatively charged blood elements, in particular platelets (Romson *et al.*, 1980). This is confirmed by our morphological data. The platelet-rich thrombus that is formed tends to be more resistant than a fibrin-rich thrombus to thrombolytic therapy (Jang *et al.*, 1989) and this may account for reperfusion failure. In this study 25% of dogs failed to reperfuse after the initial dose of rt-PA. Shebuski and colleagues (1988), observed that in rabbits, 17% of rt-PA treated animals failed to reperfuse; Fitzgerald *et al.* (1989) observed an 18% failure rate in dogs, and Kerins *et al.* (1989) a 33% failure rate in patients.

Administration of rt-PA elicits thrombolysis by preferential conversion of thrombus associated plasminogen to plasmin. Plasmin then initiates the degradation of fibrin which results in clot lysis. Paradoxically, thrombolytic therapy can itself induce platelet activation, both directly through plasmin and indirectly by the release of thrombin during clot lysis (Eisenberg *et al.*, 1987; Kerins *et al.*, 1989). Platelet activation leads to the release of both proaggregatory and vasoconstricting products, such as $TXA_2$ and 5-HT. This might explain the reduction in the amplitude of the pulsatile coronary blood flow observed upon administration of rt-PA. Activated platelets also release a fast acting plasminogen activator inhibitor (PAI-1) into the circulation which attenuates fibrinolytic activity and potentiates re-occlusion. On initial rt-PA infusion, t-PA saturates PAI-1, and also circulates as free t-PA and in complexes with protease inhibitors. Termination of rt-PA infusion causes a marked increase in PAI-1 activity and a reduction in fibrinolytic activity; these changes are associated with an increased risk of clinical myocardial infarction and re-occlusion (Lucore & Sobel, 1988). Gold and colleagues (1986) have demonstrated that by continuing the rt-PA infusion for a further 4 h after recanalisation, circulating levels of free t-PA are maintained and the rate of re-occlusion is reduced from 50% to 14%. Our results appear to confirm these observations as 25% of animals failed to reperfuse on initial infusion of rt-PA, but 100% recanalisation of the second thrombus was achieved after a second infusion of rt-PA. This is probably due to the fact that PAI-1 would still be complexed and there would be more free circulating t-PA which effectively increased fibrinolytic activity.

Although all thrombi lysed after the second infusion of rt-PA there were differences between the groups in time taken for thrombolysis to occur (Figure 4). The saline group showed no significant difference in time taken to reperfuse compared with the first rt-PA infusion.

Aspirin, a cyclo-oxygenase inhibitor, was administered at a dose that we have previously shown to prevent the formation of the platelet activating eicosanoids, $PGH_2$ and $TXA_2$ (McAuliffe *et al.*, 1992). Aspirin, in this study, did not enhance the rate of thrombolysis, there was no apparent difference between the saline and aspirin groups. This is not altogether unexpected, as aspirin as well as having anti-thrombotic properties, can itself be thrombogenic by inhibiting the production of prostacyclin ($PGI_2$) and $PGD_2$ (Sheng-Kun *et al.*, 1991; McAuliffe *et al.*, 1992). These two eicosanoids ($PGI_2$ and $PGD_2$) inhibit platelet aggregation and relax vascular smooth muscle. Clinical studies have also failed to demonstrate any benefit when aspirin was used as an adjunct to thrombolytic therapy (ISIS-2, 1988).

ZD1542 inhibits the formation of $TXA_2$ and blocks the effect of $PGH_2$ on the TP-receptor (Brownrigg *et al.*, 1992). This causes redirection of arachidonic acid metabolism towards increased production of $PGD_2$ and $PGI_2$, both of which are capable of inhibiting platelet aggregation and causing vasodilatation (McAuliffe *et al.*, 1993a). Administration of ZD1542 as an adjunct to rt-PA significantly reduced the time to recanalisation compared with both the saline group and the aspirin group ($P<0.05$). This indicates that redirection of arachidonic acid metabolism by ZD1542 might prove clinically advantageous, therefore ZD1542 may be a more effective adjunct to rt-PA thrombolytic therapy than cyclo-oxygenase blockade by aspirin.

5-HT is concentrated in the dense granules of the platelet and is released upon platelet activation. It acts as an amplification signal for other platelet agonists, causing further platelet activation, and vasoconstriction (De Clerck *et al.*, 1991). The selective $5\text{-}HT_2$ antagonist, ZM170809 (Blackburn *et al.*, 1988; 1990), was administered at a dose that we have shown to prevent thrombus formation in a stenosed coronary artery model with damaged endothelium (McAuliffe *et al.*, 1993b). In this present study, ZM170809 was a more effective adjunct than aspirin, at reducing the time to recanalisation ($P<0.05$) and preventing reocclusion.

Golino *et al.* (1988) have shown that re-occlusion, after discontinuation of rt-PA, is mediated by $TXA_2$ and 5-HT. We have demonstrated that re-direction of arachidonic acid metabolism with ZD1542 and antagonism of the $5\text{-}HT_2$ receptor with ZM170809 significantly enhances thrombolysis both in the time taken to lyse the thrombus and in the prevention of re-occlusion. Aspirin in this study did not enhance thrombolysis or prevent re-occlusion. This indicates that the elimination of $TXA_2$ and $PGH_2$ does not result in a beneficial anti-thrombotic effect when the production of $PGI_2$ and $PGD_2$ are compromised.

Since ZM170809 and ZD1542 have different modes of action, a combination would be expected to act synergistically further enhancing thrombolysis and preventing re-occlusion. This is currently being investigated.

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# Comparative pharmacology of recombinant rat $AT_{1A}$, $AT_{1B}$ and human $AT_1$ receptors expressed by transfected COS-M6 cells

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**1** Currently available antagonists and agonists cannot distinguish between angiotensin $AT_1$ receptor subtypes.

**2** We synthesized a series of compounds selected on the basis of having the most diverse structural features with respect to losartan (DuP753), the prototype non-peptide $AT_1$ receptor antagonist. Using a radioligand-receptor binding assay and membranes prepared from COS-M6 cells transfected with individual $AT_1$ receptor subtypes, we determined whether any of these compounds could distinguish between the receptor subtypes.

**3** The diversity of the structural features of this series of compounds was reflected by the wide range of affinities ($pIC_{50}$ values) displayed towards competing with [$^{125}$I]-Sar$^1$Ile$^8$ angiotensin II for binding to the $AT_1$ receptors.

**4** Direct comparisons of the $pIC_{50}$ values of individual compounds for rat $AT_{1A}$, $AT_{1B}$ and human $AT_1$ receptors revealed only minor differences.

**5** It is concluded that compounds based structurally on losartan are unlikely to distinguish between these receptors.

**Keywords:** Recombinant receptors; radioligand binding; receptor subtypes; non-peptide antagonists; comparative pharmacology; rat $AT_{1A}$ receptor; rat $AT_{1B}$ receptor; human $AT_1$ receptor; angiotensin II (AII); losartan (DuP753)

## Introduction

The existence of multiple receptors for angiotensin peptides, particularly subtypes of the angiotensin II (AII) receptor, has been postulated for some time, based on the findings of numerous studies of the structure-activity relationships of various agonist and antagonist peptide analogues of AII in a variety of tissues (Peach, 1977). Four years ago, the receptors for AII were pharmacologically characterized, by use of radioligand receptor binding, into two types (Whitebread *et al.*, 1989; Chiu *et al.*, 1989) and assigned more recently the nomenclature, $AT_1$ and $AT_2$ (Bumpus *et al.*, 1991). This characterization is based on the differential selectivity of the non-peptide AII receptor antagonists, losartan and PD123177 and also the peptides CGP42112A and *p*-aminophenylalanine$^6$AII (Timmermans *et al.*, 1991) for these receptors. The $AT_1$ subtype fully justifies recognition as a receptor, but the status of the $AT_2$ subtype beyond that of a binding site is less clear.

Whilst receptors have been defined traditionally by their ligand binding characteristics and function, the advent of molecular biology has allowed identification of apparently different subtypes based on their amino acid sequences rather than their binding profiles and function. The receptor designated $AT_1$ on the basis of its binding properties and linkage through phospholipase C to raise intracellular calcium has been cloned from cultured bovine adrenal glomerulosa cells (Sasaki *et al.*, 1991), rat aortic vascular smooth muscle cells (Murphy *et al.*, 1991), a rat kidney cDNA library (Iwai *et al.*, 1991), a rat liver cDNA library and a human lung cDNA library (K.T. Pun, personal communication), a human lymphocyte genomic library (Furuta *et al.*, 1992) and a human liver cDNA library (Takayanagi *et al.*, 1992). These receptors possess the seven transmembrane domain structure typical of other G-protein coupled receptors. There is an 8% difference in amino acid sequence between the bovine adrenal and rat vascular $AT_1$ receptors, although the single polypeptide encoded by the cDNAs are of identical length. These differences may be attributed to either or both the species concerned and perhaps differences in the function of these receptors related to their anatomical location. The cloning of an $AT_1$ receptor from a rat adrenal cDNA library with an amino acid sequence 96% homologous to the receptor cloned from rat vascular smooth muscle cells but showing substantial difference in the 5′ and 3′ non-coding regions led to the use of the terms $AT_{1A}$ for the latter and $AT_{1B}$ for the adrenal receptor (Iwai & Inagami, 1992). Further evidence for particular anatomical localization and, therefore, increasing justification for their consideration as potentially functionally important subtypes came from the observation that the $AT_{1B}$ receptor also appeared to be preferentially expressed in the rat pituitary gland (Kakar *et al.*, 1992). In contrast, so far, subtypes of the human $AT_1$ receptor have not been found.

Although these subtypes of $AT_1$ receptors had structural differences and were distributed differentially, currently available antagonists and agonists cannot distinguish between them (Murphy *et al.*, 1991; Kakar *et al.*, 1992; Takayanagi *et al.*, 1992). The aim of the present study was therefore to determine whether, from a group of chemically diverse selective '$AT_1$ receptor antagonists', we could find a compound that displayed selectivity for one of three types of angiotensin $AT_1$ receptor so far cloned, rat $AT_{1A}$, $AT_{1B}$ and human $AT_1$. It was important that all three receptors should be studied under identical conditions if subtle differences were to be detected. Therefore, individual cDNAs encoding specific angiotensin receptor subtypes were transfected into COS-M6 cells which then transiently express the receptor. Competition curves for the different drugs were constructed from a radioligand-receptor binding assay and membranes prepared from transfected COS-M6 cells. COS-M6 cells do not express endogenous angiotensin receptors. Thus, we were able to compare and contrast the pharmacology of these $AT_1$ receptors under identical experimental conditions.

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## Methods

### *Amplification and purification of plasmid DNA encoding $AT_1$ receptors*

The rat $AT_{1A}$ receptor was isolated by combined PCR/hybridisation screening from a size-selected rat liver cDNA library, constructed in the mammalian expression vector pCDM8 (Invitrogen). The Rat $AT_{1B}$ receptor was cloned from a rat adrenal cDNA library into pCDM8. The human $AT_1$ receptor was obtained by hybridisation screening of a human lung cDNA library in λgt11 (Clontech), and subcloned into the vector pCDNA1 (Invitrogen). In all three clones, full length receptor expression is driven from the human cytomegalovirus (CMV) promoter-enhancer. Each clone was transformed into the *E. coli* host strain MC1061/P3. All subsequent amplification and purification of the plasmid DNA from the bacteria was identical for each of the receptor subtypes.

Twenty ml of 2YT medium (containing 50 μg $ml^{-1}$ kanamycin, 40 μg $ml^{-1}$ ampicillin and 12.5 μg $ml^{-1}$ tetracycline) was inoculated with the *E. coli* strain MC1061/P3 containing the relevant plasmid. Bacteria were left to grow at 37°C in an orbital shaker (300 r.p.m.). The growth of the bacteria was monitored until the optical density of the medium was approximately 0.5 at $A_{600\,nm}$; 10 ml of medium was then removed and used to innoculate a further 500 ml of 2YT medium (containing the same antibiotics as outlined above). Bacteria were left to grow overnight. Purification of the plasmid DNA was then carried out with a QIAGEN plasmid kit (Hybaid).

### *Transfection of the plasmid DNA encoding specific angiotensin receptor subtypes into COS-M6 cells*

COS-M6 monkey fibroblast cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) containing 10% v/v foetal calf serum, 2 mM glutamine, 100 u $ml^{-1}$ penicillin G, 100 μg $ml^{-1}$ streptomycin and 0.25 μg $ml^{-1}$ amphotericin at 37°C in a humidified atmosphere of air/$CO_2$ (19:1). The day prior to transfection, cells were seeded at a density of 8 million cells/800 ml flask, so that they were subconfluent the next day. A transfection solution was prepared by mixing DEAE dextran (6 mg), plasmid DNA (30 μg) and chloroquine (100 μM) with 15 ml of serum-free DMEM. After washing the cells twice (25 ml) with serum-free DMEM, the transfection solution was added to the cells and left for 3 h. The transfection solution was then removed and the cells washed again (2 × 25 ml) with serum-free medium; 15 ml of 'shock-solution' (phosphate buffered saline containing 10% v/v dimethylsulphoxide) was then added to the cells for 2 min. After washing with DMEM containing serum (25 ml), 50 ml of medium was added to the cells and the cells left for 3 days, after which, membranes were prepared.

### *Preparation of membranes*

Cultured COS-M6 cells were washed with ice-cold phosphate buffered saline, harvested with a Costar scraper and collected by centrifugation (1000 *g* for 10 min). The cells were then homogenised (Polytron; maximal setting, 10 s) in ice-cold 50 mM Tris 5 mM EDTA buffer (pH 7.4) and centrifuged for 10 min at 1000 *g*. The supernatant was then removed and the remaining pellet subjected to a further homogenization (Polytron; maximal setting, 10 s) and centrifuged for 10 min at 1000 *g*. The combined supernatants from each homogenisation were then centrifuged at 30,000 *g* for 35 min. The resultant pellet was resuspended in 4 ml of buffer, an aliquot removed for protein determination and the remainder frozen in 0.25 ml aliquots at − 40°C.

### *Radioligand-receptor binding assay*

Total reaction volume was 300 μl, consisting of 150 μl membranes (30–50 μg protein; previously been shown to display 5–20,000 c.p.m. specific binding), 75 μl [$^{125}$I]-Sar$^1$Ile$^8$ AII (0.4 nM) and 75 μl of varying concentrations of competitor or assay buffer. All drugs were dissolved in 100 μl absolute ethanol, 100 μl 1 M NaOH and made up to 10 ml with $H_2O$ to produce 1 mM stock solutions. Further dilutions of drugs were carried out in assay buffer which consisted of 100 mM NaCl, 50 mM Tris-Base (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM bacitracin and 0.1% w/v BSA (protease-free). Membranes and radioligand were also diluted in assay buffer. Nine or ten concentrations of competitor were examined, each concentration in duplicate per assay. Membranes were equilibrated with competitor for 60 min at room temperature prior to the addition of radioligand for a further 90 min. Membrane bound radioligand was separated from free radioligand by vacuum filtration and washing (100 mM NaCl; 10 mM $MgCl_2$, approx 5 ml per assay tube) over glass fibre filters using a Skatron cell harvester. Filters were pretreated with 0.1% v/v polyethylenimine in order to obtain low filter blanks. After filtration the filters were counted in a γ-counter (LKB, Clinigamma, Sweden).

### *Protein determination*

The method of Lowry *et al.* (1951) was followed, with bovine serum albumin used as standard.

### *Data analysis*

Non-specific binding was measured in the presence of 10 μM unlabelled AII and did not exceed 10% of the total binding. An iterative curve fitting progamme, GRAPHPAD (ISI Software), was used to determine the $pIC_{50}$ values for the competition curves.

### *Materials*

Ampicillin, angiotensin II, bacitracin, bovine serum albumin (protease-free), chloroquin, DEAE-dextran, kanamycin and tetracycline were purchased from Sigma Chemicals (Poole, Dorset); [$^{125}$I]-Sar$^1$Ile$^8$ angiotensin II from New England Nuclear (DuPont, Stevenage); Dulbecco's modified Eagle's medium was purchased from Flow Laboratories (Irvine, Scotland); 2YT medium, antibiotic/antimycotic mixture and glutamine from Gibco (Paisley, Scotland); foetal calf serum from Sera Lab (Crawley, Sussex). Qiagen plasmid kits were purchased from Hybaid (Teddington, Middlesex). The rat $AT_{1A}$, and human $AT_1$ receptor cDNAs were cloned in the Department of Molecular Science, Glaxo Group Research. Drs Kevin Catt and Adrian Clark provided the cDNA clone-encoding the rat $AT_{1B}$ receptor. The mammalian expression vectors pCDM8 and pCDNA1 were obtained from Invitrogen (San Diego, CA, U.S.A.). The following antagonists described in this report were synthesized in the Department of Medicinal Chemistry, Glaxo Group Research, Ware, Hertfordshire: L158809 (5,7-dimethyl-2-ethyl-3-[[2′-(1H-tetrazol-5-yl) [1,1′]-biphenyl-4-yl]-methyl]-3H-imidazo[4, 5-b]pyridine), EXP3174 (2-n-butyl-4-chloro-1-[ (2′-(1H-tetrazol-5-yl) biphenyl-4-yl) methyl] imidazole-5-carboxylic acid), SKF108566 ((E)-alpha-[ [2-butyl-1-[ (4-carboxyphenyl)methyl]-1H-imidazol-5-yl] methylene]-2-thiophenepropanoic acid), GR137977 (1-[3-bromo-2-[2- (1H-tetrazol-5-yl) -phenyl]-benzofuran-5-yl-methyl]-2-butyl-4-methyl-1, 4, 6, 7-tetrahydro-imidazo-[4,5-E][1,4]-diazepine-5,8-dione), valsartan (3-methyl-2-[pentanoyl-[2′-(1H-tetrazol-5-yl)-biphenyl-4-ylmethyl]-amino]-2S-butyric acid), losartan (2-propyl-4-pentafluoroethyl-1-[ (2′-(1H-tetrazol-5-yl) biphenyl-4-yl) methyl] imidazole-5-carboxylic acid), GR117289 (1-[ [3-bromo-2-[2-(1H-tetrazol-5-yl)phenyl]-5-benzofuranyl] methyl]-2-butyl-4-chloro-1H-imidazole-5-carboxylic acid), GR131452 (5-butyl-2-isopropyl-4-[2′-(1H-tetrazol-5-yl)-

**Table 1** Potency of losartan and related structural analogues in competing with [$^{125}$I]-Sar$^1$Ile$^8$ AII for binding to recombinant rat $AT_{1A}$, $AT_{1B}$ and human $AT_1$ receptors

| | *Rat $AT_{1A}$* | | *Rat $AT_{1B}$* | | *Human $AT_1$* | |
|---|---|---|---|---|---|---|
| *Competitor* | *$pIC_{50}$* | *Hill slope* | *$pIC_{50}$* | *Hill slope* | *$pIC_{50}$* | *Hill slope* |
| L158809 | 9.71 ± 0.07 | 1.03 ± 0.12 | 9.32 ± 0.11 | 1.30 ± 0.10 | 9.68 ± 0.16 | 1.18 ± 0.24 |
| EXP 3174 | 8.78 ± 0.06 | 1.04 ± 0.13 | 8.72 ± 0.16 | 1.09 ± 0.10 | 8.88 ± 0.01 | 0.93 ± 0.08 |
| SKF108566 | 8.47 ± 0.08 | 0.94 ± 0.08 | 8.67 ± 0.07 | 1.05 ± 0.07 | 8.78 ± 0.07 | 0.88 ± 0.08 |
| GR137977 | 8.20 ± 0.07 | 0.80 ± 0.16 | 8.17 ± 0.13 | 1.11 ± 0.10 | 8.22 ± 0.17 | 1.10 ± 0.10 |
| Valsartan | 8.03 ± 0.03 | 1.20 ± 0.13 | 8.26 ± 0.01 | 1.17 ± 0.03 | 8.30 ± 0.11 | 1.04 ± 0.20 |
| Losartan | 7.93 ± 0.08 | 0.95 ± 0.09 | 8.18 ± 0.02 | 0.97 ± 0.02 | 8.20 ± 0.06 | 0.88 ± 0.04 |
| GR117289 | 8.00 ± 0.05 | 1.26 ± 0.19 | 8.08 ± 0.06 | 0.95 ± 0.11 | 8.04 ± 0.10 | 1.20 ± 0.15 |
| GR131452 | 7.40 ± 0.11 | 1.16 ± 0.10 | 7.46 ± 0.01 | 1.24 ± 0.16 | 7.53 ± 0.02 | 0.98 ± 0.09 |
| GR157249 | 7.14 ± 0.12 | 0.98 ± 0.02 | 7.64 ± 0.05 | 1.06 ± 0.08 | 7.52 ± 0.05 | 0.90 ± 0.06 |
| GR127612 | 6.85 ± 0.06 | 1.01 ± 0.05 | 7.11 ± 0.09 | 1.24 ± 0.13 | 7.00 ± 0.05 | 1.01 ± 0.01 |
| GR109793 | 6.27 ± 0.06 | 1.01 ± 0.09 | 6.66 ± 0.03 | 0.91 ± 0.02 | 6.78 ± 0.06 | 0.91 ± 0.02 |
| PD123177 | > 5.00 | | > 5.00 | | > 5.00 | |

Membranes were preincubated with the above compounds for 1 h then incubated for a further 1.5 h in the presence of 0.1 nM [$^{125}$I]-Sar$^1$Ile$^8$ AII, prior to separation of bound from free radioligand. Data are expressed as mean $pIC_{50}$ ± s.e.mean ($n$ = 3).

biphenyl-4-ylmethyl] -2H-pyrazole-3-carboxylic acid), GR-157249 (N-[2-[3-bromo-5-(2-ethyl-4, 5-dimethyl-6-oxo-6H-pyrimidin-1-ylmethyl)-benzofuran-2-yl]-phenyl]-C, C, C,-trifluoro-methanesulphonamide), GR127612 (1-[3-bromo-2-[2-(2H-tetrazol-5-yl)-phenyl]-benzofuran-5-ylmethyl] -5-butyl-1H-pyrazole-3-carboxylic acid), GR109793 (4'-(2-butyl-benzolmidazol-1-ylmethyl)-biphenyl-2-carboxylic acid), PD123177 (1-[(4-amino-3-methylphenyl)methyl]-5-(diphenylacetyl)-4, 5, 6, 7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid).

## Results

Each competitor was examined three times versus each receptor subtype. The diversity of the structural features of this series of compounds was reflected by the wide range of affinities ($pIC_{50}$ values) displayed towards competing for binding to the $AT_1$ receptors (Table 1). The potency series L158809 > EXP3174 = SKF108566 > GR137977 = valsartan = losartan = GR117289 > GR131452 = GR157249 > GR127612 > GR109793 >>> PD123177 was observed for all three $AT_1$ receptors screened. Direct comparisons of the $pIC_{50}$ values of individual compounds for the rat $AT_{1A}$, $AT_{1B}$ and human $AT_1$ receptors revealed only minor differences; affinities for any given antagonist varied by no more than three fold between the receptor subtypes.

## Discussion

Many of the physiological actions of AII appear to be mediated through $AT_1$ receptors coupled to phospholipase C. Recently, two subtypes of the rat $AT_1$ receptor have been cloned and termed $AT_{1A}$ and $AT_{1B}$ receptors (Murphy *et al.*, 1991; Iwai & Inagami, 1992). The encoded amino acid sequences of both receptors are 359 amino acids in length and exhibit a high degree of homology (96%). There are 17 amino acid differences in the amino acid sequences between the two rat $AT_1$ receptors which are dispersed throughout the whole sequence, seven in the extracellular, three in the transmembrane and seven in the intracellular domains. At present only one human $AT_1$ receptor has been cloned. It is also 359 amino acids in length and exhibits a high degree of amino acid sequence homology (94%) to the rat $AT_{1A}$ receptor. Again, differences in amino acids relative to the rat $AT_{1A}$ receptor, 20 in all, are dispersed throughout the whole receptor, nine in the extracellular, six in the transmembrane and five in the intracellular domains. The amino acid differences in the intracellular domains of these receptors, particularly serine and threonine residues in the carboxy terminal, may reflect the potential to undergo differential phosphorylation which may lead to differences in the regulation and coupling of these receptors.

The transmembrane domains of G-protein coupled receptors have been associated with formation of the ligand binding site for some time (for review see Kobilka, 1992). Recently, it has been reported that the extracellular domains may also contribute to the binding of ligands (Watling & Krause, 1993). Differences in amino acids in these regions of the $AT_1$ receptors therefore raise the possibility that they may be pharmacologically distinguishable. Such a possibility is illustrated by the finding that a single amino acid difference in the seventh transmembrane domain between rat and human 5-$HT_{1B}$ receptors is responsible for a distinct difference in the affinities of several ligands for these two receptors (Oksenberg *et al.*, 1992).

The recent development of non-peptide AII receptor antagonists has unequivocally demonstrated the existence of at least two AII receptor subtypes in various tissues, previously unidentifiable by use of peptide analogues of AII (Timmermans *et al.*, 1991). Losartan is regarded as the prototype non-peptide angiotensin $AT_1$ receptor antagonist. Structurally this compound is a functionalised imidazole linked to an acidic tetrazole moiety by a biphenyl spacer. Since the advent of losartan many non-peptide antagonists of angiotensin II have been described. In an attempt to identify a compound (ligand) which has selectivity for one of the $AT_1$ receptor subtypes, we have evaluated a series of compounds selected on the basis of having the most diverse structural features with respect to losartan. The compounds chosen have one or more of the following features: a different heterocycle from the imidazole, an alicyclic replacement for the imidazole, different functional groups in the imidazole or its replacement, an alternative spacer to a biphenyl, a different acidic group from the tetrazole. The $AT_2$ receptor antagonist PD123177 was also included in the set for comparison (for structures see Figure 1).

The diversity of the structural features of this series of compounds was reflected by the wide range of affinities ($pIC_{50}$ values) displayed towards competing for binding to the $AT_1$ receptors. The potency series L158809 > EXP3174 = SKF108566 > GR137977 = valsartan = losartan = GR117289 > GR131452 = GR157249 > GR127612 > GR109793 >>> PD123177 was observed for all three $AT_1$ receptors screened. Direct comparisons of the $pIC_{50}$ values of individual compounds for the rat $AT_{1A}$, $AT_{1B}$ and human $AT_1$ receptors revealed only minor differences, indicating that none of the compounds screened displaced differential selectivity. As a cautionary note, it should be appreciated that the $pIC_{50}$ values given here may not be a good measure of the absolute affinities of these compounds, due to the presence of 0.1% w/v BSA in the binding assay. This is particularly

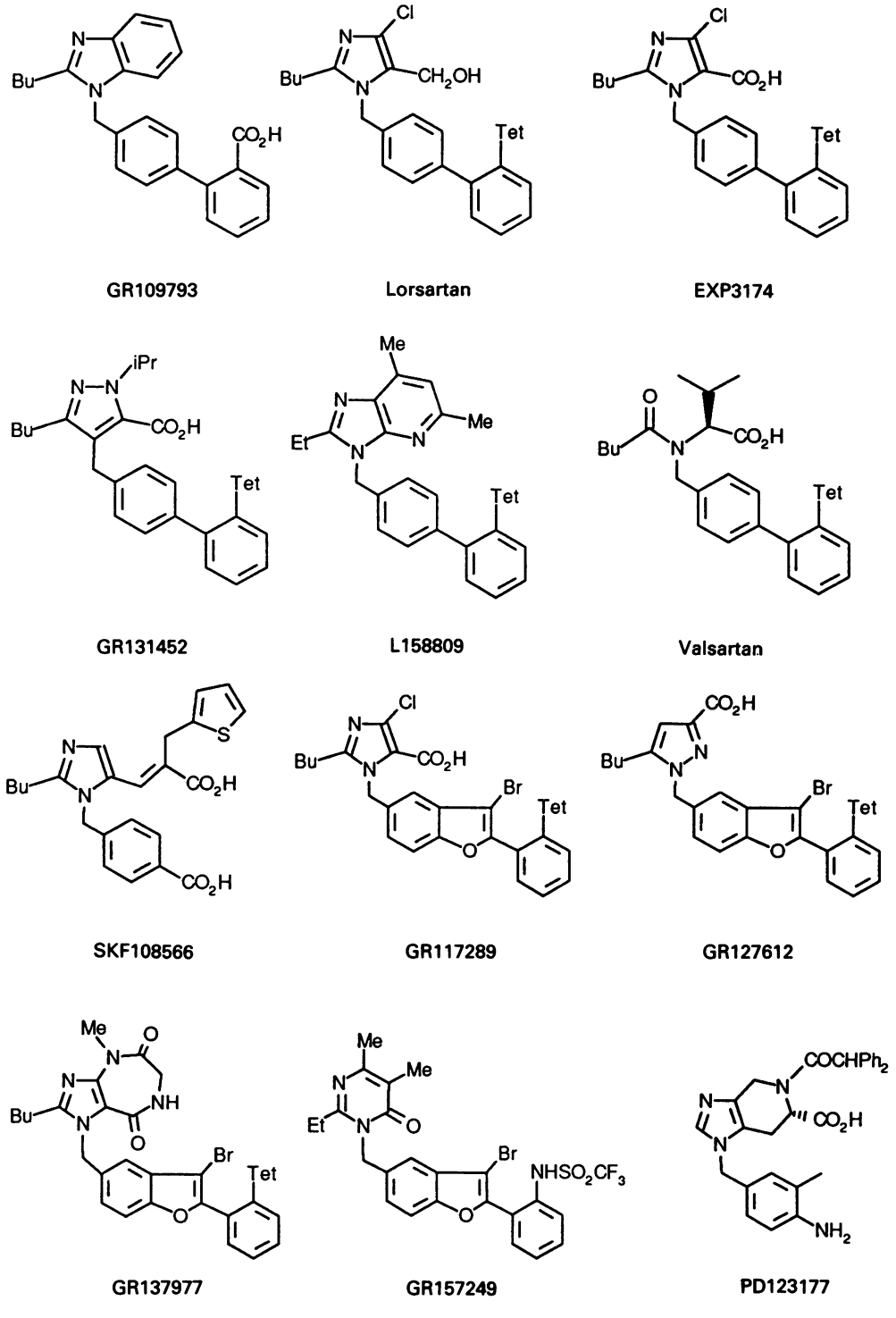


**Figure 1** Structures of the $AT_1$ receptor non-peptide antagonists synthesized.

relevant to the diacidic non-peptide compounds, for example EXP3174 and GR117289, where it has been observed that removal (Chiu *et al.*, 1991) or significant reduction of BSA to 0.001% w/v (Robertson *et al.*, 1992) in the binding assay markedly increased their apparent affinities. This has been attributed to a direct interaction of diacidic non-peptide compounds with BSA (Chiu *et al.*, 1991).

Previously, using a limited number of conventional angiotensin II peptide analogues and the two prototype non-peptide antagonists losartan and PD123177, several groups have reported the pharmacological profile of an individual cloned $AT_1$ receptor (Murphy *et al.*, 1991; Kakar *et al.*, 1992; Takayanagi *et al.*, 1992). A comparison of these limited data reveals that all three clones appear to display essentially indistinguishable affinities for these compounds. Our studies, using a much wider range of non-peptide compounds, but this time under identical experimental conditions, indicated that compounds based structurally on losartan are unlikely to distinguish between these receptors.

However, further compounds could be synthesized which would discriminate either between these receptors already cloned or others yet to be identified. There are many precedents with G-protein coupled receptors for multiple subtypes detected using molecular biology techniques prior to establishing compounds able to distinguish them. The cloned $AT_1$ receptors studied here appear to couple only to mobil-

ization of intracellular calcium. The observation of a losartan-sensitive receptor (that is an $AT_1$ receptor) subtype, coupled to inhibition of adenylate cyclase in rat liver, already suggests at least one further subtype should be identifiable although it may not necessarily differ at the ligand binding site (Bauer *et al.*, 1991). A final and important point relevant to the role of these receptors in man is that since these compounds bind with equal affinity to both human and rat angiotensin $AT_1$ receptors, experimental data obtained using these compounds in the rat may predict their effects in man.

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# The use of antagonists to characterize the receptors mediating depolarization of the rat isolated vagus nerve by α,β-methylene adenosine 5′-triphosphate

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**1** We have previously found that the $P_{2x}$-purinoceptor agonist, α,β-methylene adenosine 5′-triphosphate (α,β-methylene ATP), depolarizes the rat cervical vagus nerve, measured with a 'grease-gap' extracellular recording technique. This effect was attenuated by the $P_2$ purinoceptor antagonist, suramin. In the present study we have investigated in more detail the antagonism produced by suramin and have also investigated the actions of two other putative $P_2$ purinoceptor antagonists, cibacron blue and pyridoxal-phosphate-6-azophenyl-2′, 5′-disulphonic acid (iso-PPADS). Furthermore, we have studied the interactions between suramin and cibacron blue or iso-PPADS in an attempt to determine whether these antagonists act at a common receptor site.

**2** Suramin ($1 \times 10^{-5}$–$1 \times 10^{-4}$ M) produced reversible, concentration-related rightward displacements of the concentration-effect curve to α,β-methylene ATP. Schild analysis of this antagonism yielded a $pA_2$ value of 5.90 with a slope value of 0.47.

**3** Cibacron blue ($3 \times 10^{-5}$–$1 \times 10^{-4}$ M) also antagonized depolarizations induced by α,β-methylene ATP. The antagonistic effects of cibacron blue were slow to reach equilibrium but could be readily reversed on washout. At low concentations for antagonism, cibacron blue ($1 \times 10^{-5}$ M and $3 \times 10^{-5}$ M) produced enhancement of the maximal response to α,β-methylene ATP. At the highest concentration tested ($1 \times 10^{-4}$ M) the concentration-effect curve to α,β-methylene ATP was shifted to the right in a parallel manner, yielding a $pK_B$ estimate of 4.96.

**4** Iso-PPADS ($1 \times 10^{-6}$–$1 \times 10^{-5}$ M) produced a concentration-related depression in the maxima of the concentration-effect curves to α,β-methylene ATP. Analysis of these data by a double reciprocal plot yielded a $pK_B$ estimate of 6.02. This profile of insurmountable antagonism could not be attributed to irreversible binding of iso-PPADS to the receptor since the effect of iso-PPADS could be reversed on washing, albeit slowly.

**5** In the presence of suramin ($1 \times 10^{-4}$ M), cibacron blue ($1 \times 10^{-4}$ M) produced no further rightward displacement of the α,β-methylene ATP concentration-effect curve. The mean agonist concentration-ratios in the presence of suramin or cibacron blue alone (11.7 and 10.3, respectively) were not significantly different from the mean concentration-ratio in the presence of both antagonists (11.8). This finding suggests that high concentrations of α,β-methylene ATP activate a receptor population which is resistant to blockade by either antagonist.

**6** The antagonistic effect of iso-PPADS ($1 \times 10^{-5}$ M) was partially attenuated by suramin ($1 \times 10^{-4}$ M). It is possible that this interaction reflects a slow dissociation of iso-PPADS from the receptor with which suramin and α,β-methylene ATP interact.

**7** Suramin, cibacron blue or iso-PPADS had no marked effect on depolarization produced by 5-hydroxytryptamine (5-HT, $1 \times 10^{-7}$–$3 \times 10^{-5}$ M), indicating their specificity in antagonizing responses to α,β-methylene ATP.

**8** Responses to α,β-methylene ATP were not antagonized by 8-para-sulphophenyltheophylline ($3 \times 10^{-5}$ M), ondansetron ($1 \times 10^{-7}$ M), bicuculline ($1 \times 10^{-5}$ M), phentolamine ($1 \times 10^{-6}$ M) or hexamethonium ($1 \times 10^{-4}$ M), which are antagonists at $P_1$-purinoceptors, $5\text{-}HT_3$ receptors, $GABA_A$ receptors, α-adrenoceptors and nicotinic cholinoceptors, respectively, thereby excluding the involvement of these receptors. Indomethacin ($3 \times 10^{-6}$ M) had no effect on responses to α,β-methylene ATP.

**9** The results obtained with three purinoceptor antagonists confirm and extend our original supposition that α,β-methylene ATP-induced depolarization of the rat vagus nerve is mediated predominantly via $P_2$ purinoceptors, thought to be of the $P_{2x}$ subtype. The finding that responses induced by high concentrations of agonist were resistant to blockade by suramin and cibacron blue, but could be attenuated by iso-PPADS, adds further weight to our speculation that the purinoceptor population in the rat vagus nerve is heterogeneous.

**Keywords:** Rat vagus nerve; α,β-methylene ATP; $P_2$ purinoceptors; suramin; pyridoxal-phosphate-6-azophenyl-2′,5′-disulphonic acid (iso-PPADS); cibacron blue

## Introduction

$P_2$ purinoceptors mediate the actions of adenosine 5′-triphosphate (ATP) on many physiological systems including most vascular and visceral smooth muscles and certain neurones in the peripheral and central nervous systems (see Burnstock, 1990 for review). On the basis of different rank orders of potencies of several analogues of ATP, this group of receptors has been divided into four subtypes, designated $P_{2X}$, $P_{2Y}$, $P_{2T}$ and $P_{2U}$ (Cusack, 1993). Indeed, recently the $P_{2Y}$ and $P_{2U}$ receptor genes have been cloned (Lustig *et al.*, 1993; Webb *et al.*, 1993). Other $P_2$ purinoceptors that do not fit

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neatly into this classification system have also been described (e.g. O'Connor *et al.*, 1991; Pintor *et al.*, 1993).

However, an understanding of the distribution and functional significance of these receptors has been hindered by the lack of selective antagonists for each of these receptors (see Fedan & Lamport, 1990, for review). The most widely studied $P_2$ purinoceptor antagonist, suramin, does not discriminate between the known $P_2$ receptor subtypes (Cusack, 1993). Cibacron blue, an anthraquinone sulphonic acid derivative formerly called reactive blue 2, reportedly shows selectivity for $P_{2Y}$ receptors over $P_{2X}$ receptors (Burnstock & Warland, 1987; Reilly *et al.*, 1987), at least within a limited concentration-range. Another sulphonic acid derivative, pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) antagonizes $P_{2X}$ purinoceptor-mediated responses in both the guinea-pig and rabbit vas deferens (Lambrecht *et al.*, 1992; McLaren *et al.*, 1993). An isomer of this compound, pyridoxalphosphate-6-azophenyl-2',5'-disulphonic acid (iso-PPADS) is now commercially available. However, the activity of these agents at other purinoceptor subtypes has not been described.

Detailed studies on the effect of $P_2$ purinoceptor antagonists on neuronal preparations have not been reported, and this can be readily attributed to the paucity of simple quantitative assay systems for the accurate measurement of antagonist effects. Recently, we described for the first time the depolarizing effect of ATP and analogues of ATP on the rat isolated cervical vagus nerve (Trezise *et al.*, 1993). In this technically convenient assay system, the $P_{2X}$ purinoceptor selective agonist, α,β-methylene ATP, was the most potent nucleotide tested and the depolarizing effects of this agent were antagonized by suramin, albeit in a manner which was not entirely consistent with simple competitive kinetics between agonist and antagonist. On this basis we proposed the involvement of a $P_{2X}$ purinoceptor.

The aims of the present study were threefold. First, to study in more detail the effect of suramin on responses of the vagus nerve to α,β-methylene ATP in an attempt to understand the profile of antagonism observed with this agent. Secondly, to evaluate the effects of the putative $P_2$ purinoceptor antagonists, cibacron blue and iso-PPADS, with a view to providing corroborative evidence for the involvement of $P_2$ purinoceptors and finally, to study the interaction between suramin and the other two $P_2$ purinoceptor antagonists to establish whether or not they act at a common receptor site.

## Methods

Extracellular recordings of agonist-induced depolarizations were made from segments of rat isolated cervical vagus nerve, according to the method of Ireland & Tyers (1987). Briefly, male AHA Wistar rats (200–270 g) were stunnned by a blow to the head, decapitated, and the two cervical vagus nerves rapidly excised. Segments of nerve, approximately 15–20 mm long, were desheathed under a dissecting microscope, and transferred to heated (27°C) two-compartment Perspex baths such that approximately 50% of the nerve lay in the first compartment, while the remainder projected through a greased slot (Dow-Corning high vacuum grease) into the second. The d.c. potential between the two compartments was measured with silver-silver chloride electrodes connected to the preparation through agar-saline/filter paper bridges. Signals were amplified, filtered (0.5 Hz) and displayed on a chart recorder (Lectromed Multitrace 8). Each compartment of the bath was perfused continuously with Krebs solution, preheated to 27°C and gassed with 95% $O_2$/5% $CO_2$, at a rate of 1–2 ml $min^{-1}$. Drugs were applied at known concentrations into the perfusate of the first compartment only.

### *Experimental protocols*

After a 30 min equilibration period the viability of each preparation was assessed by exposure to a near maximal concentration of 5-hydroxytryptamine (5-HT, $1 \times 10^{-5}$ M) for 2 min. Preparations that depolarized by less than 250 μV were rejected (< 10% of preparations). Repeated exposures to 5-HT ($1 \times 10^{-5}$ M) for 2 min at 30 min intervals were performed until reproducible depolarizations were obtained. After a further 30 min re-equilibration period, concentration-effect curves to either α,β-methylene ATP or 5-HT were constructed non-cumulatively using serially increasing concentrations. Each concentration of agonist was applied for 2–2.5 min during which time a peak effect was reached. An interval of 45 min was left between agonist applications since in preliminary experiments depolarizing responses to α,β-methylene ATP showed tachyphylaxis if shorter intervals were employed. Use of this protocol precluded construction of more than one concentration-effect curve in any one preparation. Since there was little variation in the sensitivity to the agonist between control preparations from different animals, the effects of antagonists were determined by comparing with respective time- and vehicle-matched control curves from other preparations obtained from either the same or from different animals. Antagonist contact times of 45 min were employed.

In some experiments the time taken for antagonists to achieve equilibrium was investigated. In these studies preparations were exposed repeatedly to a near maximal ($EC_{80}$) concentration of α,β-methylene ATP ($3 \times 10^{-5}$ M) at 45 min intervals until reproducible responses were obtained (within 15%). The antagonist was then added to the perfusate and responses to α,β-methylene ATP were then re-determined (at 45 min intervals). Once reproducible responses to α,β-methylene ATP in the presence of the antagonist were observed, the antagonist was removed from the perfusate. To assess the reversibility of the antagonist, responses to α,β-methylene ATP were determined throughout a 90–135 min washout period.

### *Analysis of results*

The depolarization induced by agonists was measured as the peak change (μV) in the d.c. potential between the two compartments, and expressed as a percentage of the response to 5-HT ($1 \times 10^{-5}$ M). To quantify agonist potency, the concentration of agonist required to produce 50% of the maximal response was determined. This is referred to as the $EC_{50}$ value. Antagonist effects were quantified, where possible, by measuring lateral displacements of agonist concentration-effect curves at the $EC_{50}$ level of the control concentration-effect curve to give concentration-ratios for antagonist-treated and control, vehicle-treated preparations. To determine whether antagonists had any effect upon the maximal response to the agonist the maximum response in the presence of the antagonist was expressed as a percentage of the maximum response in the control preparation. The data with suramin were analysed using the method of Arunlakshana & Schild (1959). Since cibacron blue only caused a parallel shift of the agonist concentration-effect curve at $1 \times 10^{-4}$ M, an estimate of $pK_B$ was derived from the concentration-ratios obtained at this antagonist concentration. An apparent $pK_B$ value for antagonist effects of iso-PPADS was calculated using a double reciprocal plot followed by the equation:

$$pK_B \text{ estimate} = -\log \frac{[A]}{(\text{slope} - 1)}$$

where [A] = the molar concentration of antagonist and slope refers to the slope of the double reciprocal plot of equieffective agonist concentrations (Kenakin, 1993).

For studies on the equilibration of the antagonists the first response to α,β-methylene ATP was assigned a value of unity and subsequent responses were expressed as a percentage of this value. The changes in the response to α,β-methylene ATP following addition or removal of the antagonist were corrected for spontaneous changes in the amplitude of the res-

ponse of the respective time- and vehicle-matched control preparation.

All data are expressed either as arithmetic mean ± s.e.mean or geometric mean with 95% confidence limits where appropriate. $n$ refers to the number of preparations. Differences between groups were assessed by Student's $t$ test or an analysis of variance and considered significant when $P<0.05$.

### *Drugs and solutions*

The composition of the Krebs solution was as follows (mM in de-ionised water): NaCl 118, $NaHCO_3$ 25, KCl 4.7, $MgSO_4.7H_2O$ 0.6, $KH_2PO_4$ 1.2, D-glucose 11.1, $CaCl_2.6H_2O$ 1.3. The following drugs were used: 5-hydroxytryptamine creatinine sulphate (5-HT), α,β-methylene ATP lithium salt, cibacron blue 3GA, (+) bicuculline, hexamethonium bromide, phentolamine hydrochloride (all Sigma), suramin (Bayer), pyridoxalphosphate-6-azophenyl-2', 5'-disulphonic acid (iso-PPADS, Cookson Chemicals Ltd), 8-para-sulphophenyltheophylline (Research Biochemical Incorporated), ondansetron hydrochloride (Glaxo Group Research). All drugs were dissolved and diluted to the required concentration in Krebs solution, and stored on ice.

## Results

α,β-Methylene ATP ($1\times10^{-6}$–$3\times10^{-4}$ M) induced concentration-related depolarization of the rat isolated vagus nerve. The maximum amplitude of this effect was 202.2 ± 7.1% of the response to 5-HT ($1\times10^{-5}$ M) and the mean $EC_{50}$ value was $1.11\times10^{-5}$ M ([0.95–1.29]; $n=43$).

### *Antagonist effects of suramin*

In the presence of suramin ($1\times10^{-5}$–$1\times10^{-4}$ M) the concentration-effect curves to α,β-methylene ATP were displaced to the right (see Figure 1). Analysis of the concentration-ratios by the method of Arunlakshana & Schild (1959) yielded a $pA_2$ value of 5.90 ± 0.71 for suramin with a slope value that was significantly less than unity (0.47 ± 0.22, $P<0.05$).

### *Antagonist effects of cibacron blue*

Cibacron blue, at a concentration of $1\times10^{-5}$ M and $3\times10^{-5}$ M, tended to increase the maximal response to α,β-methylene ATP. The higher concentrations ($3\times10^{-5}$–$1\times10^{-4}$ M), displaced the agonist concentration-effect curves to the right (Figure 2). At $1\times10^{-4}$ M, the mean concentration-ratio was 10.3 [6.1–17.2] which corresponded to a $pK_B$ estimate of 4.96 ± 0.11 ($n=5$).

### *Antagonist effect of iso-PPADS*

Iso-PPADS ($1\times10^{-6}$–$1\times10^{-5}$ M) depressed the maximal response to α,β-methylene ATP in a concentration-related manner (Figure 3). When analysed by a double reciprocal plot according to the method of Kenakin (1993) these data yielded a $pK_B$ estimate of 6.02 ± 0.10.

### *Kinetics of antagonist equilibration and washout*

The antagonist effect of suramin ($1\times10^{-4}$ M) reached apparent equilibrium in less than 90 min and could be readily reversed on washout (<45 min; Figure 4). In contrast, cibacron blue ($1\times10^{-4}$ M) was very slow to reach equilibrium (>2 h) but could be fully reversed on washout for 90 min (Figure 4). Iso-PPADS ($1\times10^{-6}$ M) was found to reach apparent equilibrium in less than 90 min (Figure 4). After

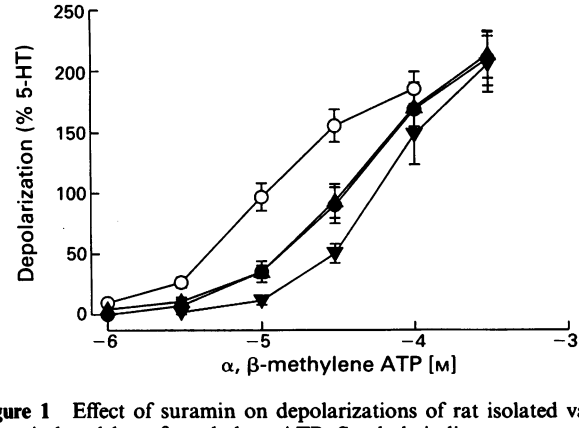


**Figure 1** Effect of suramin on depolarizations of rat isolated vagus nerve induced by α,β-methylene ATP. Symbols indicate responses to α,β-methylene ATP in the presence of vehicle (○) or in the presence of $1\times10^{-5}$ M (▲), $3\times10^{-5}$ M (●) or $1\times10^{-4}$ M suramin (▼). Each point represents the mean of $n$ determinations with s.e.mean. ($n$ = 4–12). The abscissa scale represents the log molar concentration of drug and the ordinate scale the depolarizing responses expressed as percentages of the depolarization induced by 5-HT ($1\times10^{-5}$ M).

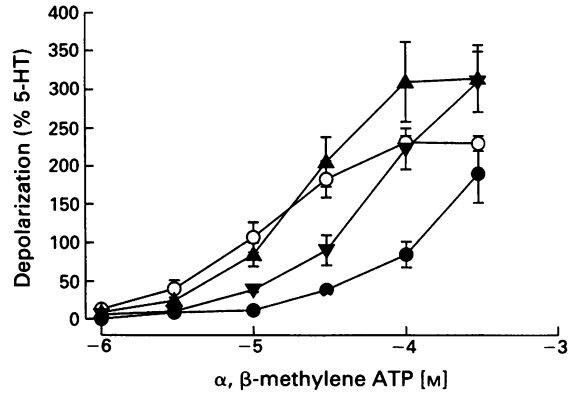


**Figure 2** Effect of cibacron blue on depolarization of rat isolated vagus nerve induced by α,β-methylene ATP. Symbols indicate responses to α,β-methylene ATP in the presence of vehicle (○) or in the presence of $1\times10^{-5}$ M (▲), $3\times10^{-5}$ M (▼) or $1\times10^{-4}$ M cibacron blue (●). Each point represents the mean of $n$ determinations with s.e.mean ($n$ = 4–6). The abscissa scale represents the log molar concentration of drug and the ordinate scale the depolarizing responses expressed as percentages of the depolarization induced by 5-HT ($1\times10^{-5}$ M).

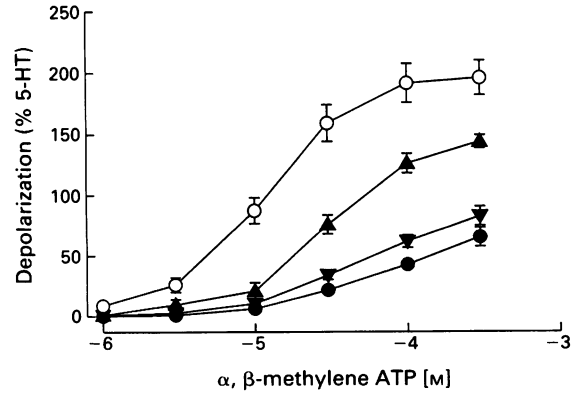


**Figure 3** Effect of iso-PPADS on depolarization of rat isolated vagus nerve induced by α,β-methylene ATP. Symbols indicate responses to α,β-methylene ATP in the presence of vehicle (○) or in the presence of $1\times10^{-6}$ M (▲), $3\times10^{-6}$ M (▼) or $1\times10^{-5}$ M iso-PPADS (●). Each point represents the mean of $n$ determinations with s.e.mean ($n$ = 6–12). The abscissa scale represents the log molar concentration of drug and the ordinate scale the depolarizing responses expressed as percentages of the depolarization induced by 5-HT ($1\times10^{-5}$ M).

90 min washout the antagonist effect of iso-PPADS ($1 \times 10^{-6}$ M) was fully reversed.

### *Combination antagonism; suramin and cibacron blue*

When administered in combination, the antagonist effects of suramin ($1 \times 10^{-4}$ M) and cibacron blue ($3 \times 10^{-5}$–$1 \times 10^{-4}$ M) were not additive (Figure 5). The displacement of the concentration-effect curve to α,β-methylene ATP in the presence of suramin alone (mean concentration ratio 11.7 [3.1–44.3], $n = 4$) was not significantly different from the displacement in the presence of suramin and $3 \times 10^{-5}$ M cibacron blue (6.8 [3.7–12.4], $n = 4$) or suramin and $1 \times 10^{-4}$ M cibacron blue (11.8 [6.6–21.0], $n = 4$).

### *Combination antagonism; suramin and iso-PPADS*

In the presence of suramin ($1 \times 10^{-4}$ M) the antagonist effect of iso-PPADS ($1 \times 10^{-5}$ M) was partially reversed (see Figure 6) but because of the nature of the antagonism produced by iso-PPADS no attempt was made to quantify this reversal.

### *Specificity of putative $P_2$ purinoceptor antagonists*

To determine the specificity of the putative $P_2$ purinoceptor antagonists for responses induced by α,β-methylene ATP the effects of these agents were tested against depolarizations to 5-HT ($1 \times 10^{-7}$–$3 \times 10^{-5}$ M). In control preparations, 5-HT evoked concentration-related depolarizations with a mean $EC_{50}$ value of $7.2 \times 10^{-7}$ M [5.4–9.6; $n = 12$]. Suramin ($1 \times 10^{-4}$ M) had no effect on either the potency (mean concentration-ratio 1.2 [0.9–1.7]) or the maximal response of 5-HT (95.5 ± 17.3%; $n = 4$). Similarly, at the lower concentrations tested neither iso-PPADS ($1$–$3 \times 10^{-6}$ M) nor cibac-

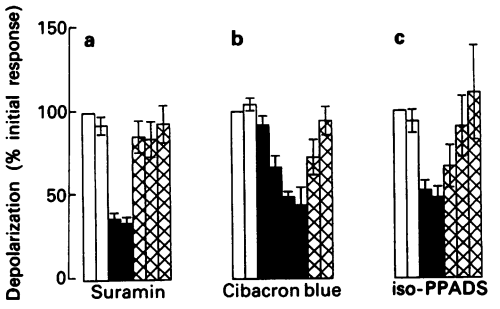


**Figure 4** The equilibration and washout of $P_2$ purinoceptor antagonists in the rat isolated vagus nerve, as determined by the antagonism of depolarizations to α,β-methylene ATP. Data shown are responses expressed as a percentage of the initial depolarization response induced by α,β-methylene ATP ($3 \times 10^{-5}$ M). Panels (a) and (b) and (c) illustrate the effect of suramin ($1 \times 10^{-4}$ M), cibacron blue ($1 \times 10^{-4}$ M) and iso-PPADS ($1 \times 10^{-6}$ M), respectively, on depolarizations to an $EC_{80}$ concentration of α,β-methylene ATP ($3 \times 10^{-5}$ M). In each case the first open column indicates the control response (assigned a value of 1) and the second column a subsequent response 45 min later. At this time point the antagonist was added to the perfusate. The solid and hatched columns represent responses in the presence of antagonist and after washout of the antagonist, respectively. The amplitude of these responses have been corrected for the spontaneous decline of the control response observed with time. All values shown are the mean of $n$ determinations and the vertical bars represent the s.e.mean ($n = 6$).

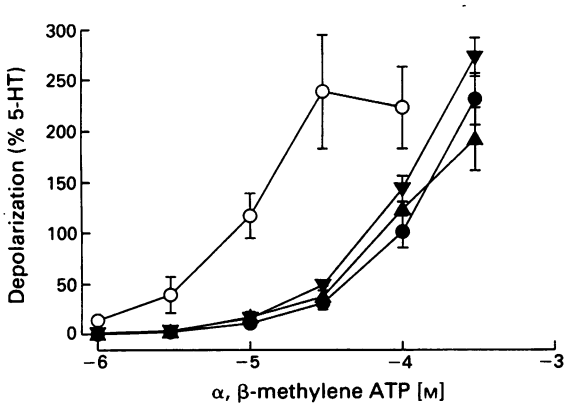


**Figure 5** Effect of a combination of suramin and cibacron blue on depolarizations of rat isolated vagus nerve induced by α,β-methylene ATP. Symbols indicate response to α,β-methylene ATP in the presence of vehicle (○) or in the presence of $1 \times 10^{-4}$ M suramin alone (▲), $1 \times 10^{-4}$ M suramin plus $3 \times 10^{-5}$ M cibacron blue (▼) or $1 \times 10^{-4}$ M suramin plus $1 \times 10^{-4}$ M cibacron blue (●). Each point represents the mean of $n$ determinations with s.e.mean ($n = 6$). The abscissa scale represents the log molar concentration of drug and the ordinate scale the depolarizing responses expressed as percentages of the depolarization induced by 5-HT ($1 \times 10^{-5}$ M).

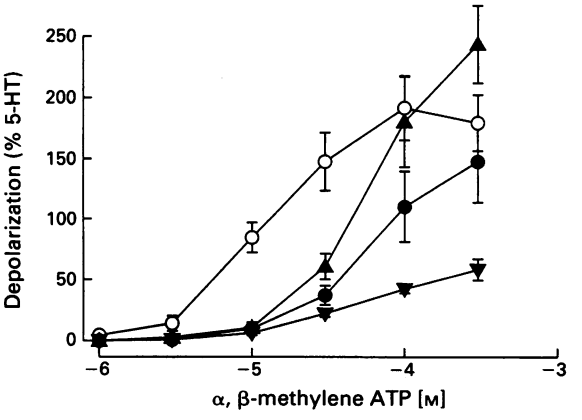


**Figure 6** Effect of a combination of suramin and iso-PPADS on depolarizations of rat isolated vagus nerve induced by α,β-methylene ATP. Symbols indicate responses to α,β-methylene ATP in the presence of vehicle (○) or in the presence of $1 \times 10^{-4}$ M suramin alone (▲), $1 \times 10^{-5}$ M iso-PPADS (▼) or $1 \times 10^{-4}$ M suramin plus $1 \times 10^{-5}$ M iso-PPADS (●). Each point represents the mean of $n$ determinations with s.e.mean ($n = 6$). The abscissa scale represents the log molar concentration of drug and the ordinate scale the depolarizing responses expressed as percentages of the depolarization induced by 5-HT ($1 \times 10^{-5}$ M).

**Table 1** Effect of antagonists and inhibitors on depolarizations of rat isolated nerve to α,β-methylene ATP

| *Drug* | *Concn* [M] | *Mean CR* | *95% CI* | *Mean max* | *s.e.mean* | n |
|---|---|---|---|---|---|---|
| Indomethacin | $3 \times 10^{-6}$ | 1.1 | 0.4–2.8 | 91.9 | 3.9 | 4 |
| Bicuculline | $1 \times 10^{-5}$ | 1.4 | 0.6–3.5 | 87.8 | 7.2 | 4 |
| 8pSPT | $3 \times 10^{-5}$ | 1.0 | 0.3–4.1 | 100.3 | 11.3 | 4 |
| Hexamethonium | $1 \times 10^{-4}$ | 1.0 | 0.4–2.3 | 97.3 | 15.7 | 5 |
| Phentolamine | $1 \times 10^{-6}$ | 1.0 | 0.3–3.2 | 82.6 | 9.0 | 5 |
| Ondansetron | $1 \times 10^{-7}$ | 1.0 | 0.5–1.9 | 98.8 | 5.3 | 4 |

Values shown are geometric mean concentration-ratios (CRs) with 95% confidence intervals (CI) and relative maximum depolarizations (arithmetic mean ± s.e.mean) when compared to time-matched control preparations. $n$ refers to the number of different nerve preparations. 8pSPT = 8-para-sulphophenyltheophylline.

ron blue ($1-3 \times 10^{-5}$ M) attenuated responses to 5-HT. At higher concentrations both iso-PPADS ($1 \times 10^{-5}$ M) and cibacron blue ($1 \times 10^{-4}$ M) produced a small but significant rightward shift in the curve to 5-HT (mean concentration-ratios 2.2 [1.9–2.7] and 2.6 [2.0–3.5], respectively) but had no significant effect on the maximal response (87.0 ± 6.6% and 94.4 ± 7.2%, respectively; both $n = 4$).

### *Effects of other antagonists/inhibitors*

A range of antagonists specific to receptor types other than $P_2$ purinoceptors were tested against responses to α,β-methylene ATP (see Table 1). Bicuculline ($1 \times 10^{-5}$ M), 8-parasulphophenyltheophylline ($3 \times 10^{-5}$ M), hexamethonium ($1 \times 10^{-4}$ M), phentolamine ($1 \times 10^{-6}$ M) and ondansetron ($1 \times 10^{-7}$ M) all failed to antagonize responses to α,β-methylene ATP (Table 1). Similarly, the cyclo-oxygenase inhibitor, indomethacin ($3 \times 10^{-6}$ M), did not attenuate the depolarization responses to α,β-methylene ATP.

## Discussion

The most important finding of the present study was that three chemically dissimilar $P_2$ purinoceptor antagonists, namely suramin, cibacron blue and iso-PPADS, specifically antagonized depolarizations of the rat isolated vagus nerve to α,β-methylene ATP.

Suramin produced concentration-related rightward displacements of the concentration-effect curve to α,β-methylene ATP. However, the slope of the Schild plot was less than would be anticipated if this agent acted as a simple competitive antagonist at equilibrium with a homogeneous receptor population activated by α,β-methylene ATP. Since we have shown that in the rat vagus nerve, equilibration of suramin was achieved relatively rapidly (<90 min) it seems unlikely that an insufficient contact time for the antagonist explains the shallow slope of the Schild regression. The most obvious explanation that the agonist may have been activating a heterogeneous receptor population is discussed later.

In this study there was little or no increase in the apparent maximum response to α,β-methylene ATP in the presence of suramin. However, we have found that depolarizations produced by ATP in the rat vagus nerve were markedly augmented by suramin (Trezise *et al.*, 1993). Furthermore, augmentation by suramin of contractile responses of certain smooth muscles (Hoyle *et al.*, 1990; Blakeley *et al.*, 1991; Mallard *et al.*, 1992) and of membrane currents in neurones of the rat medial habenula (Edwards *et al.*, 1992) induced by α,β-methylene ATP has been reported frequently. Interestingly, at low concentrations, cibacron blue did augment responses of the rat vagus nerve to α,β-methylene ATP. At these concentrations the antagonist did not significantly affect depolarizations to 5-HT. It is, therefore, tempting to try to interpret these findings in terms of specific interactions with purinoceptors. One simple explanation would be that both suramin and cibacron blue antagonize an inhibitory receptor activated by high concentrations of the agonist, thereby revealing the 'true' maximum excitatory response. Indeed, in the mouse vas deferens there are data to suggest that α,β-methylene ATP activates such an inhibitory receptor (Blakeley *et al.*, 1991). However, using a wide range of ATP analogues we could find no evidence for a purinoceptor that mediates hyperpolarization of the vagus nerve and, therefore, we feel that this explanation is unlikely (Trezise *et al.*, 1993). Another possibility is that suramin and cibacron blue may bind to a subunit on the purinoceptor to facilitate allosterically agonist effects. In skeletal muscle, suramin has already been shown to modulate nicotinic receptor function by what appears to be an allosteric mechanism (Henning *et al.*, 1992). An interaction of cibacron blue and suramin with ATPase or GTPase enzymes important in either the transduction of purinoceptor-mediated responses or the metabolism of the agonist, could cause augmentation of responses to α,β-methylene ATP (Wills & Wormall, 1950; Butler *et al.*, 1988; Hourani & Chown, 1989). However, no definitive evidence to support any of these hypotheses was obtained in the present study and therefore the mechanism(s) underlying this phenomenon remains unclear.

At higher concentrations ($3 \times 10^{-5}$–$1 \times 10^{-4}$ M), cibacron blue antagonized responses of the rat vagus nerve to α,β-methylene ATP. It could be argued that this finding suggests an action of α,β-methylene ATP at $P_{2Y}$ purinoceptors, since, at the same concentration-range, cibacron blue selectively antagonizes $P_{2Y}$-compared to $P_{2X}$-mediated responses in the rabbit mesenteric artery (Burnstock & Warland, 1987). However, in other smooth muscle preparations these concentrations of cibacron blue attenuate responses mediated not only via $P_{2Y}$ receptors but also via $P_{2X}$ receptors (Reilly *et al.*, 1987; Fedan & Lamport, 1990) and via α-adrenoceptors and cholinoceptors (Fedan & Lamport, 1990). Taken together, these findings indicate that cibacron blue is not a selective $P_{2Y}$-purinoceptor antagonist. Thus, given our previous observation that the selective $P_{2Y}$-purinoceptor agonist 2-methylthioATP is only a very weak agonist in the rat vagus nerve (Trezise *et al.*, 1993), it seems unlikely that a $P_{2Y}$ receptor is involved in the depolarizing response to α,β-methylene ATP.

To determine whether cibacron blue and suramin were antagonizing responses to α,β-methylene ATP by blocking a common receptor site, the profile of antagonism of the combination of these two antagonists was investigated. In theory, if the two antagonists interact with the same population of receptors it can be shown by resultant analysis that the separate displacements of the agonist concentration-effect curve by each antagonist are additive (Paton & Rang, 1965). Surprisingly, in the presence of suramin ($1 \times 10^{-4}$ M), cibacron blue ($3 \times 10^{-5}$–$1 \times 10^{-4}$ M) produced no further shift in the α,β-methylene ATP concentration-effect curve. The most plausible explanation for this finding is that, at high concentrations, the agonist activates a population of receptors that is resistant to blockade by both cibacron blue and suramin. This observation could explain the low slope of the Schild regression observed with suramin. Interestingly, from radioligand binding experiments it is clear that in several other rat tissues, including vas deferens, spleen and striatum, α,β-methylene ATP recognises more than one site (Michel & Humphrey, 1993). Furthermore, we have previously demonstrated that other purines, including ATP, depolarize the rat vagus nerve via activation of receptors that are insensitive to suramin (Trezise *et al.*, 1993). Thus, a suramin-resistant component of responses evoked by high concentrations of α,β-methylene ATP is not entirely unexpected.

The putative $P_2$ purinoceptor antagonist, iso-PPADS, also inhibited depolarizations of the rat vagus nerve induced by α,β-methylene ATP. This represents the first full report of the antagonist activity of this compound at $P_2$ purinoceptors. The parent isomer, PPADS, reportedly antagonizes purinoceptor-mediated responses of the rabbit (Lambrecht *et al.*, 1992) and guinea-pig vas deferens (McLaren *et al.*, 1993). In the guinea-pig vas deferens, other actions of PPADS unrelated to purinoceptor antagonism were also apparent including smooth muscle cell membrane depolarization (McLaren *et al.*, 1993). In the present study, the highest concentration of iso-PPADS tested ($1 \times 10^{-5}$ M) had no detectable depolarizing effect *per se* and produced only a small antagonism of responses to 5-HT, indicating a degree of selectivity for purinoceptors.

The concentration-related depression in the maximum response to α,β-methylene ATP produced by iso-PPADS is a profile of antagonism classically observed with irreversible antagonists (Furchgott, 1966). To test whether iso-PPADS was acting as an irreversible antagonist in the rat vagus nerve, the reversal of the antagonism of responses to α,β-methylene ATP on washout of iso-PPADS was investigated. In these experiments, it was evident that the inhibitory effect of iso-PPADS could be reversed by continual washout, albeit

more slowly than the washout of the antagonism produced by suramin. Importantly, these findings indicate that iso-PPADS is not an irreversible antagonist at purinoceptors in the vagus nerve.

However, it remains tenable that the depression of the concentration-effect curve to α,β-methylene ATP produced by iso-PPADS is a consequence of a slow dissociation of the antagonist from the receptor. We attempted to address this issue by performing experiments similar to those described for other antagonists that give profiles of insurmountable antagonism. In rabbit aorta, for example, the antagonism of contractions to angiotensin II by slowly-dissociating non-peptide angiotensin antagonists can be partially reversed by co-administration of a more rapidly dissociating (competitive) antagonist that interacts with the same receptor (e.g. Robertson *et al.*, 1992; Wienen *et al.*, 1993). Theoretically, the agonist can more readily equilibrate with receptors occupied by the rapdily-dissociating antagonist than those occupied by the slowly-dissociating antagonist. The antagonist that dissociates rapidly thus provides a degree of 'protection' for the receptor from the slowly dissociating antagonist, and consequently a larger agonist response is evoked. By analogy, it might be anticipated that if suramin dissociates more rapidly from the receptor than iso-PPADS then a similar phenomenon of receptor protection would be observed. When suramin and iso-PPADS were co-administered the depolarizing response of the rat vagus nerve to α,β-methylene ATP appeared to be antagonized less than when iso-PPADS was given alone. It is tempting, therefore, to interpret this finding as evidence that iso-PPADS acts as a slowly-reversible purinoceptor antagonist in this preparation leading to a pseudo-equilibrium situation between agonist and antagonist. However, since suramin itself tended to produce an increase in the response to high concentrations of α,β-methylene ATP, notably in our previous experiments (Trezise *et al.*, 1993), this interpretation is equivocal. Moreover, such a conclusion from this finding would assume that iso-PPADS and suramin interact at a common site on the receptor to antagonize responses to the agonist. Although preliminary findings from radioligand binding experiments on the rat vas deferens suggest that both suramin and iso-PPADS do compete for common binding sites occupied by α,β-methylene ATP (Khakh, Michel & Humphrey, unpublished observations), there is no evidence from analagous studies in the vagus nerve that this is the case. Thus, in conclusion, although the evidence is consistent with the concept that iso-PPADS acts as a slowly-reversible purinoceptor antagonist in the rat vagus nerve, further experiments are required to confirm this.

The inability of 8-para-sulphophenyltheophylline, ondansetron, bicuculline, phentolamine and hexamethonium to antagonize depolarization responses to α,β-methylene ATP indicates that $P_1$ purinoceptors, 5-$HT_3$ receptors, $GABA_A$ receptors, α-adrenoceptors and nicotinic cholinoceptors, respectively, are not involved in this response. The concentrations of each antagonist employed in the present study have been shown previously to antagonize responses of the rat vagus nerve, or of other preparations, mediated via activation of these receptor types (Ireland & Tyers, 1987; Butler *et al.*, 1990). In other systems the effects of purinoceptor agonists are mediated via the release of prostaglandins (see Burnstock, 1989 for review). However, indomethacin was without effect on depolarizations produced by α,β-methylene ATP, demonstrating that cyclo-oxygenase products are not involved in this response.

In summary, we have shown that three $P_2$ purinoceptor antagonists of diverse chemical structure specifically antagonize depolarization responses of the rat vagus nerve induced by α,β-methylene ATP. A $P_1$ purinoceptor antagonist was inactive. Thus, these observations confirm and extend our original proposal that in this neuronal preparation, responses to α,β-methylene ATP are mediated predominantly via specific $P_2$ purinoceptors. The relatively high potency of α,β-methylene ATP suggests that its effects are mediated predominantly by $P_{2X}$ purinoceptors (Trezise *et al.*, 1993). However, the findings that responses induced by high concentrations of agonist were resistant to blockade by suramin and cibacron blue, but could be attenuated by iso-PPADS, adds further weight to our proposal that the purinoceptor population is not entirely homogeneous in this preparation.

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# The effect of nitric oxide synthase inhibition on the plasma fibrinolytic system in septic shock in rats

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**1** We have investigated the effect of pretreatment of rats with nitric oxide (NO) synthase inhibitor, $N^G$-nitro-L-arginine methyl ester (L-NAME) on the *E. coli* lipopolysaccharide (LPS)-induced changes in the plasma fibrinolytic system, platelet count, fibrinogen level, as well as in gross and microscopic pathophysiological changes indicative of disseminated intravascular coagulation (DIC) in rats.

**2** *E. coli* LPS (6 mg $kg^{-1}$, i.p.) produced a decrease in the levels of plasma fibrinogen and a drop in the blood platelet count 6 h after administration. The decrease in fibrinogen but not the drop in platelet count was reversed by pretreatment with L-NAME (30 mg $kg^{-1}$, i.p., 24 h and 15 min before administration of LPS).

**3** Pretreatment with L-NAME antagonized the LPS-induced activation of fibrinolysis as measured by changes in the euglobulin clot lysis time (ECLT) and enhanced the LPS-induced rise in the plasma level of plasminogen activator inhibitor (PAI). In animals pretreated with L-NAME there was also a marked reduction in the histological changes indicative of DIC.

**4** We propose that L-NAME can act as a protective agent in LPS-induced DIC, and this protection is due to an increased generation of PAI following inhibition of NO synthase.

**Keywords:** Lipopolysaccharide; fibrinolysis; disseminated intravascular coagulation (DIC); plasminogen activator inhibitor (PAI); nitric oxide synthase inhibition

## Introduction

Administration of lipopolysaccharide (LPS) *in vivo* induces septic shock, characterized by hypotension, vascular injury, and disseminated intravascular coagulation (DIC), which leads to a fatal dysfunction of various organs (Proctor, 1986). Both the early and late phase hypotension after LPS administration are associated with over-production of nitric oxide (NO) within the vasculature (Thiemermann & Vane, 1990; Nava *et al.*, 1991; Petros *et al.*, 1991; Szabo *et al.*, 1993) the latter stage being characterized by the induction of NO synthase in various organs (Salter *et al.*, 1991). Although inhibitors of NO synthase may be beneficial in clinical treatment of endotoxaemia (Petros *et al.*, 1991), it is not yet clear whether such treatment could affect LPS-induced DIC. Our previous studies (Korbut *et al.*, 1990; Lidbury *et al.*, 1990) showed that NO was highly effective in protecting platelets against the release of plasminogen activator inhibitor (PAI), the level of which in plasma has been found to contribute to the pathogenesis of DIC in septicaemic patients (Paramo *et al.*, 1988). We were, therefore, interested in whether the protective effects of NO synthase inhibitors were in part related to their ability to influence DIC formation. We have examined the effects of pretreatment with the NO synthase inhibitor, $N^G$-nitro-L-arginine methyl ester (L-NAME, Moore *et al.*, 1989) on the LPS-induced changes in euglobulin clot lysis time (ECLT), platelet count and fibrinogen and plasminogen activator inhibitor (PAI) levels in the rat.

## Methods

Male Wistar rats (220–270 g) were injected with $N^G$-nitro-L-arginine methyl ester (30 mg $kg^{-1}$, i.p.) or vehicle, 24 h and 15 min before administration of *E. coli* LPS (6 mg $kg^{-1}$, i.p.) or vehicle (saline). Six h later, animals were anaesthetized (pentobarbitone, 40 mg $kg^{-1}$, i.p.) and blood samples taken from the carotid artery into tri-sodium citrate (3.15% w/v) at a ratio of 9:1. Animals were then killed by exsanguination and examined *post-mortem* for gross and microscopic (following staining with haematoxylin-eosin) pathophysiological changes indicative of DIC. Plasma from the blood samples were prepared by centrifugation (14.900 *g* for 3 min, Biofuge A, Heraeus) and used to assess euglobulin clot lysis time (ECLT), platelet count, fibrinogen levels and plasminogen activator inhibitor (PAI) activity. ECLT was assayed according to the method of Von Kaulla & Schultz (1958), as described previously (Lidbury *et al.*, 1990). The levels of fibrinogen and PAI were measured with commercially available kits.

### *Materials*

*E. coli* endotoxin (LPS serotype 0127:B8) and $N^G$-nitro-L-arginine methyl ester hydrochloride were obtained from Sigma. Assay kits for fibrinogen and PAI were purchased from MeDiTech, Inc. (U.S.A.) and Biopool (Sweden), respectively.

### *Statistics*

Mean values ± s.e.mean were compared by ANOVA and $P<0.05$ taken as significant.

## Results

### *Effects on coagulation system*

LPS treatment produced a decrease in the levels of plasma fibrinogen (Figure 1) and a drop in the blood platelet count by 64 ± 11% from 480 ± 30 × $10^3/mm^3$ in controls to 172 ± 21 × $10^3/mm^3$ ($n = 15$). The decrease in fibrinogen (Figure 1) but not the drop in platelet count was reversed by pretreatment with L-NAME. L-NAME had no effect on plasma fibrinogen levels in control animals (Figure 1).

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Administration of LPS significantly activated fibrinolysis, as measured by shortening of the ECLT (Figure 2), and significantly increased plasma PAI activity (Figure 3). Pretreatment with L-NAME antagonized the LPS-induced activation of fibrinolysis (Figure 2) and enhanced the LPS-induced rise in plasma PAI (Figure 3). L-NAME had no effect on either the plasma fibrinolytic activity or PAI levels in control animals (Figures 2 and 3).

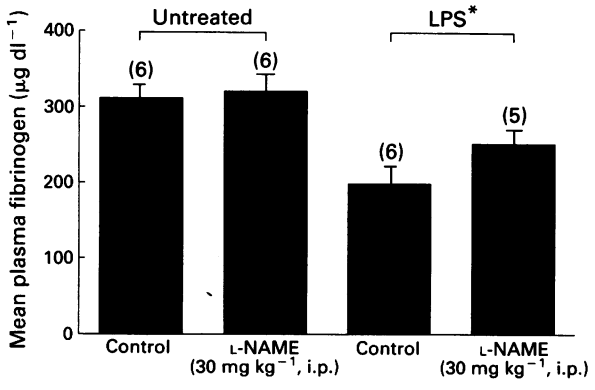


**Figure 1** Effect of $N^G$-nitro-L-arginine methyl ester (L-NAME, 30 mg $kg^{-1}$, i.p., 24 h and 15 min before lipopolysaccharide, LPS) on the decrease in plasma fibrinogen produced by *E. coli* LPS (6 mg $kg^{-1}$, i.p.). $^*P<0.05$ as compared to corresponding control. Numbers in parentheses indicate number of experiments.

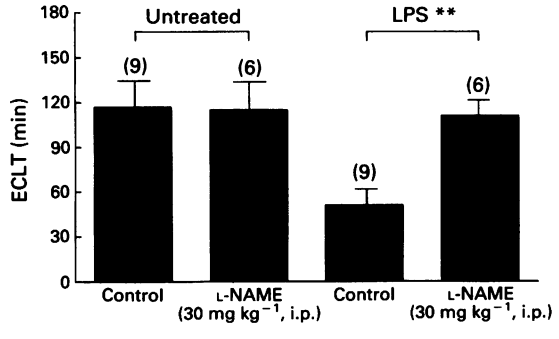


**Figure 2** Effect of $N^G$-nitro-L-arginine methyl ester (L-NAME, 30 mg $kg^{-1}$ i.p., 24 h and 15 min before lipopolysaccharide, LPS) on the shortening of euglobulin clot lysis time (ECLT) produced by *E. coli* LPS (6 mg $kg^{-1}$, i.p.). $^{**}P<0.01$ as compared to corresponding control. Numbers in parentheses indicate number of experiments.

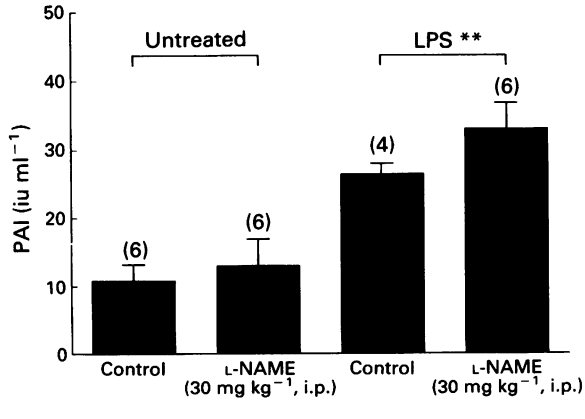


**Figure 3** Effect of $N^G$-nitro-L-arginine methyl ester (L-NAME, 30 mg $kg^{-1}$ i.p., 24 h and 15 min before lipopolysaccharide, LPS) on the increase of plasminogen activator inhibitor (PAI) levels produced by *E. coli* LPS (6 mg $kg^{-1}$, i.p.). $^{**}P<0.01$ as compared to corresponding control. Numbers in parentheses indicate number of experiments.

### *Histological effects*

In animals treated with LPS there were pathological changes including diffuse haemorrhagic congestion of the small intestine and ecchymotic haemorrhages in visceral organs including the heart, lungs spleen and kidneys. Microscopic examination revealed numerous fibrin microthrombi and leukocyctic and erythrocytic aggregates in small blood vessels of the lung, heart, spleen, kidneys and intestine ($n = 7$).

When animals were pretreated with L-NAME there was a marked reduction in the pathological changes seen following treatment with LPS. Very little haemorrhagic congestion of the intestine was seen and ecchymotic haemorrhages in the visceral organs were largely absent.

## Discussion

Disseminated intravascular coagulation (DIC), apart from causing hypotension, is considered to be an important cause of circulatory shock and death evoked by endotoxin (Bick & Kunkel, 1992). DIC is characterized by an abnormal simultaneous activation of the blood coagulation and fibrinolytic systems with resultant formation of microthrombi, obstruction of blood flow as well as internal haemorrhage. Here, we show that L-NAME, a potent inhibitor of NOS (Moore *et al.*, 1990) prevents the reduction in plasma fibrinogen level produced by LPS and partially protects against the accompanying haemorrhagic congestion of the intestine and viscera. In adddition, L-NAME significantly suppressed the activation of the plasma fibrinolytic system induced by LPS, as assessed by changes in the ECLT. This antifibrinolytic effect of L-NAME correlated well with the L-NAME-induced rise of the plasma level of PAI. It is worthy of note that L-NAME was effective at a moderate dose of 30 mg $kg^{-1}$, which is similar to the dose of $N^G$-monomethyl-L-arginine, another inhibitor of NO synthase, that is beneficial in combatting the fall in blood pressure produced by intravenous administration of LPS (Nava *et al.*, 1991). In these experiments a tenth of the dose of $N^G$-monomethyl-L-arginine was ineffective whereas a 10 times higher dose (300 mg $kg^{-1}$) accelerated and enhanced the fall in blood pressure. Interestingly, in our experiments L-NAME attenuated most of the symptoms of DIC induced by LPS, suggesting that thrombocytopenia and associated intravascular platelet aggregation may not be a crucial event in this disorder.

The mechanism by which LPS causes DIC has been a matter of considerable debate and a number of pathophysiological actions of LPS have been implicated as the primary cause (Bick & Kunkel, 1992). These include, stimulation of platelet aggregation, increased procoagulant activity of monocytes and macrophages and increased cellular release of platelet activating factor and thromboxane $A_2$, as well as the stimulation of fibrinolysis through activation of Factor XII and the release of plasminogen pro-activator (van Deventer *et al.*, 1990; Quax *et al.*, 1990). In addition, recent studies point to augmented plasma levels of PAI contributing to the pathogenesis of DIC in septicaemic patients (van Deventer *et al.*, 1990). Thus, LPS-induced DIC is a very complex phenomenon. The results of our study demonstrate that the beneficial effects produced by inhibition of NO synthase on the symptoms of LPS-induced DIC correlate with an increased generation of PAI, which is in agreement with our previous observation that NO inhibits the release of PAI (Korbut *et al.*, 1990; Lidbury *et al.*, 1990). Possibly, the increased generation of PAI in septicaemia is a compensatory mechanism against excessive activation of the fibrinolytic system produced by NO. This could well be of great importance, considering that the level of PAI is critical in regulating the fibrinolytic system. We would, therefore, sug-

gest that L-NAME can act as a protective agent in LPS-induced DIC, and this protection is due to an increased generation of PAI following inhibition of NO synthase.

Further studies on the beneficial effects of NO synthase inhibitors in other types of circulatory shock, including those produced by haemorrhage and trauma may provide new insights into the understanding of the fibrinolytic system and its regulation in health and disease.

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# Effect of a 5-lipoxygenase inhibitor and leukotriene antagonist (PF 5901) on antigen-induced airway responses in neonatally immunized rabbits

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**1** The effect of a single intratracheal dose (10 mg) of PF 5901 (2-[3(1-hydroxyhexyl) phenoxymethyl] quinoline hydrochloride, a specific inhibitor of the 5-lipoxygenase pathway of arachidonic acid metabolism and a leukotriene $D_4$ antagonist) on airway changes induced in response to *Alternaria tenuis* aerosol challenge was assessed in adult rabbits neonatally immunized. Leukotriene generation was determined *in vivo* by measuring leukotriene $B_4$ ($LTB_4$) levels in bronchoalveolar lavage (BAL) fluid and *ex vivo* by measuring calcium ionophore-stimulated production of $LTB_4$ in whole blood.

**2** While PF 5901 (10 mg) had no significant effect on the acute bronchoconstriction induced by antigen, this dose was sufficient to inhibit significantly the increase in airway responsiveness to inhaled histamine 24 h following antigen challenge ($P<0.05$).

**3** Total leucocyte infiltration into the airways induced by antigen, as assessed by bronchoalveolar lavage, was significantly inhibited by pretreatment with PF 5901 (10 mg). However, the pulmonary infiltration of neutrophils and eosinophils induced by antigen was unaltered by prior treatment with PF 5901 (10 mg).

**4** PF 5901 (10 mg) had no effect on *ex vivo* $LTB_4$ synthesis in whole blood. However, the antigen-induced increase in $LTB_4$ levels in BAL 24 h following challenge was significantly inhibited ($P<0.05$).

**5** We suggest from the results of the present study that the antigen-induced airway hyperresponsiveness to inhaled histamine in immunized rabbits is mediated, at least in part, by products of the 5-lipoxygenase metabolic pathway, and is not dependent on the extent of eosinophil or neutrophil influx into the airway lumen.

**Keywords:** Antigen; leukotrienes; inflammation; airway hyperresponsiveness

## Introduction

Asthma is considered a combination of features including airway obstruction with spontaneous and drug-induced reversibility, increased airway responsiveness to exogenous and endogenous stimuli and airway inflammation. A number of animal models have been established to mimic various aspects of the allergic asthmatic response (including airway hyperresponsiveness) and to investigate the mechanisms contributing to such changes. One such model is the neonatally immunized rabbit. It has been shown that rabbits neonatally immunized with antigen and adjuvant within 24 h of birth and repeatedly exposed to antigen during the first 3 months of life, preferentially produce IgE antibodies (Pinckard *et al.*, 1972). As adults these rabbits respond to antigen exposure by eliciting both an acute and late-onset airways obstruction (Larsen *et al.*, 1987; Shampain *et al.*, 1982), exacerbations of airway responsiveness (Bloom *et al.*, 1988) and pulmonary inflammatory cell infiltration (Marsh *et al.*, 1985). Given the similarities between this model and allergic asthma in man, the rabbit has been used as a test system to investigate the many and varied mechanisms contributing to allergic inflammation and asthma (Marsh *et al.*, 1985; Murphy *et al.*, 1986; Coyle *et al.*, 1989; 1990; Riccio *et al.*, 1993).

Metabolism of arachidonic acid via the enzymes phospholipase $A_2$ and 5-lipoxygenase leads to the formation of the leukotrienes, 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and 5-hydroxyeicosatetraenoic acid (5-HETE), all of which exert potent inflammatory effects. Leukotrienes belong to a family of structurally-related compounds of which the most active are the sulphidopeptide, cysteinyl-containing leukotrienes (leukotriene $C_4$ ($LTC_4$), $LTD_4$ and $LTE_4$) and the dihydroxy acid $LTB_4$. The peptido-leukotrienes have been shown to induce many of the features of asthma both in animals and man, including bronchoconstriction, mucus hypersecretion, increased vascular permeability (reviewed in Barnes *et al.*, 1988), pulmonary inflammatory cell recruitment (Laitinen *et al.*, 1993) and airway hyperresponsiveness (Arm *et al.*, 1988; O'Hickey *et al.*, 1991). $LTB_4$ and 5-HETE are potent stimulators of leucocytes, including the chemotaxis, chemokinesis, adherence and aggregation of polymorphonuclear leucocytes (Ford-Hutchinson *et al.*, 1980; Ford-Hutchinson, 1991). *In vitro* 5-HPETE potentiates histamine release from human basophils (Peters *et al.*, 1982).

Peptido-leukotrienes have been detected in nasal secretions after allergen challenge *in vivo* (Creticos *et al.*, 1984; Freeland *et al.*, 1989), in bronchoalveolar lavage (BAL) fluid after local antigen challenge (Diaz *et al.*, 1989), urine after inhaled antigen provocation (Taylor *et al.*, 1989) and in pooled plasma from subjects with acute asthma (Zawrzewski *et al.*, 1985). Peptido-leukotrienes have also been detected in BAL fluid (Wardlaw *et al.*, 1989), sputum (Lam *et al.*, 1988), urine (Taylor *et al.*, 1989), plasma (Okubo *et al.*, 1987) and nasal secretions (Ferreri *et al.*, 1988) of asthmatic patients. In addition, fragments of lung (Dahlen *et al.*, 1983) and peripheral blood leucocytes from allergic asthmatic subjects (Mita *et al.*, 1986) have been shown to release leukotrienes after specific antigen challenge *in vitro*.

Asthmatic airways are capable of generating leukotrienes after exposure to inhaled antigen (Miadonna *et al.*, 1990; Wenzel *et al.*, 1990) and during acute asthmatic attacks

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(Taylor *et al.*, 1989). In view of these findings, a large number of studies have been carried out in human volunteers using a range of drugs interfering with the synthesis or actions of leukotrienes to modify airway responses to antigen (Britton *et al.*, 1987; Hui *et al.*, 1991; Taylor *et al.*, 1991; O'Shaughnessy *et al.*, 1993) and nasal responses to antigen (Knapp, 1990). To date however, there is little information on the influence such drugs have on antigen-induced airways inflammation.

In the present study we have investigated the ability of a specific leukotriene synthesis inhibitor and $LTD_4$ antagonist, PF 5901 (2-[3(1-hydroxyhexyl)phenoxymethyl]quinoline hydrochloride) (Van Inwegen *et al.*, 1987; Evans *et al.*, 1991) to influence acute bronchoconstriction, $LTB_4$ production, pulmonary cell infiltration and airway hyperresponsiveness to inhaled histamine induced by antigen in spontaneously breathing rabbits. Preliminary accounts of this work have been presented to the American Thoracic Society (Herd & Page, 1993) and to the British Pharmacological Society (Herd *et al.*, 1994).

## Methods

### *Animals*

New Zealand White (NZW) rabbits (Froxfield Farms, Petersfield, Hampshire) of either sex were used throughout the study. The immunization protocol of neonatal rabbits was that described previously (Minshall *et al.*, 1993). Rabbits were injected intraperitoneally (0.5 ml) within 24 h of birth with *Alternaria tenuis* extract in aluminium hydroxide ($Al(OH)_3$) moist gel and saline in the ratio of 2:1:1. Antigen and adjuvant administration was repeated weekly for the first month and then biweekly for the following two months. The methods described in this study were subject to Home Office approval and performed under the Animals (Scientific Procedures) Act, 1986.

### *Pulmonary function measurements*

Immunized, adult rabbits (2.0–3.75 kg) were pre-medicated with diazepam (2.5 mg $kg^{-1}$, i.p.) and were subsequently administered Hypnorm (0.4 ml $kg^{-1}$, intramuscularly), a regime which produces neuroleptanalgesia and is recommended for recovery experiments in laboratory rabbits (Flecknall, 1987). Neuroleptanalgesia was maintained throughout the course of the experiment by administration of 0.2–0.3 ml Hypnorm i.m. approximately every 30 min (Flecknall, 1987). Animals were intubated with a cuffed endotracheal tube (3.0 mm internal diameter; Mallinckrodt Laboratories, Athlone, Ireland), which was connected to a heated (37°C) Fleisch pneumotachograph (size 00). Measurements of flow, pleural pressure, transpulmonary pressure (the difference between thoracic and pleural pressure) and tidal volume were made according to methods previously described (Minshall *et al.*, 1993). Measurements of total lung resistance ($R_L$) and dynamic compliance ($C_{dyn}$) were calculated by an online respiratory analyser (Pulmonary Monitoring System PMS Version 5.1 Mumed Ltd., London).

### *Experimental protocol*

On Day 1, airway responsiveness to aerosolized histamine was determined as a measure of lung function, as previously described (Herd *et al.*, 1992). Following each 2 min aerosol of histamine, animals were disconnected from the nebulizer and attached to the Fleisch tube. The following ten breaths were recorded and the mean value calculated. Cumulative dose-response curves were established and the provocation concentration (PC) of histamine which produced 50% increase in $R_L$ ($PC_{50}$) and 35% decrease in $C_{dyn}$ ($PC_{35}$) was determined for each rabbit. These calculated parameters were used as indices of airway responsiveness.

On day 2, a suspension of PF 5901 (10 mg in a volume of 0.5 ml (40% polyethylene glycol (PEG-400) and 60% saline)) or vehicle alone (40% PEG-400 and 60% saline) was instilled directly into the lung via a cannula passed into the airway to the point of the bifurcation (via the endotracheal tube) 1 h prior to the commencement of the antigen challenge. Each antigen challenge consisted of a 2 min aerosol of saline followed by 5 consecutive aerosols of 2 min duration of *Alternaria tenuis* (10,000 PNU $ml^{-1}$), after which time respiratory function was recorded as described above.

On Days 3 and 5, airway responsiveness to histamine was determined as on Day 1.

### *Bronchoalveolar lavage*

Bronchoalveolar lavage (BAL) was performed immediately following histamine challenge on Days 1, 3 and 5. Saline (5 ml) was injected into the lungs through a polyethylene catheter (via the endotracheal tube) and then immediately aspirated and collected. Both total and differential cell counts were enumerated from BAL fluid as previously described (Herd *et al.*, 1992; Minshall *et al.*, 1993).

### *$LTB_4$ measurement*

On two occasions, immediately preceding both drug instillation and antigen challenge (i.e. 1 h apart), 2 ml blood was sampled from the marginal ear vein and collected into heparin sodium-containing tubes. Duplicate 500 µl aliquots were equilibrated for 10 min at 37°C. Calcium ionophore A23187 (10 µg) was added to each duplicate to provide a final concentration of 15 µg $ml^{-1}$ and incubated for a further 30 min at 37°C. The samples were then centrifuged (10,000 *g*, 2 min, 4°C) and the resultant plasma frozen at −20°C for subsequent analysis of the concentration of $LTB_4$. Two × 1 ml of BAL fluid were aliquoted following each lavage and each similarly frozen at −20°C for subsequent analysis. Using the anti-$LTB_4$ antiserum described in the Drugs and Chemicals section, $LTB_4$ was quantified by radioimmunoassay (RIA) as previously described (Salmon *et al.*, 1982).

### *Analysis of results*

For the lung function studies, statistical analysis was performed on $\log_{10}$ transformed data ($PC_{50}$ and $PC_{35}$) in order to normalize the distribution of the data and to allow the application of parametric statistics. Two-way analyses of variance for repeated measures were employed to analyze histamine potency data and $LTB_4$ levels in BAL. Student's *t* test was used to analyze the bronchoconstriction data ($R_L$ and $C_{dyn}$) (expressed as percentage change) and paired *t* test was used for the ionophore-stimulated whole blood $LTB_4$ generation. The total and differential cell counts were analyzed by Kruskall-Wallis one-way analysis of variance. Tukey's HSD test was used to determine differences in means when multiple comparisons were made for the parametric data (with analysis of variance) and distribution-free multiple comparisons were used to determine differences in means when multiple comparisons were made for the non-parametric data (Kruskall-Wallis one-way analysis of variance). Results were considered significant if $P<0.05$.

### *Drugs and chemicals*

The drugs and chemicals used were: *Alternaria tenuis* extract (Batch No. M1-152-7P16; 40,000 PNU $ml^{-1}$, 1 mg $ml^{-1}$; Greer Laboratories Inc. Lenoir, NC, U.S.A.); aluminium hydroxide ($Al(OH)_3$) moist gel (FSA Laboratory Supplies, Loughborough, Leicestershire); histamine diphosphate, calcium ionophore A23187, chromotrope 2R (Sigma Chemical Co., Poole, Dorset); haematoxylin (BDH Chemicals, Poole, Dor-

set); diazepam (Valium 5 mg ml$^{-1}$; Roche Products Ltd., Welwyn Garden City, Hertfordshire); Hypnorm (a mixture of fentanyl citrate 0.315 mg ml$^{-1}$ and fluanisone 10 mg ml$^{-1}$; Janssen Pharmaceutical Ltd., Grove, Oxfordshire); PF 5901 (2-[3(1-hydroxyhexyl)phenoxymethyl]quinoline hydrochloride, a gift from The Purdue Frederick Company, Norwalk, CT, U.S.A.); polyethylene glycol 400 (Fisons Scientific Equipment, Loughborough, Leicestershire); sterile pyrogen-free 0.9% sodium chloride solution (saline; Baxter Healthcare Ltd., Thetford, Norfolk). All reagents were of analytical grade. For *in vivo* studies, all solutions were prepared in saline.

### *Drugs and chemicals for $LTB_4$ assay*

Rabbit anti-$LTB_4$ antiserum (Advanced Magnetics Inc., Metachem Diagnostics Ltd., Northampton, Northamptonshire) characterized by cross-reactivity $<<1.0\%$ for all leukotrienes and prostanoids tested other than $LTB_4$ (100%) and the $LTB_4$ enantiomers 5(S), 12(**R**)-diHETE (6.7%) and 5(S), 12(S)-diHETE (2.0%); analyte tracer [$^3$H]-$LTB_4$ (Amersham International plc, Amersham, Buckinghamshire), specific activity 7.5–8.0 TBq mmol$^{-1}$; unlabelled standard $LTB_4$, Tris base and hydrochloride (Sigma Chemical Co., Poole, Dorset); gelatin, charcoal and sodium azide (BDH Chemicals, Poole, Dorset); dextran 150 (Fisons Scientific Equipment, Loughborough, Leicestershire).

## Results

### *Airway responsiveness*

Baseline absolute values of airway resistance ($R_L$) or dynamic compliance ($C_{dyn}$) were not significantly different between vehicle and drug-treated groups on any of the experimental days (Table 1). Furthermore, no significant difference was observed between airway responsiveness ($PC_{50}$ $R_L$ or $PC_{35}$ $C_{dyn}$) to inhaled histamine at 3 months in immunized rabbits pretreated with either vehicle or PF 5901 (10 mg) (Tables 2a and 2b). The combined mean values are $PC_{50}$ $R_L$: 21.88 ± 1.33 mg ml$^{-1}$ ($n = 20$); $PC_{35}$ $C_{dyn}$: 19.50 ± 1.26 mg ml$^{-1}$ ($n = 20$).

### *Acute bronchoconstriction*

Bronchoconstriction induced by antigen was not significantly different in groups of immunized rabbits pretreated with vehicle or PF 5901 (10 mg) directly administered into the airway (increases in $R_L$: vehicle 11.0 ± 5.1% ($n = 10$), PF 5901 18.0 ± 6.0% ($n = 10$); falls in $C_{dyn}$: vehicle − 28.3 ± 5.2% ($n = 10$), PF 5901 − 20.5 ± 4.8% ($n = 10$)).

### *Airway hyperresponsiveness*

Antigen challenge to immunized rabbits pretreated with vehicle caused a significant decrease in histamine $PC_{50}$ ($R_L$) and histamine $PC_{35}$ ($C_{dyn}$) values 24 h and 72 h later (Tables 2A and 2B; Figures 1a and 1b). In immunized rabbits pretreated with a single dose of PF 5901 (10 mg) directly instilled into the lungs, histamine $PC_{50}$ ($R_L$) and histamine $PC_{35}$ ($C_{dyn}$) values were not significantly altered 24 h or 72 h following antigen challenge (Tables 2A and 2B; Figures 1a and 1b). PF 5901 (10 mg) significantly inhibited antigen-induced airway hyperresponsiveness at 24 h following antigen challenge ($R_L$ and $C_{dyn}$) compared with the vehicle-treated group ($P < 0.05$) (Tables 2A and 2B; Figures 1a and 1b).

### *Bronchoalveolar lavage*

The mean pretreatment cell counts from immunized rabbits were not significantly different in rabbits administered either vehicle or PF 5901 (10 mg) (Table 3). Total leucocyte counts were significantly elevated in BAL fluid 24 h and 72 h following antigen exposure in rabbits pretreated with vehicle ($P < 0.05$). In rabbits pretreated with PF 5901 there were no significant differences between total cell numbers prior to or at any of the measured time points following antigen challenge (Table 3). The antigen-induced increase in total cell infiltration in vehicle-treated animals 24 h following challenge was significantly greater than that observed in the drug-treated group at the same time point ($P < 0.05$, Table 3). The number of neutrophils in BAL fluid was significantly elevated 1 h, 24 h and 72 h following antigen exposure in both treatment groups and there were no significant differences between the groups at any of the time points (Table 3). Similarly, there was a significant increase in the number of eosinophils observed in BAL fluid 24 h and 72 h following antigen in both vehicle-and PF 5901 (10 mg)-treated groups ($P < 0.05$). There were no significant differences in eosinophil numbers between the groups at any of the measured time points (Table 3); 24 h after antigen challenge mononuclear cell numbers were significantly reduced in rabbits pretreated with PF 5901 (10 mg), which was significantly different from that observed in the vehicle control group at 24 h ($P < 0.05$, Table 3).

### *$LTB_4$ measurements*

There was no significant difference in *ex vivo* ionophore-stimulated $LTB_4$ production between rabbits pretreated with either PF 5901 (10 mg) or vehicle (Table 4a). In contrast, the antigen-induced increase in $LTB_4$ levels detected in BAL fluid 24 h following challenge was significantly inhibited by PF 5901 (10 mg) (Table 4b).

## Discussion

Allergen exposure to allergic asthmatics often results in transient exacerbation of airway responsiveness to inhaled histamine (Cockcroft *et al.*, 1977; Hargreave *et al.*, 1986), which has been suggested as a model of asthma. The current study

**Table 1** Baseline lung function prior to, 24 h and 72 h following antigen challenge in immunized rabbits pretreated with vehicle or PF 5901 (10 mg): (**A**) total lung resistance ($R_L$) and (**B**) dynamic compliance ($C_{dyn}$)

| | *Baseline $R_L$ (cm$H_2O$ l$^{-1}$ s$^{-1}$)* | | |
|---|---|---|---|
| **A** | *Pre* | *24 h* | *72 h* |
| Vehicle | 39.2 ± 2.8 (10) | 35.3 ± 3.8 (10) | 35.6 ± 4.8 (8) |
| PF 5901 | 36.8 ± 3.6 (10) | 32.4 ± 3.4 (10) | 34.0 ± 3.3 (8) |
| | *Baseline $C_{dyn}$(ml cm$H_2O^{-1}$)* | | |
| **B** | *Pre* | *24 h* | *72 h* |
| Vehicle | 6.2 ± 0.8 (10) | 6.4 ± 0.4 (10) | 6.4 ± 0.7 (8) |
| PF 5901 | 5.8 ± 0.4 (10) | 6.1 ± 0.8 (10) | 5.4 ± 0.5 (8) |

Values represent mean ± s.e.mean for the number of replicates shown in parentheses.

**Table 2** Effect of vehicle or PF 5901 (10 mg) on airway responsiveness to inhaled histamine prior to, 24 h and 72 h following antigen challenge in immunized rabbits: (A) total lung resistance ($PC_{50}$) and (B) dynamic compliance ($PC_{35}$)

| A | | *Histamine $PC_{50}$* (mg ml$^{-1}$) | |
|---|---|---|---|
| *PF 5901* | *Pre* | *24 h* | *72 h* |
| Vehicle | 23.71 ± 1.48 (10) | 9.34 ± 1.34 (10)*‡ | 8.85 ± 1.66 (9)* |
| 10 mg | 20.18 ± 1.53 (10) | 17.02 ± 1.44 (10) | 13.96 ± 1.49 (9) |
| **B** | | *Histamine $PC_{35}$* (mg ml$^{-1}$) | |
| *PF 5901* | *Pre* | *24 h* | *72 h* |
| Vehicle | 21.43 ± 1.34 (10) | 4.29 ± 1.09 (10)*‡ | 5.83 ± 1.26 (8)* |
| 10 mg | 17.70 ± 1.44 (10) | 12.25 ± 1.32 (10) | 8.20 ± 1.37 (9) |

Values represent mean ± s.e.mean for the number of replicates shown in parentheses in both (A) and (B).
*$P<0.05$ compared with Pre control group.
‡$P<0.05$ compared with drug-treated group.

**Table 3** Total and differential cell numbers recovered from bronchoalveolar lavage fluid (BAL), Pre, 1 h, 24 h and 72 h following antigen exposure in immunized rabbits pretreated with PF 5901 (vehicle and 10 mg)

| | | × $10^5$ cells ml$^{-1}$ | | |
|---|---|---|---|---|
| | *Total* | *Neutrophils* | *Eosinophils* | *Mononuclear cells* |
| Vehicle Pre ($n=9$) | 2.04 (0.30–5.65) | 0.156 (0–0.914) | 0.0080 (0–0.0638) | 1.88 (0.30–5.65) |
| 1 h ($n=6$) | 2.40 (0.50–4.80) | 0.743 (0.063–1.608)* | 0.0550 (0–0.2880) | 1.60 (0.43–2.90) |
| 24 h ($n=8$) | 5.71 (1.31–16.25)*‡ | 3.397 (0.572–11.050)* | 0.4070 (0.0520–1.3810)* | 1.91 (0.65–4.18)‡ |
| 72 h ($n=6$) | 6.33 (1.20–25.90)* | 3.474 (0.150–17.094)* | 0.0635 (0–0.1900)* | 2.79 (0.94–8.81) |
| PF 5901 Pre ($n=9$) | 2.99 (0.30–7.35) | 0.247 (0–0.765) | 0.0020 (0–0.0213) | 2.75 (0.30–7.13) |
| 1 h ($n=8$) | 3.23 (0.50–8.95) | 0.808 (0.095–2.026)* | 0.0770 (0–0.3190) | 2.34 (0.40–7.38) |
| 24 h ($n=8$) | 3.69 (0.45–8.95) | 2.234 (0.248–5.281)* | 0.2130 (0.0225–0.5700)* | 1.25 (0.18–3.49)* |
| 72 h ($n=7$) | 3.86 (0.50–5.90) | 1.339 (0.035–3.885)* | 0.1463 (0–0.4640)* | 2.38 (0.45–3.89) |

Values represent mean with range in parentheses.
*$P<0.05$ compared with Pre control
‡$P<0.05$ compared with drug-treated group.

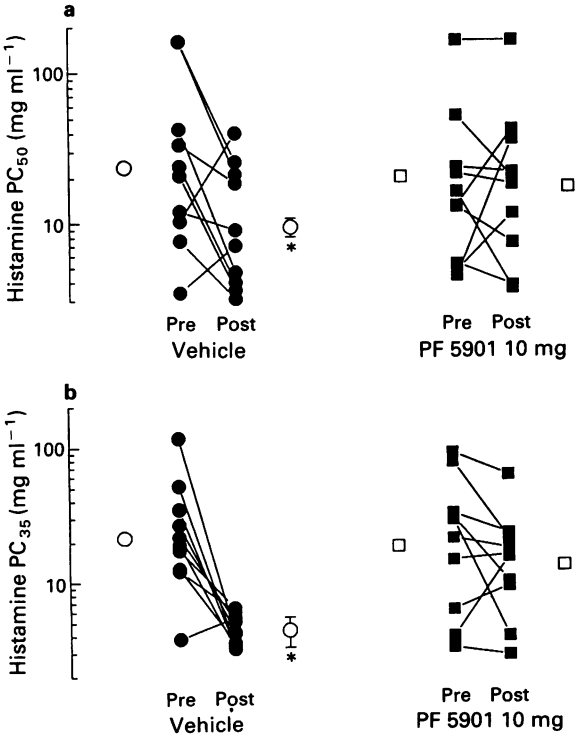


**Figure 1** Effect of vehicle (○, ●) ($n=10$) and PF 5901 (10 mg) (□, ■) ($n=10$) on antigen-induced airway hyperresponsiveness in immunized rabbits 24 h following antigen challenge. Closed symbols represent individual animal data and open symbols represent mean ± s.e.mean. (a) Histamine $PC_{50}$ is the concentration of histamine required to cause a 50% increase in airway resistance ($R_L$): (b) Histamine $PC_{35}$ is the concentration of histamine required to cause a 35% decrease in dynamic compliance ($C_{dyn}$). *$P<0.05$ compared with pre-antigen control.

has demonstrated that exposure of allergic rabbits to an aerosol of antigen results in airway hyperresponsiveness to inhaled histamine 24 h later as described previously by others (Shampain *et al.*, 1982; Coyle *et al.*, 1989; 1990) which persisted at 72 h. Such changes in airway responsiveness were associated with an influx of inflammatory cells into the airways as assessed by BAL and accompanied by an increase in the levels of $LTB_4$ measured in BAL fluid 24 h later. This extends previous observations showing that the lipoxygenase pathway of arachidonic acid metabolism is activated secondary to antigen/antibody interactions and that products of the lipoxygenase pathway contribute to various facets of the allergic response.

The acute antigen-induced bronchoconstriction in the rabbit appears not to be mediated via the release of 5-lipoxygenase products since no significant effect was achieved with prior treatment with PF 5901. Alternatively, a pretreatment time of 1 h prior to antigen exposure may be insufficient for access by PF 5901 to the leukotriene-generating cells in the lung. Similar findings have recently been reported in monkeys and conscious sheep (Wegner *et al.*, 1993). These results differ from observations in man where peptido-leukotriene antagonists or leukotriene synthesis inhibitors attenuate the bronchoconstriction induced by allergen (Britton *et al.*, 1987; Taylor *et al.*, 1991; O'Shaughnessy *et al.*, 1993). Nonetheless, the magnitude of antigen-induced bronchoconstriction in the rabbit model is minimal and it is possible that lipoxygenase products could contribute to bronchoconstriction following a greater antigen burden.

The ability of antigen to induce airway hyperresponsiveness in the neonatally immunized rabbit was significantly attenuated by prior treatment with PF 5901. There was a trend toward airway hyperresponsiveness with respect to the compliance component in the presence of PF 5901. This observation may reflect differences in the contribution of lipoxygenase products in the upper and lower airways. It is

**Table 4** Leukotriene $B_4$ ($LTB_4$) levels in (**A**) A23187-stimulated whole blood prior to, and 1 h post antigen challenge and (**B**) bronchoalveolar lavage (BAL) fluid prior to, and 1 h, 24 h and 72 h post antigen challenge, in immunized rabbits pretreated with vehicle or PF 5901 (10 mg)

| **A** | *$LTB_4$ level* ($ng\,ml^{-1}$) | |
|---|---|---|
| | *Pre* | *1 h post* |
| Vehicle ($n = 8$) | 27.71 ± 5.29 | 32.47 ± 7.49 |
| PF 5901 ($n = 8$) | 34.09 ± 7.55 | 35.32 ± 6.85 |

| **B** | *$LTB_4$ level* ($ng\,ml^{-1}$) | | | |
|---|---|---|---|---|
| | *Pre* | *1 h post* | *24 h post* | *72 h post* |
| Vehicle | 0.256 ± 0.08 | 1.009 ± 0.35* | 1.264 ± 0.33 | 2.339 ± 1.18 |
| *n* | 8 | 8 | 8 | 8 |
| PF 5901 | 0.22 ± 0.1 | 0.33 ± 0.09 | 0.945 ± 0.35 | 0.994 ± 0.35 |
| *n* | 8 | 8 | 8 | 6 |

Values represent mean ± s.e.mean.
*$P < 0.05$ compared with drug-treated group at the same time point.

possible that with a higher dose of the drug more complete inhibition of airway hyperresponsiveness could have been achieved. As this drug has been shown to have no effect on airway responsiveness to histamine following saline challenge, this inhibitory action of PF 5901 is not attributable to histamine $H_1$ antagonism or via some non-specific effect on airway responsiveness (Herd *et al.*, 1992). PF 5901 was originally described as a 5-lipoxygenase inhibitor (Van Inwegen *et al.*, 1987) and is now recognised as being a 5-lipoxygenase-activating protein (FLAP) inhibitor similar to the prototype FLAP inhibitor MK 886 (Evans *et al.*, 1991). Following cell stimulation, leucocyte 5-lipoxygenase is translocated from its cytosolic location to a cell membrane site (Rouzer & Kargman, 1988) and it has been reported that the involvement of FLAP is a requirement for the activation of 5-lipoxygenase (Dixon *et al.*, 1990; Miller *et al.*, 1990). However, recent findings in guinea-pig airways *in vitro* imply that activation of 5-lipoxygenase by antigen can occur with a lesser requirement for FLAP involvement (Yeadon *et al.*, 1993b), possibly by inducing an increase in intracellular calcium of smaller magnitude than that induced by calcium ionophores. In addition, the potency of some leukotriene synthesis inhibitors differs between cell types and in the same cell type against different stimuli (Noonan *et al.*, 1992). This implies that it is not axiomatic that inhibition of leukotriene generation in one cell type by PF 5901 will be accompanied by inhibition of leukotriene generation in all cells in the lungs. PF 5901 is also a moderately potent antagonist of $LTD_4$ (Van Inwegen *et al.*, 1987). Which of the actions of PF 5901 is responsible for the inhibition of antigen-induced airway hyperresponsiveness in the present study has not yet been determined. In atopic subjects the $LTD_4$ receptor antagonist ICI 204,219 was found to inhibit significantly allergen-induced airway hyperresponsiveness in response to inhaled histamine (Taylor *et al.*, 1991) and a recent study reports that the selective leukotriene antagonist, ONO-1078 reduces airway responsiveness to methacholine in asthmatic volunteers (Fujimura *et al.*, 1993). In contrast, the oral FLAP inhibitor MK-886 was without effect on allergen-induced increases in airway responsiveness to histamine (Friedman *et al.*, 1993).

The present study shows that PF 5901 is acting to inhibit the 5-lipoxygenase pathway in the cells responsible for the generation of $LTB_4$, as the increased levels of $LTB_4$ in BAL following antigen exposure were significantly reduced in animals pretreated with topical PF 5901. Given that $LTB_4$ has been found to induce airway hyperresponsiveness in allergic dogs (O'Byrne *et al.*, 1985) and allergic guinea-pigs (Pretolani *et al.*, 1993) and the related state of hyperalgesia (Levine *et al.*, 1984), it is plausible that this action of PF 5901 contributes to its ability to inhibit antigen-induced airway hyperresponsiveness. However, the precise cellular source of $LTB_4$ in the airways remains to be determined. No inhibition by PF 5901 of ionophore-stimulated release of $LTB_4$ from peripheral blood leucocytes was noted *ex vivo*, consistent with the poor systemic bioavailability of this drug (Serajuddin *et al.*, 1988). The lower limit of detectability of $LTB_4$ by this RIA is 0.5 pg $ml^{-1}$, thus values of 1 ng $ml^{-1}$ in BAL of immunoreactive $LTB_4$ represent highly significant amounts of analyte. No signal in the RIA was obtained from either BAL taken prior to administration of PF 5901 or its vehicle, or the components of the vehicle when added to standard curves in the RIA. Since the specificity of the antiserum is extremely high for $LTB_4$, it is likely that the measurements accurately reflect the concentration of $LTB_4$ in BAL, or another related material whose cross-reactivity with the antiserum has not been determined.

PF 5901 did not inhibit the influx of eosinophils into the airways induced by antigen despite inhibiting the associated airway hyperresponsiveness. This observation is in accord with the suggestion above that PF 5901 may block the development of airway hyperresponsiveness by inhibiting generation of $LTB_4$ in BAL, without significant inhibition of peptidoleukotriene generation. Each of the cells involved in the process of airway inflammation release a distinct profile of lipid mediators following cell stimulation. For example, human eosinophils form predominantly peptido-leukotrienes (Henderson *et al.*, 1984) in contrast to neutrophils which produce mainly $LTB_4$ (Henderson & Klebanoff, 1983). It is possible that access of the drug was limited to the neutrophil as it is recruited to the lung at an earlier time point than the eosinophil.

The direct acting 5-lipoxygenase inhibitor, BW B70C, prevents pulmonary eosinophilia following antigen inhalation in guinea-pigs (Yeadon *et al.*, 1993a), and it has recently been shown that inhalation of $LTE_4$ can induce eosinophil accumulation in the lungs of asthmatics (Laitenen *et al.*, 1993). Thus, the inhibition by PF 5901 of both $LTB_4$ generation in BAL and airways hyperresponsiveness, but not of eosinophil accumulation secondary to antigen challenge, suggests that in the rabbit, the peptido-leukotrienes may be important for cell recruitment and $LTB_4$ for airway hyperresponsiveness. To date no such studies have reported the effect in man of 5-lipoxygenase inhibitors or $LTD_4$ antagonists on pulmonary cell recruitment. Furthermore, these observations support the idea that cell infiltration and airway hyperresponsiveness may be dissociated events as has been suggested by others. In guinea-pigs, capsaicin will inhibit antigen-induced airway hyperresponsiveness without modifying eosinophil infiltration (Ladenius & Biggs, 1989; Matsuse *et al.*, 1991) and cytokines can cause pulmonary eosinophilia without airway hyperresponsiveness (Kings *et al.*, 1990). Low dose antigen in guinea-pigs has been reported to induce eosinophil accumulation in BAL in the absence of any alteration in airways responsiveness, and at a higher dose of antigen, ketotifen, AH

21132, dexamethasone and aminophylline were shown to inhibit the pulmonary eosinophilia and not the associated airway hyperresponsiveness (Sanjar *et al.*, 1990). Furthermore, a recent study reports that eosinophil recruitment into the airways of mice is not sufficient to induce airway hyperresponsiveness (Vianna *et al.*, 1993). Together these results suggest that the presence of eosinophils within the airway lumen may not be the sole prerequisite for the development of airway hyperresponsiveness. Such an interpretation is consistent with clinical observations that airway hyperresponsiveness may be present in asthmatics without an observed eosinophil infiltrate (Lundgren *et al.*, 1988) and conversely, chronic eosinophilic bronchitis is not always associated with airway hyperresponsiveness (Gibson *et al.*, 1989). Furthermore, a recent study reports no correlation betweeen baseline airway inflammation and increased airway responsiveness in mild asthmatics (Boulet *et al.*, 1993). However, it is possible that eosinophil numbers could be similar in both control and drug-treated groups and yet activation state different. Therefore, without measuring various markers of eosinophil priming in both groups, we cannot rule out the participation of this cell type.

In conclusion, antigen-induced airway hyperresponsiveness in neonatally immunized rabbits can be inhibited by topical airway administration of PF 5901, an effect that may not be related to the associated pulmonary infiltration of eosinophils.

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# Dual effects of mastoparan on intracellular free $Ca^{2+}$ concentrations in human astrocytoma cells

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**1** The effect of mastoparan, a wasp venom toxin, on intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) was examined in human astrocytoma cells. Mastoparan inhibited $[Ca^{2+}]_i$ induced by carbachol (100 μM) in a concentration-dependent manner in the absence of extracellular $Ca^{2+}$, consistent with our previous results showing that mastoparan inhibits phosphoinositide hydrolysis in human astrocytoma cells.

**2** In contrast, mastoparan itself increased $[Ca^{2+}]_i$ and augmented carbachol-induced increase in the $[Ca^{2+}]_i$ in the presence of extracellular $Ca^{2+}$, suggesting that mastoparan elicited $Ca^{2+}$ influx from the extracellular medium. The increase appeared to be maximum at extracellular $Ca^{2+}$ concentrations of 0.1–0.2 mM. The higher concentrations of extracellular $Ca^{2+}$ depressed the influx.

**3** Pertussis toxin did not affect mastoparan-induced inhibition of $[Ca^{2+}]_i$ in the absence of extracellular $Ca^{2+}$, consistent with the previous results that pertussis toxin did not affect mastoparan-induced inhibition of phosphoinositide hydrolysis.

**4** Pertussis toxin augmented mastoparan-induced increase in $[Ca^{2+}]_i$ in the presence of extracellular $Ca^{2+}$, suggesting that pertussis toxin substrate(s) seems to be inhibitory for $Ca^{2+}$ influx induced by mastoparan.

**5** Verapamil, nifedipine and diltiazem (each 10 μM), L-type $Ca^{2+}$ antagonists, did not affect mastoparan-induced $Ca^{2+}$ influx. However, verapamil (10 μM) slightly inhibited the increase in $[Ca^{2+}]_i$ induced by carbachol in the presence of mastoparan.

**6** The results obtained in the present study indicate that mastoparan has two opposite effects on $[Ca^{2+}]_i$ in human astrocytoma cells and possibly has at least two sites of action.

**Keywords:** Mastoparan; calcium ions; phospholipase C; inositol phosphate; calcium channel; pertussis toxin

## Introduction

Mastoparan is a wasp venom toxin with a structure of 14 amino-acids. It is well known that mastoparan liberates histamine from mast cells (Hirai *et al.*, 1979), a release that is mediated via an activation of phosphatidylinositol 4,5-bisphosphate ($PIP_2$)-specific phospholipase C (Okano *et al.*, 1985), resulting in an accumulation of inositol 1,4,5-trisphosphate ($IP_3$) and intracellular $Ca^{2+}$ mobilization. The effect of mastoparan on mast cells disappears after treatment of the cells with pertussis toxin, suggesting that mastoparan activates pertussis toxin-sensitive G-protein, such as $G_i$ and $G_o$ (Higashijima *et al.*, 1987). Mastoparan has been reported to increase GTPase activity and [$^{35}$S]-GTPγS binding of purified $G_o$ or $G_i$ in phospholipid vesicles (Higashijima *et al.*, 1988). Recent lines of evidence suggest that mastoparan directly interacts with a G-protein in a manner similar to an activated receptor by agonists (Higashijima *et al.*, 1990; Tomita *et al.*, 1991). The interacting site of mastoparan with G-protein is assumed to be around the 4th cysteine residue from the C-terminal of pertussis toxin-sensitive G-protein (Higashijima *et al.*, 1990).

On the other hand, 1321N1 human astrocytoma cells express muscarinic cholinoceptors (Masters *et al.*, 1985), $H_1$-histamine (Nakahata *et al.*, 1986), bradykinin (Hepler *et al.*, 1987), thromboxane $A_2$ (Nakahata *et al.*, 1989) and endothelin (Yanai *et al.*, 1992) receptors, which are coupled to $PIP_2$-specific phospholipase C mediated via a pertussis toxin-insensitive G-protein. We have shown that mastoparan inhibits phosphoinositide hydrolysis in human astrocytoma cells (Nakahata *et al.*, 1990), although it stimulates phosphoinositide hydrolysis in mast cells (Higashijima *et al.*, 1987). The results are consistent with the reports by Wojcikiewicz & Nahorski (1989) and Gusovsky *et al.* (1991) that mastoparan inhibits phosphinositide hydrolysis in permeabilized SH-SY5Y and HL-60 cells, respectively. Mastoparan-induced inhibition of phosphoinositide hydrolysis in human astrocytoma cells was resistant to pertussis toxin treatment (Nakahata *et al.*, 1990), indicating that the action of mastoparan may not be mediated via a pertussis toxin-sensitive G-protein.

In the present study, we analyzed intracellular free $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$) using fura-II for examining whether mastoparan inhibits an increase in $[Ca^{2+}]_i$ induced by carbachol in human astrocytoma cells. The results obtained suggest that mastoparan has two opposite actions on $[Ca^{2+}]_i$ in human astrocytoma cells; one is a decrease in $[Ca^{2+}]_i$ that supports the inhibitory effect of mastoparan on phosphoinositide hydrolysis, the other is an increase in $[Ca^{2+}]_i$ which might be due to some $Ca^{2+}$-channel opening.

## Methods

### *Cell culture*

Human astrocytoma cells (1321N1) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% foetal bovine serum, 50 units $ml^{-1}$ of penicillin and 50 μg $ml^{-1}$ of streptomycin in a 37°C humidified incubator in an atmosphere of 95% $O_2$ and 5% $CO_2$.

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### *Measurement of intracellular free $Ca^{2+}$ concentrations with fura II*

Human astrocytoma cells cultured on a 150 mm dish were washed three times with a modified Tyrode solution (composition, mM: NaCl 137, KCl 2.7, $MgCl_2$ 1.0, $CaCl_2$ 0.18, glucose 5.6, HEPES 10, pH 7.4). The cells were freed from the dish by treatment with 0.1% collagenase and 1.0% bovine serum albumin (BSA) in 10 ml of the modified Tyrode solution for 15 min at 37°C. Cells liberated from the dish were collected into a 50 ml tube and centrifuged at 250 *g* for 1 min. Cells were washed once with 10 ml of the modified Tyrode solution. The cells ($10^6-5\times10^6$ ml$^{-1}$) were treated with 1 μM fura-2-AM at 37°C for 15 min and then were centrifuged at 250 *g* for 1 min in order to remove the remaining fura 2-AM, and washed twice with the modified Tyrode solution. The cells were suspended in the modified Tyrode solution in the concentrations of $10^6-5\times10^6$ ml$^{-1}$, and 1–2 ml of the cell suspension were used for fura 2 assay. Fluorescence of fura 2 at 510 nm by excitation waves at 340 and 380 nm was monitored simultaneously by a spectrofluorometer (Hitachi, F-2000). The maximum ratio of fluorescence at 510 nm by the excitation wave of 340 nm to that by 380 nm was obtained in the presence of 0.1% triton X-100, and the minimum fluorescence ratio was obtained in the presence of 2 mM EGTA. Free calcium concentrations were calculated by using the $K_d$ of fura II to $Ca^{2+}$ ions as 224 nM.

### *Materials*

Foetal bovine serum was obtained from Cell Culture Laboratory (Cleveland, OH, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Mastoparan was from Peptide Institute (Osaka, Japan). Fura 2-AM was from Dojindo (Kumamoto, Japan). Carbachol, verapamil, diltiazem and nifedipine was from Sigma (St. Louis, MO, U.S.A.), Pertussis toxin was purchased from Funakoshi (Tokyo, Japan). EGTA was from Nakari Chemicals (Kyoto, Japan). Triton X-100 was from Wako Pure Chemicals (Tokyo, Japan). Collagenase was obtained from Warthington Biochemical Corp. (Freehold, NJ, U.S.A.). Other chemicals and drugs were of reagent grade or highest quality available.

### *Data analysis*

The results obtained in separate experiments were expressed as mean ± s.e., and a significant difference ($P<0.05$) was determined with Student's *t*-test.

## Results

A resting level of $[Ca^{2+}]_i$ in fura 2-loaded human astrocytoma cells was 50–100 nM in the presence of extracellular $Ca^{2+}$ ions. In the absence of extracellular $Ca^{2+}$ ions, i.e. in the presence of 2 mM EGTA, the resting level of $[Ca^{2+}]_i$ was approximately 30 to 50 nM. Carbachol (100 μM) increased $[Ca^{2+}]_i$ in the absence of extracellular $Ca^{2+}$ ions, as shown in Figure 1. The results indicate that carbachol could mobilize intracellular $Ca^{2+}$ ions from intracellular storage sites, consistent with an activation of phosphoinositide hydrolysis and $IP_3$ accumulation by carbachol, previously described by Masters *et al.* (1985).

Mastoparan (30 μM) potently inhibited carbachol-induced increase in $[Ca^{2+}]_i$ (Figure 1). Mastoparan also inhibited the histamine (100 μM)-induced increase in $[Ca^{2+}]_i$ (data not shown). Figure 2 shows the concentration-inhibition curve for mastoparan on the carbachol-induced increase in $[Ca^{2+}]_i$. The inhibition curve for mastoparan is similar to that for mastoparan on carbachol-induced phosphoinositide hydrolysis (Nakahata *et al.*, 1990). On the other hand, mastoparan itself (30 μM) increased $[Ca^{2+}]_i$ in the presence of extracellular $Ca^{2+}$ ions (Figure 3). Interestingly, mastoparan augmented carbachol-induced increase in $[Ca^{2+}]_i$ in the presence of extracellular $Ca^{2+}$ ions (Figure 3). In the presence of extracellular $Ca^{2+}$ ions, mastoparan concentration-dependently increased $[Ca^{2+}]_i$, and also augmented the carbachol-induced increase in $[Ca^{2+}]_i$ (Figure 4). The effect of mastoparan on $[Ca^{2+}]_i$ in the presence of extracellular $Ca^{2+}$ ions was completely different from its effect in the absence of extracellular $Ca^{2+}$ ions, suggesting that mastoparan has at least two sites of action on $[Ca^{2+}]_i$. As there is a possibility that mastoparan could activate $Ca^{2+}$-influx from extracellular space across plasma membranes, we examined whether extra-

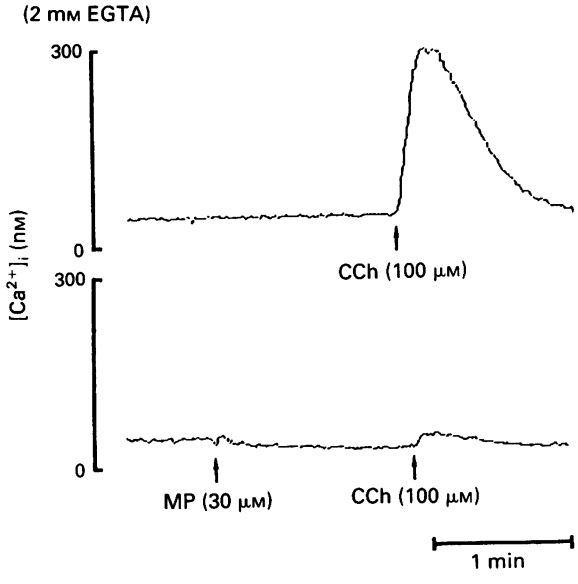


**Figure 1** Effect of mastoparan on intracellular free $Ca^{2+}$ concentrations in the absence of extracellular $Ca^{2+}$ ions. The cells loaded with fura 2 were suspended in the modified Tyrode solution. EGTA (2 mM final concentration) was added 225 s before addition of carbachol (CCh, 100 μM). Mastoparan (30 μM) was added 100 s before addition of carbachol. Mastoparan inhibited carbachol-induced increase in $[Ca^{2+}]_i$.

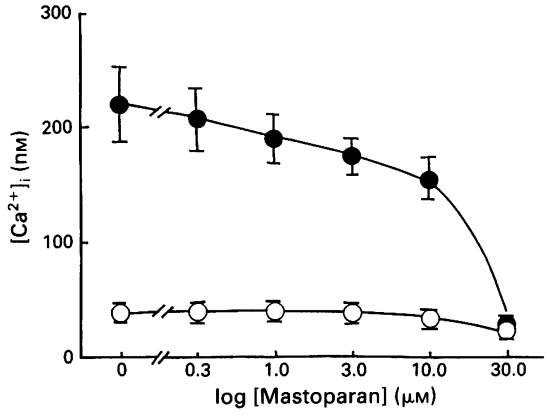


**Figure 2** Concentration-dependent inhibition by mastoparan of carbachol-induced increase in intracellular free $Ca^{2+}$ concentrations in the presence of 2 mM EGTA. Experiments were performed as in Figure 1, except that the concentrations of mastoparan were varied. Each point represents the mean value ± s.e. of six separate experiments. Abscissa scale: concentration of mastoparan (μM). Ordinate scale: intracellular free $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$). (○) Mastoparan alone; (●) carbachol (100 μM) in the presence of various concentrations of mastoparan.

cellular $Ca^{2+}$ ion concentrations ($[Ca^{2+}]_o$) could regulate mastoparan-induced $Ca^{2+}$ mobilization or not (Figure 5). Mastoparan (30 μM) alone increase $[Ca^{2+}]_i$ in the presence of extracellular $Ca^{2+}$ ions with a peak in the presence of 0.1 to 0.2 mM $[Ca^{2+}]_o$. Higher concentrations of $[Ca^{2+}]_o$ did not potentiate, but rather inhibited, mastoparan-induced increase in $[Ca^{2+}]_i$, indicating that the plasma membrane of astrocytoma cells was still intact after addition of mastoparan. The carbachol-induced increase in $[Ca^{2+}]_i$ in the presence of mastoparan (30 μM) was not changed in various $[Ca^{2+}]_o$. Because mastoparan did not increase $[Ca^{2+}]_i$ in the absence of extracellular $Ca^{2+}$ ions, mastoparan is assumed to mobilize $Ca^{2+}$ ions from extracellular space.

Pertussis toxin ADP-ribosylated $G_i$ or $G_o$ protein; then signal transduction involved in $G_i$ or $G_o$ was inhibited by treatment of the cells with pertussis toxin (Ui, 1984). Mastoparan-induced inhibition of the increase in $[Ca^{2+}]_i$ induced by carbachol was not affected by pertussis toxin treatment in the absence of extracellular $Ca^{2+}$ ions (Figure 6), consistent with the previous results that pertussis toxin

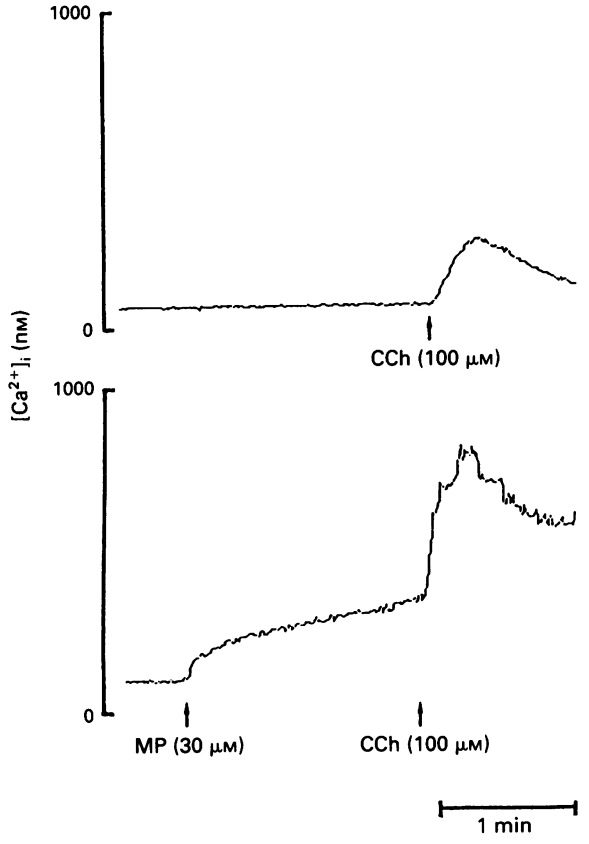


**Figure 3** Effect of mastoparan on intracellular free $Ca^{2+}$ concentrations in the presence of extracellular $Ca^{2+}$ ions. The cells loaded with fura 2 were suspended in the modified Tyrode solution containing 0.18 mM $CaCl_2$. Mastoparan (30 μM) was added 175 s before addition of carbachol (CCh, 100 μM).

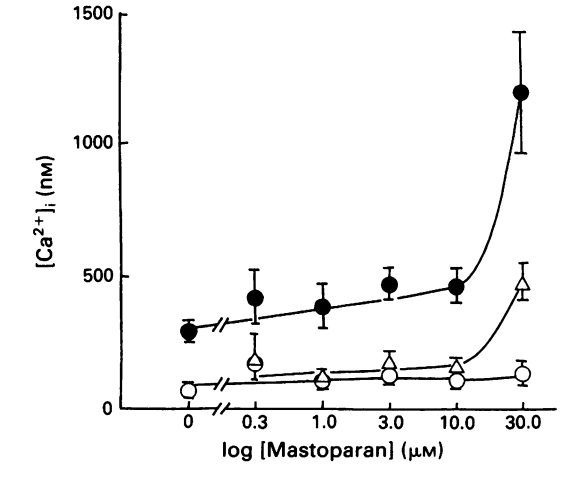


**Figure 4** Concentration-dependency of mastoparan-induced increase in intracellular free $Ca^{2+}$ ion concentrations in the presence of extracellular $Ca^{2+}$ ions. Experiments were performed as in Figure 3, except that concentrations of mastoparan were varied. (○) $[Ca^{2+}]_i$ before addition of mastoparan; (△) $[Ca^{2+}]_i$ after addition of mastoparan; (●) $[Ca^{2+}]_i$ after addition of carbachol (100 μM) and mastoparan. Abscissa scale: concentration of mastoparan (μM). Ordinate scale: intracellular free $Ca^{2+}$ concentrations. Each point represents the mean value ± s.e. of four separate experiments.

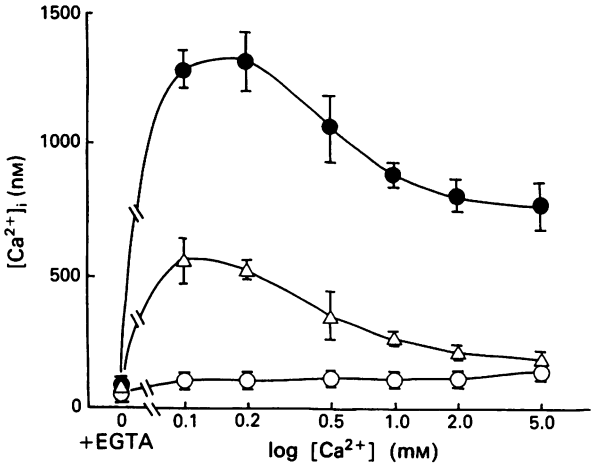


**Figure 5** Extracellular $Ca^{2+}$ dependency of mastoparan-induced increase in intracellular $Ca^{2+}$ concentration. Fura 2-loaded cells were incubated in the indicated extracellular $Ca^{2+}$ concentrations or 2 mM EGTA, then 30 μM mastoparan was added to the medium. After the response reached a steady-state, 100 μM carbachol was added to the medium. (○) $[Ca^{2+}]_i$ before addition of mastoparan; (△) $[Ca^{2+}]_i$ after addition of mastoparan; (●) $[Ca^{2+}]_i$ after addition of carbachol and mastoparan. Note that the peak increase in $[Ca^{2+}]_i$ by mastoparan appeared between 0.1 and 0.2 mM extracellular $Ca^{2+}$ concentration. Each point represents the mean value ± s.e. of three separate experiments.

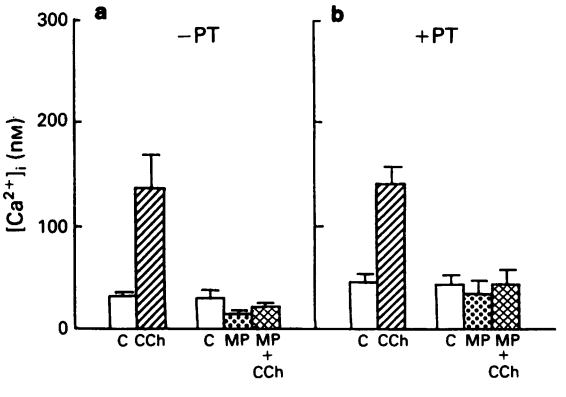


**Figure 6** Effect of pertussis toxin (PT) on intracellular free $Ca^{2+}$ concentrations induced by carbachol and mastoparan in the absence of extracellular $Ca^{2+}$. Cells were treated with (a) or without (b) pertussis toxin (100 ng $ml^{-1}$) for 18 h. Intracellular free $Ca^{2+}$ concentrations were monitored as described in Figure 1. Open column: control; hatched column: carbachol (CCh, 100 μM); stippled column: mastoparan (MP, 30 μM); cross hatched column: carbachol (CCh, 100 μM) after mastoparan (MP, 30 μM). Each point represents the mean value ± s.e. of four separate experiments. Note that the inhibitory effect of mastoparan was unaffected by pertussis toxin treatment.

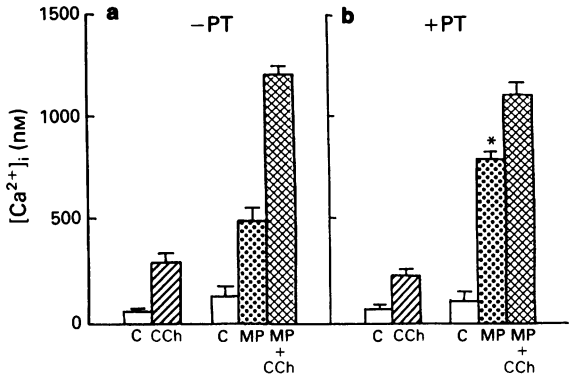


**Figure 7** Effect of pertussis toxin on intracellular free $Ca^{2+}$ concentrations induced by carbachol and mastoparan in the presence of extracellular free $Ca^{2+}$ ions. Cells were treated with (a) or without (b) pertussis toxin (100 ng $ml^{-1}$) for 18 h. Intracellular free $Ca^{2+}$ concentrations were monitored as described in Figure 3. Open column: control; hatched column: carbachol (CCh, 100 μM); stippled column: mastoparan (MP, 30 μM); cross hatched column: carbachol (CCh, 100 μM) after mastoparan (MP, 30 μM). Each point represents the mean value ± s.e. of four separate experiments. *Significant difference between the response of the cells with pertussis toxin treatment and the response of the cells without pertussis toxin treatment. Note that pertussis toxin augmented mastoparan-induced increase in $[Ca^{2+}]_i$.

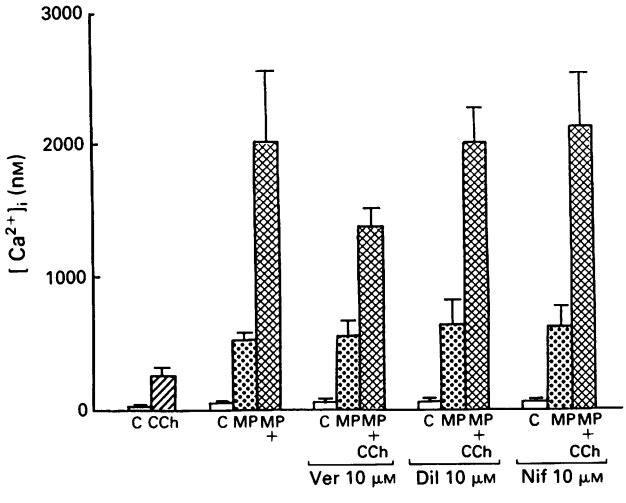


**Figure 8** Effects of $Ca^{2+}$ antagonists on mastoparan-induced increase in free $Ca^{2+}$ concentrations. Cells loaded with fura 2-AM were preincubated with verapamil (Ver, 10 μM), diltiazem (Dil, 10 μM), nifedipine (Nif, 10 μM) or vehicle for 5 min; then the mastoparan-induced increase in $[Ca^{2+}]_i$ was observed. After the response reached a steady-state, carbachol (CCh, 100 μM) was added. Open column: control; stippled column: mastoparan (MP, 30 μM); cross-hatched column: carbachol (CCh, 100 μM) after mastoparan (MP, 30 μM). Each point represents the mean value ± s.e. of three separate experiments. Note that carbachol-induced increase in $[Ca^{2+}]_i$ in the presence of mastoparan was slightly inhibited by verapamil.

did not modify the inhibitory action on carbachol-induced phosphoinositide hydrolysis (Nakahata *et al.*, 1990). In the presence of extracellular $Ca^{2+}$ ions, the mastoparan-induced increase in $[Ca^{2+}]_i$ was augmented by treatment of the cells with pertussis toxin (Figure 7). The results suggest that a pertussis toxin substrate may inhibit $Ca^{2+}$ influx from extracellular space.

As there is a possibility that the mastoparan-induced increase in $[Ca^{2+}]_i$ may be dependent on influx of $Ca^{2+}$ ions through a Ca-channel, the effects of Ca-channel antagonists on mastoparan-induced increase in $[Ca^{2+}]_i$ were examined (Figure 8). Verapamil, diltiazem and nifedipine at a concentration of 10 μM did not affect mastoparan-induced increase in $[Ca^{2+}]_i$, suggesting that $Ca^{2+}$-influx by mastoparan might not be due to the opening of L-type $Ca^{2+}$-channels. On the other hand, carbachol-induced $Ca^{2+}$-influx in the presence of mastoparan was somewhat reduced by 10 μM verapamil (Figure 8), although verapamil did not affect the carbachol-induced increase in $[Ca^{2+}]_i$ (data not shown). The results suggest that in the presence of mastoparan, carabachol is able to stimulate the opening of some $Ca^{2+}$-channel.

## Discussion

The present study demonstrated that mastoparan has two opposite effects on $[Ca^{2+}]_i$ in human astrocytoma cells; one is an inhibitory action that is explained by the inhibition of $PIP_2$-specific phospholipase C and a decreased accumulation of $IP_3$ (Nakahata *et al.*, 1990); the other is $Ca^{2+}$ influx from extracellular space. $Ca^{2+}$ influx from extracellular space induced by mastoparan was not inhibited by treatment of the cells with pertussis toxin, indicating that there is no involvement of pertussis toxin-sensitive G-protein in mastoparan-induced $Ca^{2+}$ influx. Interestingly, pertussis toxin rather potentiated $Ca^{2+}$ influx induced by mastoparan. Therefore, pertussis toxin-sensitive G-protein may inhibit the $Ca^{2+}$ influx induced by mastoparan in human astrocytoma cells, instead of an activation of influx. Pertussis toxin did not abolish mastoparan-induced inhibition of an increase in $[Ca^{2+}]_i$ induced by carbachol in the absence of extracellular $Ca^{2+}$ ions. It is suggested that one of the target proteins of mastoparan was a G-protein α-subunit (Higashijima & Ross, 1991). Recent lines of evidence indicated that a small molecular G-protein (substrate for botulinum toxin C3 exoenzyme, rho/rac) was affected by mastoparan (Koch *et al.*, 1991), although the G-proteins activated by mastoparan are usually considered as pertussis toxin-substrates (Higashijima & Ross, 1991).

Mastoparan itself increased $[Ca^{2+}]_i$ (Figure 2). Mastoparan is a amphiphylic peptide and exists in plasma membranes by interacting with phospholipids (Higashijima *et al.*, 1983); when it is applied to cells, there is a possibility that mastoparan-induced increase in $[Ca^{2+}]_i$ by fura 2 assay is merely an exclusion of fura 2 from inside the cells to outside. In fact, Tanimura *et al.* (1991) demonstrated the presence of lactate dehydrogenase (LDH) in the extracellular space after treatment of rat acinar cells with mastoparan, suggesting that mastoparan could change the membrane permeability for proteins or small molecules. However, this is not the case in the present experiments, because higher concentrations of extracellular $Ca^{2+}$ ions did not augment mastoparan-induced increase in fura-2 fluorescence. Furthermore, Yule & Williams (1991) demonstrated that mastoparan induces oscillations of cytosolic $Ca^{2+}$ in rat pancreatic acinar cells, showing that mastoparan could increase cytosolic $Ca^{2+}$ without exclusion of fura 2.

The carbachol-induced increase in $[Ca^{2+}]_i$ after mastoparan in the presence of extracellular $Ca^{2+}$ ions, was inhibited by 10 μM verapamil. As diltiazem and nifedipine did not inhibit the increase, the $Ca^{2+}$ channel involved may not be of the L-type. Since verapamil did not inhibit carbachol-induced increase in $[Ca^{2+}]_i$ (data not shown), mastoparan may change the state of the cell membranes so that some $Ca^{2+}$-channel is active. But the situation is abnormal, because the increased level of $[Ca^{2+}]_i$ is too high.

Mastoparan is believed to activate G-protein (Higashijima & Ross, 1991). The present study showed two different effects of mastoparan on $[Ca^{2+}]_i$. If both effects of mastoparan involve G-protein(s), these G-protein(s) could not be pertussis toxin-substrates in human astrocytoma cells. In fact, mastoparan activates GTPγS binding in membrane preparations derived from human astrocytoma cells after treatment

with pertussis toxin (Nakahata *et al.*, unpublished observations). Although mastoparan may directly activate G-protein(s), it has been shown to activate or inhibit several biological systems but not G-protein(s). Kikkawa *et al.* (1992) reported that mastoparan activates nucleoside diphosphate kinase, which in turn activates G-protein(s). Raynor *et al.* (1991) reported that mastoparan inhibits protein kinase C, Ca/CaM kinase II, Na-K ATPase and the Na pump. It is interesting that phosphoinositide 3,4,5-trisphosphate is accumulated by mastoparan in human neutrophils (Norgauer *et al.*, 1992), and mastoparan activates arachidonic acid release without inositol phosphate accumulation in Swiss 3T3 cells (Gil *et al.*, 1991). Denker *et al.* (1991) reported that mastoparan activates nucleotidase of which the molecular mass is 85 kDa from bovine brain. The accumulated evidence suggests that there might be cell specificity and multifunctional effects in the action of mastoparan.

The present results indicate that mastoparan has two opposite effects on $[Ca^{2+}]_i$ in human astrocytoma cells, and hence that mastoparan possibly has at least two sites of action for regulation of $[Ca^{2+}]_i$ in human astrocytoma cells.

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# Differential effects of lumenal L-arginine and $N^G$-nitro L-arginine on blood flow and water fluxes in rat ileum

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**1** The role of endogenous mucosal nitric oxide (NO) in the local regulation of $H_2O$ absorption and blood flow in rat ileum was studied by perfusing L-arginine (L-Arg) (0.1–1.0 mM) and $N^G$-nitro L-arginine (L-NOARG) (0.01–1.0 mM) through the lumen. D-Arginine (D-Arg) or L-Arg (1 mM), combined with L-NOARG, were used to determine if any of the measured intestinal effects of L-NOARG were exerted through NO formation.

**2** Net and unidirectional $H_2O$ fluxes and effective mucosal blood flow were measured using $^3H_2O$ and [$^{14}$C]-inulin in the perfusate. Mucosal NO formation was measured as the appearance of lumenal $NO_2^-$.

**3** L-NOARG, beginning at a concentration of 0.1 mM, decreased net $H_2O$ absorption, but had only minor effects on unidirectional $H_2O$ fluxes or on blood flow. L-NOARG increased blood pressure, beginning at a concentration of 0.5 mM.

**4** L-Arg had no significant effects on net $H_2O$ absorption or blood pressure, and only minor effects on unidirectional $H_2O$ fluxes and blood flow.

**5** NO appearance in the lumen was marginally decreased by 1.0 mM L-NOARG, but not increased by L-Arg.

**6** Mucosal blood flow resistance paralleled systemic blood pressure suggesting that vascular effects on the mucosa were exerted only after L-NOARG had reached the general circulation.

**7** Lumenal L-Arg reversed the effects of lumenal L-NOARG on net $H_2O$ absorption and blood pressure, but D-Arg did not.

**8** It was concluded that there is tonic NO production by the rat intestinal mucosa that promotes $H_2O$ absorption, but does not affect blood flow resistance. Mucosal NO production was not related to the observed effects on mucosal function.

**Keywords:** Nitric oxide; intestine; $H_2O$ absorption; blood flow; mucosa; blood pressure; $H_2O$ fluxes

## Introduction

Nitric oxide (NO), or a related product, is produced by enterocytes, vascular tissue, smooth muscle, neurones, fibroblasts, mast cells and white blood cells (Blachier *et al.*, 1991; Nathan, 1992), all of which are found in the intestinal mucosa. The role of local NO production on intestinal mucosal function has not been well defined. Under normal conditions, NO is produced by constitutive NO synthases (cNOS) and has signalling functions. An inducible NOS (iNOS) produces larger amounts of NO following insults, and NO under these conditions can protect or damage tissues (Lopez-Belmonte *et al.*, 1993). Both cNOS and iNOS are produced in the intestine (Salter *et al.*, 1991; Boughton-Smith *et al.*, 1992). Most research concerned with the role of NO in the intestine revolves around intestinal motility, blood flow and the role of NO in preventing mucosal damage during shock stimuli. Relatively little work has focused on the role of NO in regulating intestinal absorption.

NO is produced by intestinal non-adrenergic non-cholinergic neurones and inhibits intestinal motility (Boeckxstaens *et al.*, 1991), but there are few of these neurones in the mucosa (Young *et al.*, 1992). NO is also a neural vasodilator (Amerini *et al.*, 1992) and is also produced by endothelial cells in the intestinal vasculature (Andriantsitohaina & Surprenant, 1992). The specific effect of endogenous NO on mucosal vasodilatation, however, has not been determined. NO maintains mucosal and vascular integrity at low and intermediate levels but causes damage at higher concentrations (Lopez-Belmonte *et al.*, 1993). Hence, NO has known functions in the intestine but the role of NO in many aspects of gut function requires further elucidation.

NOS produce NO from L-arginine (L-Arg) (Nathan, 1992), and production is inhibited by compounds such as $N^G$-nitro-L-arginine (L-NOARG) (Rees *et al.*, 1990). The D-isomers are inactive, and their lack of effect, when the L-isomers are active, has been used as a criterion of effects exerted through NO production. $N^G$-nitro-L-arginine and L-arginine are absorbed by the intestinal mucosa and, therefore, can have local effects as well as systemic effects, including effects on the intestine, after these compounds have been absorbed into the general circulation (Gardiner *et al.*, 1990b). A criterion for local mucosal effects would be that lumenal agents acted on mucosal functions at lower concentrations than those that exerted systemic effects. NO formed in the mucosa will diffuse into the lumen and interstitial space. NO rapidly and spontaneously forms $NO_2^-$ and $NO_3^-$ in varied proportions depending on the site of formation (Wennmalm *et al.*, 1992). Lumenal $NO_2^-$ appearance, therefore, was used as an estimate of mucosal NO production. Absorptive site blood flow, arterial blood pressure and unidirectional $H_2O$ fluxes were also measured to determine cardiovascular effects, and, because, it is sometimes possible to infer the transport mechanism that is affected by treatments from these parameters (Mailman, 1984).

In these experiments, $H_2O$ absorption and mucosal blood flow were initially considered as representative of local effects, while blood pressure was used as an example of systemic effects. The experiments presented here were designed to determine the role of mucosal NO in regulating the intestinal mucosal functions of $H_2O$ absorption and its supporting blood flow.

## Methods

### *Animal preparation*

Female Sprague Dawley rats, 287 ± 7 g, were fasted for 24 h with $H_2O$ allowed *ad libitum*. The animals were anaesthetized

with sodium pentobarbitone (50 mg $kg^{-1}$, i.p.). The trachea was cannulated. A femoral vein was cannulated for infusion of supplemental anaesthetic, as needed, and for hydration with Krebs-Ringer bicarbonate solution (KRB) at 1.2 ml $h^{-1}$. A femoral artery was cannulated with plastic tubing containing heparinized (50 u $ml^{-1}$) KRB for measurement of blood pressure. A laparotomy was performed. A 20 cm segment of ileum, with its nerve and blood supply intact, beginning about 2 cm from the ileocecal junction was cannulated with an inflow and effluent cannula. The segment was flushed with KRB at 37°C. The segment was curled back into the abdominal cavity, and the incision was closed with wound clips and covered with saline-soaked sponges and plastic wrap. Body temperature was monitored with a rectal thermistor and maintained at 38°C with a heat lamp.

The ileal lumen was perfused by a syringe pump (Sage) through a condensor that maintained the lumenal perfusate at 37°C. The perfusate was labelled KRB (composition, mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5 and $NaHCO_3$ 25) containing $^3H_2O$ (about 50,000 c.p.m. $ml^{-1}$) and [$^{14}$C]-inulin (25 mg $l^{-1}$; about 5,500 c.p.m. $ml^{-1}$). A second, continuously variable, syringe pump was connected to the inflow cannula for addition of labelled KRB in control animals or L-Arg, D-Arg and/or L-NOARG, in labelled KRB, in experimental animals. The concentration of these compounds was changed by adjusting the flow rates of one or both pumps.

### *Protocol*

After surgery, the lumenal perfusion was started (0.25 ml $min^{-1}$). The labelled KRB perfusate either contained no added agent or contained L-Arg or D-Arg (1 mM). After a 50 min equilibration period, the effluent was collected for four 10 min periods that served as initial baseline periods, so that each animal served as its own control. The second syringe pump was then started, beginnning period 1. In control animals, the second perfusate was the same as the baseline perfusate. In experimental animals, the second perfusate contained L-Arg or L-NOARG dissolved in the base perfusate. After four 10 min periods, the flow, and thus the concentration of L-Arg or L-NOARG, was changed two more times for the same periods and then turned off to reestablish control levels of flow and concentration. A final group of four 10 min periods was carried out, ending with period 16. Total perfusion rates varied between 0.25 to 0.28 ml $min^{-1}$.

L-NOARG concentrations were 0.01–1 mM and L-Arg 0.1–1 mM when added to KRB. When the base perfusion contained L-Arg or D-Arg (1 mM), the second syringe pump delivered only L-Arg or D-Arg (1 mM) in control animals or L-NOARG at 0.1, 0.5 and 1 mM in 1 mM D-Arg or L-Arg in experimental animals. At the end of the experiment, a blood sample was taken for measurement of plasma $^3H_2O$ and [$^{14}$C]-inulin. The perfused segment of gut was removed and weighed. Values were expressed per g gut.

### *Measurements*

$^3H_2O$ and [$^{14}$C]-inulin were measured by liquid scintillation counting in a Triton X-100/toluene/Liquifluor (Beckman) cocktail. Quench corrections were made by the external standard channels ratio method. Net and unidirectional $H_2O$ fluxes were measured as previously described (Mailman, 1984). In brief, net $H_2O$ absorption was determined from the inflow rate and the change in [$^{14}$C]-inulin concentration. The absorptive and secretory $H_2O$ fluxes were determined from the net $H_2O$ absorption and the change in specific activity of $^3H_2O$ due to unlabelled $H_2O$ entering the lumen from the plasma. Blood levels of $^3H_2O$ were estimated from the final plasma concentration, assuming a linear increase over time.

Effective mucosal blood flow at the site of $H_2O$ absorption was measured as absorptive site blood flow (ASBF), as previously described (Mailman, 1981). Briefly, the clearance of $^3H_2O$ from the lumen was calculated by dividing the total amount of $^3H_2O$ absorbed by the effluent concentration of $^3H_2O$. This technique equates the lumenal $^3H_2O$ concentration with plasma concentration at the mucosal absorptive site because of the rapid equilibration of $H_2O$ within the mucosa, as shown elsewhere (Mailman, 1981).

$NO_2^-$ in the lumen perfusion solutions and effluent was measured by the Greiss reaction. Preliminary experiments showed that addition of HCl, to initiate the conversion of $NO_2^-$ to NO, caused a small amount of turbidity in otherwise clear effluent. Hence, HCl (final concentration 1.2 M) and sulphanillic acid (final concentration 1.2 mM) were added first, and optical density at 540 nm was measured. Then, N-(1-naphthyl)ethylenediamine HCl (final concentration 0.3 mM) was added, and optical density was reread. The $NO_2^-$ concentrations were determined from the change in optical density in standards and samples. Net $NO_2^-$ appearance was calculated from the difference between the amounts of $NO_2^-$ in the inflow and effluent solutions. The amounts were calculated from the concentrations and flow rates in the two inflow perfusates and the effluent solution.

Blood pressure (BP) was measured by transducer and was continuously recorded on a polygraph (Narco). ASBF resistance per 100 g gut was caculated as BP $ASBF^{-1}$ $100^{-1}$.

### *Drugs*

L-Arg HCl, D-Arg HCl and L-NOARG were obtained from Sigma and dissolved in the lumenal perfusion solutions. L-Arg and L-NOARG (1 mM) decreased the pH of the Krebs solution by only 0.12 and 0.09, respectively.

### *Statistics*

The parameter values in periods after the initial control periods are expressed as a change from the mean of the initial period values. Initial values are given in the figure legends. Statistical comparisons were by two – factor ANOVA and Dunnett's (1955) or unpaired *t* test. ANOVA was used to compare all periods and treatments in KRB control animals with those perfused with L-NOARG or L-Arg. If significant differences were present within treatments, then the Dunnett *t* test was used to compare several treatments to a single control (KRB controls vs L-Arg at 0.1, 0.5, 1 mM and L-NOARG at 0.01, 0.05, 0.1 mM and 0.1, 0.5, 1 mM), at each 10 min period so as to account for any changes due to time/and or the small changes in perfusion rates and any effects of the baseline perfusion. Unpaired *t* test, after ANOVA, was used for comparing each period in D-Arg controls to D-Arg plus L-NOARG (0.1, 0.5, 1 mM) and L-Arg controls to L-Arg plus L-NOARG (0.1, 0.5, 1 mM). Values are given as mean ± s.e.mean. Significance was considered at $P<0.05$ by two-tailed comparisons. Calculations were carried out using Statview 512 on a Macintosh computer.

## Results

### *Effects of L-NOARG alone*

Net $H_2O$ absorption was not decreased by L-NOARG (0.01 or 0.05 mM) but was decreased after 20 min of 0.1 mM L-NOARG and then returned to control levels 30 min after the L-NOARG was stopped (Figure 1). The maximum decrease was to about 50% of control. In a second series of experiments, net $H_2O$ absorption was decreased by L-NOARG (0.1–1.0 mM) and returned to control levels 30 min after the L-NOARG was stopped (Figure 1). The maximum decrease was to about 50% of control, similar to the effect of 0.1 mM L-NOARG seen in the previous experiments.

Blood pressure was not significantly changed by the concentration of L-NOARG (0.1 mM) that first reduced net $H_2O$

absorption when L-NOARG was perfused at 0.01–0.1 mM (Figure 2). Blood pressure was increased after 20 min of 0.5 mM and during 1.0 mM L-NOARG, but not at 0.1 mM or the first 20 min of 0.05 mM that decreased net $H_2O$ absorption, and blood pressure remained elevated after L-NOARG was stopped (Figure 2).

L-NOARG had only minor effects on $H_2O$ fluxes or ASBF. L-NOARG caused a significant decrease in the secretory $H_2O$ flux only in one period at 1 mM and decreases in the absorptive $H_2O$ flux and ASBF in the last two periods at 1 mM and in the first period after 1 mM L-NOARG was stopped (not shown).

Lumenal $NO_2^-$ appearance was inconsistently affected by lumenal L-NOARG (not shown). $NO_2^-$ appearance was significantly decreased, relative to controls, in one period during infusion of 0.1 mM L-NOARG and in three periods at 1 mM. The significant changes represented about a 30% decrease from control. There were no significant changes in lumenal $NO_2^-$ appearance in any other experiments even if changes in net $H_2O$ absorption or blood pressure occurred (not shown).

### *Effects of L-Arg alone*

L-Arg had no significant effects on net $H_2O$ absorption or blood pressure (not shown). L-Arg significantly increased ASBF and the absorptive $H_2O$ flux only in one period (not shown). The unidirectional secretory $H_2O$ fluxes were significantly increased by L-Arg in four periods at 0.5–1.0 mM (not shown).

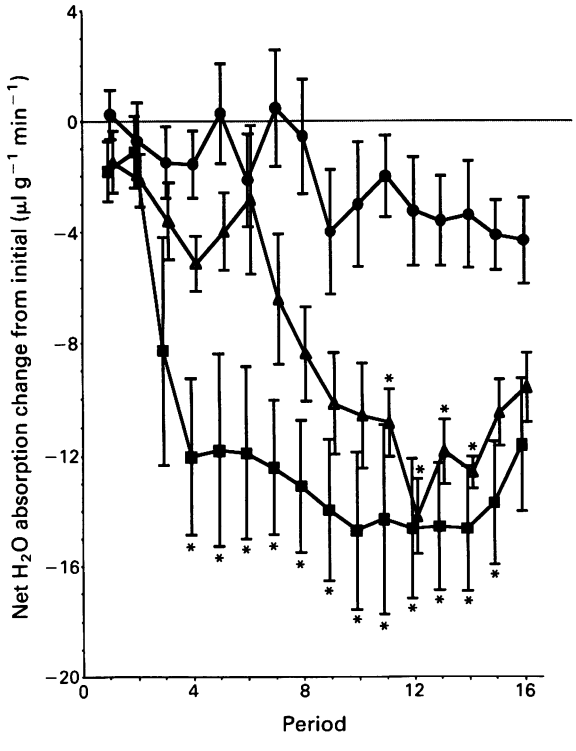


**Figure 1** Net $H_2O$ absorption from rat ileum during lumenal perfusion with Krebs solution (●) ($n = 11$) or $N^G$-nitro L-arginine (L-NOARG) at 0.01, 0.05, 0.1 mM and after stopping L-NOARG during periods 1–4, 5–8, 9–12 and 13–16, respectively (▲) ($n = 8$) or L-NOARG at 0.1, 0.5, 1 mM and after stopping L-NOARG during periods 1–4, 5–8, 9–12 and 13–16, respectively (■) ($n = 7$). Points represent 4–10 min periods at each concentration. Values are expressed as a change from initial control values. Initial values were $21.6 \pm 2.2$, $21.9 \pm 2.3$ and $26.6 \pm 2.7$ µl $g^{-1}$ $min^{-1}$ for the Krebs perfusion, 0.01–0.1 L-NOARG and 0.1–1 L-NOARG experiments, respectively. Mean ± s.e.mean. *represents a significant difference from periods in control animals, $P < 0.05$.

### *Effects of L-NOARG with D-Arg*

Net $H_2O$ absorption was decreased about 75% by L-NOARG (0.1–1.0 mM) in the presence of a background perfusion of 1 mM D-Arg, and it returned to control levels 20 min after the L-NOARG was stopped (Figure 3). The initial net $H_2O$

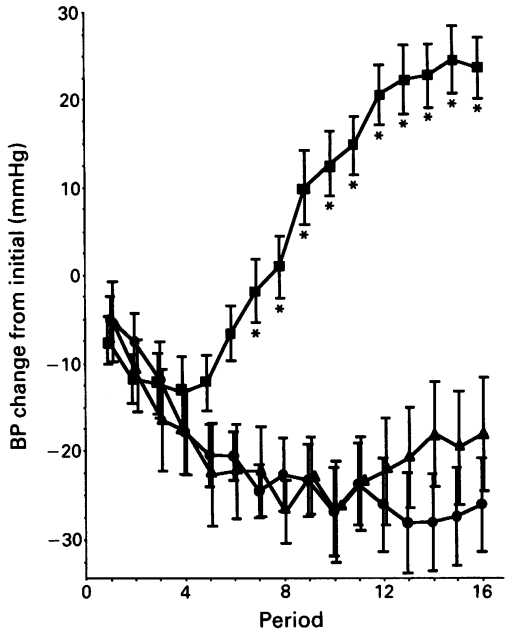


**Figure 2** Blood pressure (BP) during lumenal perfusion with Krebs solution (●) ($n = 11$) or $N^G$-nitro L-arginine (L-NOARG) at 0.01, 0.05, 0.1 mM and after stopping L-NOARG during periods 1–4, 5–8, 9–12 and 13–16, respectively (▲) ($n = 8$) or L-NOARG at 0.1, 0.5, 1 mM and after stopping L-NOARG during periods 1–4, 5–8, 9–12 and 13–16, respectively (■) ($n = 7$). Points represent 4–10 min periods at each concentration. Values are expressed as a change from initial control values. Initial values were $106 \pm 5$, $118 \pm 6$ and $110 \pm 5$ mmHg for the Krebs, 0.01–0.1 L-NOARG and 0.1–1 L-NOARG experiments, respectively. Mean ± s.e.mean. *represents a significant difference from periods in control animals, $P < 0.05$.

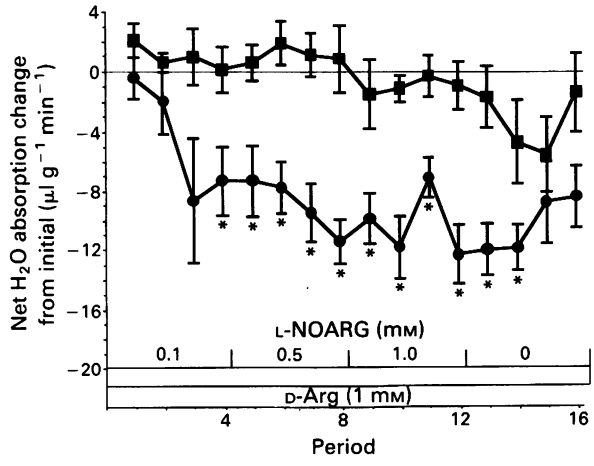


**Figure 3** Net $H_2O$ absorption during lumenal perfusion through rat ileum with 1 mM D-arginine (D-Arg) (■) ($n = 6$) or D-Arg + $N^G$-nitro L-arginine (L-NOARG) (●) ($n = 6$) at 0.1, 0.5, 1 mM and after stopping L-NOARG during periods 1–4, 5–8, 9–12 and 13–16, respectively. D-Arg and L-NOARG concentrations are shown on the X-axis. Points represent 4–10 min periods at each concentration. Values are expressed as a change from initial control values. Initial values were $16.7 \pm 2.4$ and $13.3 \pm 1.5$ µl $g^{-1}$ $min^{-1}$ for D-Arg and D-Arg + L-NOARG experiments, respectively. Mean ± s.e.mean. *represents a significant difference from periods in control animals, $P < 0.05$.

absorption during baseline perfusion with 1 mM D-Arg (Figure 3 legend) was significantly ($P<0.01$) lower than during perfusion with Krebs solution (Figure 1 legend).

Blood pressure was increased by L-NOARG (0.1–1.0 mM) in the presence of 1 mM D-Arg and remained elevated after L-NOARG was stopped (Figure 4). The responses of both net $H_2O$ absorption and blood pressure were qualitatively similar to the effects of L-NOARG alone (Figures 1 and 2) but were potentiated, in that they occurred earlier or at a lower concentration, in the presence of D-Arg.

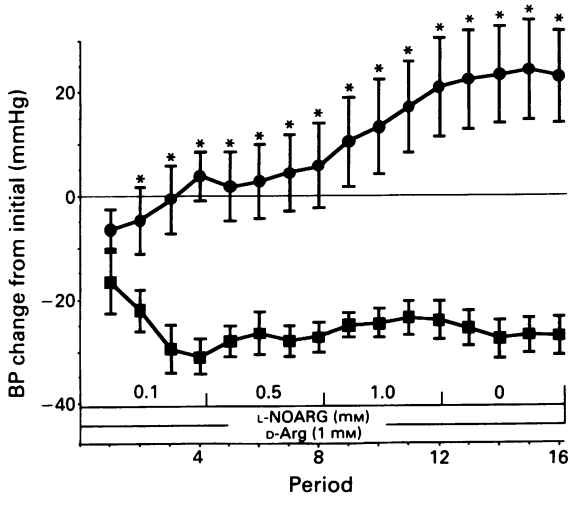


**Figure 4** Blood pressure during lumenal perfusion through rat ileum with 1 mM D-arginine (D-Arg) (■) ($n=6$) or D-Arg + $N^G$-nitro L-arginine (L-NOARG) (●) ($n=6$) at 0.1, 0.5, 1 mM and after stopping L-NOARG during periods 1–4, 5–8, 9–12 and 13–16, respectively. D-Arg and L-NOARG concentrations are shown on the X-axis. Points represent 4–10 min periods at each concentration. Values are expressed as a change from initial control values. Initial values were 117 ± 6 and 101 ± 8 mmHg for the D-Arg and D-Arg + L-NOARG experiments, respectively. Mean ± s.e.mean. *represents a significant difference from periods in control animals, $P<0.05$.

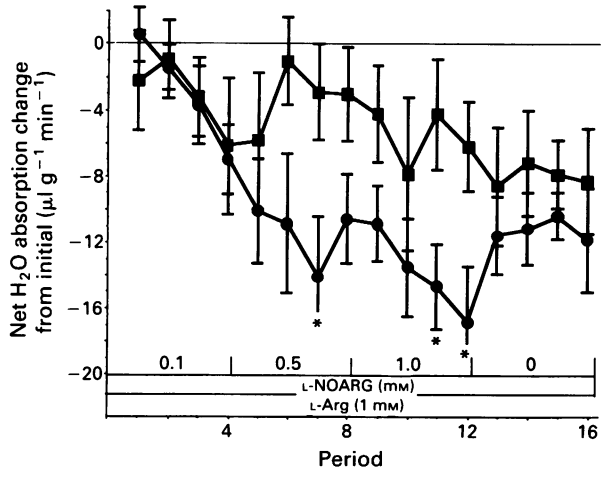


**Figure 5** Net $H_2O$ absorption from rat ileum during baseline lumenal perfusion with 1 mM L-arginine (L-Arg) (■) ($n=6$) or L-Arg + $N^G$-nitro L-arginine (L-NOARG) (●) ($n=6$) at 0.1, 0.5 and 1 and after stopping L-NOARG during periods 1–4, 5–8, 9–12 and 13–16, respectively. L-Arg and L-NOARG concentrations are shown on the X-axis. Points represent 4–10 min periods at each concentration. Values are expressed as a change from initial control values. Initial values were 19.1 ± 1.6 and 20.0 ± 2.4 µl $g^{-1}$ $min^{-1}$ for L-Arg and L-Arg + L-NOARG experiments, respectively. Mean ± s.e.mean. *represents a significant difference from periods in control animals, $P<0.05$.

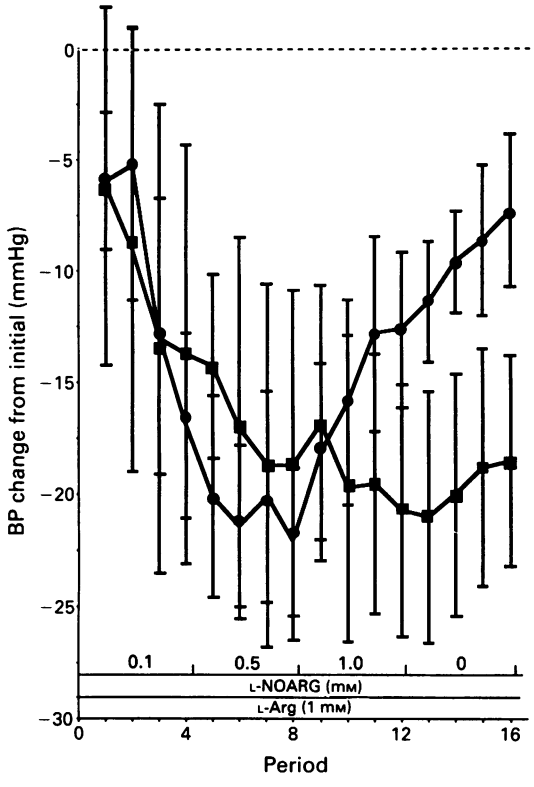


**Figure 6** Blood pressure during lumenal perfusion through rat ileum with 1 mM L-arginine (L-Arg) (■) ($n=6$) or L-Arg + $N^G$-nitro L-arginine (L-NOARG) (●) ($n=6$) at 0.1, 0.5, 1 mM and after stopping L-NOARG during periods 1–4, 5–8, 9–12 and 13–16, respectively. L-Arg and L-NOARG concentrations are shown on the X-axis. Points represent 4–10 min periods at each concentration. Values are expressed as a change from initial control values. Initial values were 108 ± 9 and 119 ± 7 mmHg for L-Arg and L-Arg + L-NOARG experiments, respectively. Mean ± s.e.mean.

There were few significant effects of L-NOARG with D-Arg on unidirectional $H_2O$ fluxes or ASBF, and these were similar to those observed with L-NOARG alone.

### *Effects of L-NOARG with L-Arg*

Net $H_2O$ absorption was inconsistently decreased by L-NOARG (0.5–1.0 mM) in the presence of a background perfusion at 1 mM L-Arg, and it returned to control levels after the L-NOARG was stoppped (Figure 5). The effects of L-NOARG in reducing net $H_2O$ absorption were inhibited by L-Arg compared to L-NOARG alone (Figure 1). The increase in blood pressure produced by L-NOARG alone (Figure 2) was completely blocked by L-NOARG (0.1–1.0 mM) in the presence of 1 mM L-Arg (Figure 6).

L-NOARG, in the presence of L-Arg, had only minor effects on unidirectional $H_2O$ fluxes or ASBF similar to L-NOARG when by itself. The absorptive $H_2O$ flux decreased only in two periods and there were no significant effects on ASBF or the secretory $H_2O$ flux.

### *Effects on ASBF resistance*

ASBF was little changed by any of the treatments. Hence, ASBF resistance paralleled blood pressure in all experiments (not shown).

## Discussion

These experiments were designed to determine if endogenous mucosal NO could regulate $H_2O$ absorption and blood flow.

Net $H_2O$ absorption and blood flow were intially considered as local effects, while blood pressure was considered a systemic effect, of agents applied lumenally. There are several cell types in the intestinal mucosa that can produce NO (Blachier *et al.*, 1991; Salter *et al.*, 1991; Nathan, 1992). Lumenal L-NOARG will inhibit tonic NO production (Rees *et al.*, 1990). L-Arg will increase NO production if endogenous L-Arg availability is rate limiting (Creager *et al.*, 1992). Lumenal agents would be presented to the mucosa at higher concentrations than those reached in the general circulation after absorption. Once in the circulation, systemic effects could include effects on the intestine. The data indicate that there is tonic mucosal NO production that increases net $H_2O$ absorption but that blood flow is not locally affected.

Lumenal L-NOARG decreased net $H_2O$ absorption. Decreases were observed beginning at concentrations of 0.1 mM. These responses suggested that tonic NO production increased net $H_2O$ absorption, and blocking NO production removed the stimulation. Lower lumenal concentrations of L-NOARG that decreased net $H_2O$ absorption did not change blood pressure, and, thus, L-NOARG had not been absorbed into the circulation in amounts sufficient to exert systemic effects. Hence, the effect of L-NOARG on net $H_2O$ absorption was local and not part of a systemic response. In experiments by others, i.a. administration of $N^G$-nitro-L-arginine methyl ester (L-NAME) did not change fluid transport in feline intestine (Kubes, 1992). This difference in results could be related to the route of administration of the NO production inhibitor or species differences.

L-NOARG decreased net $H_2O$ absorption without parallel effects on the unidirectional $H_2O$ fluxes, i.e. changes in unidirectional $H_2O$ fluxes were not significant, but the difference between them was consistently changed to favour reduced net absorption. This suggested that active absorption by enterocytes was affected. The net flux of $H_2O$ is only about 15% of the unidirectional $H_2O$ fluxes, and is due to the bias of active transcellular transport imposed on the unidirectional $H_2O$ fluxes that are affected to a greater extent by local cardiovascular events (Mailman, 1981; 1984). Thus, net $H_2O$ absorption can be significantly changed in the absence of significant changes in unidirectional $H_2O$ fluxes. Previous studies have shown that the unidirectional secretory fluxes are increased when mucosal capillary pressure is increased, thus favouring passive secretion driven by increased interstitial pressure (Mailman, 1984). Capillary pressure would be little changed by L-NOARG, in these experiments, because transmission of the increased arterial pressure to the capillaries was reduced by the increased resistance, that is largely precapillary (Kubes & Granger, 1992). The unidirectional absorptive fluxes are increased when ASBF increases, due to a washout effect of the blood flow on absorbed substances (Mailman, 1984). ASBF was little changed by L-NOARG, in these experiments, and thus, there was little significant effect on the absorptive fluxes. Some of the effect of L-Arg in increasing unidirectional $H_2O$ fluxes may be accounted for by its causing small increases in ASBF and increasing capillary pressure as precapillary resistance decreased in the face of constant arterial pressure.

NO can act through stimulation of soluble guanylate cyclases (Moncada *et al.*, 1991). Increased guanosine 3':5'-cyclic monophosphate (cyclic GMP) in enterocytes, as caused by heat-stable enterotoxin acting on particulate guanylate cylase, for example, causes intestinal secretion (Waldman & Murad, 1987). Reducing mucosal NO should reduce enterocyte cyclic GMP if these are target cells of NO. In the present experiments, reduced NO caused decreased absorption, an effect opposite to that expected from reducing cyclic GMP. However, enterocytes have relatively little soluble guanylate cyclase (Waldman & Murad, 1987), thus making it unlikely that enterocytes were acted on directly by NO. The effects of L-NOARG on transport may be due to effects on regulatory cells in the mucosa that, in turn, affect enterocytes.

Another cell type that may be involved are the fibroblasts that underlie the epithelial layer. Fibroblasts enhance the secretory response of cultured epithelial cells to agents that are known to stimulate NO release, as well as the release of other bioactive substances (Berschneider & Powell, 1992). Therefore, direct fibroblast-enterocyte interactions would cause increased absorption if NO production had been blocked by L-NOARG. Again, this response would be opposite to the observed response.

L-NOARG may act on mucosal neurones to decrease mucosal absorption. NO can interfere with cholinergic transmission (Lefebvre *et al.*, 1992), and cholinergic stimulation increases intestinal secretion or reduces absorption (Cooke, 1984). There is tonic neural inhibition of intestinal absorption (Andres *et al.*, 1985; Cooke, 1989). If NO interferes with cholinergic transmission, then L-NOARG would increase cholinergic stimuli and, thus, decrease absorption, as was observed. However, there are relatively few NO producing neurones in the mucosa (Young *et al.*, 1992), although the NO may be released by other types of cells and then act on mucosal neurones. The cell types involved and the mechanism of the decreased absorption due to lumenal L-NOARG requires further study.

Lumenal L-Arg partially blocked the effect of L-NOARG in reducing net $H_2O$ absorption but completely blocked the increased blood pressure occurring at 0.5 and 1 mM. The relatively greater effect of L-Arg in blocking the effects of L-NOARG on blood pressure, as compared to net $H_2O$ absorption, may be due to the relative concentrations of these two compounds reached in the mucosa, as compared to the circulation and/or to the relative sensitivity of the vasculature, as compared to the cells involved in the response of $H_2O$ absorption. Lumenal D-Arg did not block the effects of L-NOARG. These findings further supported the suggestion that NO was the active agent because effects on NO production are enantiomer specific. However, D-Arg potentiated the effects of L-NOARG in decreasing absorption and increasing blood pressure. D-Arg may decrease the availability of L-Arg and thus, reduce NO production. This possibility is consistent with the effect of D-Arg, by itself, in decreasing the initial baseline net $H_2O$ absorption.

The use of lumenal D-Arg or L-Arg to characterize the responses to L-NOARG as due to inhibition of NO formation assumed that their interaction occurred at NOS. Another possibility is that L-Arg or D-Arg may differentially inhibit the transport of L-NOARG across the mucosa, where local effects could occur, and then into the circulation, where systemic effects could occur. Transport was not measured in these experiments, but neither L-NOARG nor D-Arg greatly inhibited the uptake of L-Arg into endothelial cells (Bogle *et al.*, 1992). The endothelial cell transport system resembles the $y^+$ system (Mann *et al.*, 1990) that is also the major L-Arg transport pathway in the intestine (Satoh *et al.*, 1989; Cheeseman *et al.*, 1992). Hence, effects of L-Arg or D-Arg on L-NOARG transport across the mucosa are not likely and were not tested.

Although the inhibition of L-NOARG effects on $H_2O$ absorption and blood pressure by L-Arg suggested that NO was a mediator, the small effects of L-NOARG on the entry rate of $NO_2^-$ into the lumen did not support this possibility. L-NOARG caused only inconsistent decreases in lumenal $NO_2^-$ and there was no parallel between lumenal $NO_2^-$ and the effects of any treatment on $H_2O$ absorption or blood pressure. Lumenal $NO_2^-$ may not reliably represent lumenal NO entry, because $NO_2^-$ moves across the gut mucosa (Witter *et al.*, 1979; Witter & Balish, 1979). Hence, lumenal $NO_2^-$ may underestimate NO production and also be subject to variation because of changes in transport rate.

L-Arg had little or no effect on net $H_2O$ absorpton, blood pressure, ASBF or lumenal $NO_2^-$. This suggested that lumenal L-Arg was not rate limiting for NO production, in agreement with other findings (Griffith *et al.*, 1991; Creager *et al.*, 1992). It is possible that the small effects on the

unidirectional $H_2O$ fluxes were due to L-Arg acting as a transported and metabolized amino acid.

Local ASBF resistance paralleled systemic blood pressure, indicating that L-NOARG did not have a local effect on the mucosal vasculature. This is surprising because L-NOARG would have a locally higher concentration in the mucosa than after dilution in the systemic circulation. An analogous effect was observed when rats were given $N^G$-monomethyl-L-arginine (L-NMMA) or L-NAME by i.v. or drinking $H_2O$ routes (Gardiner *et al.*, 1990a,b). In these experiments, the mesenteric vasculature was more sensitive than the hindquarters to i.v. NOS antagonists and responded more rapidly. During oral administration, mesenteric blood flow was not greatly changed, relative to controls at the same time, but mesenteric conductance was decreased, and the mesenteric responses were less than those of the hindquarters. A similar effect has been observed in the renal circulation (Granger *et al.*, 1992). L-NAME, at approximately the same dose delivered to the renal circulation, had much greater effects when infused i.v. than when infused i.a. The differences were attributed to systemic effects on the kidneys, and generalized sympathetic stimulation was suggested as a possible mechanism. Local i.a. NOS antagonists increased total intestinal resistance and decreased blood flow in cats, but the blood flow distribution within the gut was not examined (Kubes & Granger, 1992). Possibly, the L-NOARG may act on the intestinal microcirculation at resistance vessels that are sufficiently distant from the mucosa that they are not affected by mucosal levels of L-NOARG but are affected by circulating levels. Other research has shown that the resistance of the mesentery was increased by i.v. NOS antagonists, but the intestinal resistance was not affected even though blood pressure increased (Pizcueta *et al.*, 1991). Alternatively, there could be local and systemic compensations that tend to maintain mucosal blood flow at constant levels and that override local effects of L-NOARG.

In summary, lumenal L-NOARG decreased net $H_2O$ absorption at lower concentrations than those that increased blood pressure, suggesting that tonic NO production by the mucosa increased $H_2O$ absorption. Lumenal L-Arg reduced the effects of L-NOARG, but D-Arg did not, further, supporting a role of NO. However, the rate of entry of $NO_2^-$ into the lumen, used as a measure of NO production, did not support a role of mucosal NO in the observed effects. A local effect of NO on mucosal blood flow resistance was not found.

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# Regulation by hypoxia of endothelin-1-stimulated phospholipase D activity in sheep pulmonary artery cultured smooth muscle cells

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**1** The aim of the study was to characterize the effects of hypoxia on agonist-stimulated phospholipase D (PLD) and phospholipase C activity of sheep pulmonary artery cultured smooth muscle cells.

**2** Endothelin-1 (ET-1), 5-hydroxytryptamine (5-HT) and the protein kinase C (PKC) activator tetradecanoylphorbol acetate (TPA), stimulated a time- and concentration-dependent increase in [$^3$H]-phosphatidylbutanol accumulation. This was abolished by pretreatment of the cells with the PKC inhibitor, Ro-318220, suggesting that agonist-stimulated phospholipase D activity is dependent upon the activation of PKC.

**3** Hypoxia ($Po_2$ 20 mmHg for 30 min) stimulated basal [$^3$H]-phosphatidylbutanol accumulation by approximately 2 fold and this activity was abolished by preincubation of the cells with 10 $\mu$M Ro-318220.

**4** In cells preincubated in low $O_2$ containing medium for 30 min, the subsequent agonist-stimulated accumulation of [$^3$H]-phosphatidylbutanol was reduced. However, the decrease in stimulation was greater for ET-1 and 5-HT than for TPA.

**5** ET-1 and TPA stimulated a time-dependent increase in protein kinase C- mediated psuedosubstrate phosphorylation. Following preincubation for 30 min in low $O_2$ containing media, basal pseudosubstrate phosphorylation increased whilst the fold stimulation by TPA and ET-1 decreased.

**6** In cells preincubated in low $O_2$ containing medium, ET-1-stimulated [$^3$H]-inositol phosphate accumulation was reduced by approximately 30– 40%. This reduction was reversed by preincubation of the cells with Ro-318220.

**7** These results suggest a role for PKC in the effects of hypoxia on PLD in pulmonary artery smooth muscle cells.

**Keywords:** Pulmonary artery smooth muscle cells; phospholipase D; endothelin-1; hypoxia; protein kinase C

## Introduction

Acute and chronic hypoxia have been shown to affect agonist-stimulated contraction and growth of pulmonary artery smooth muscle. This is due not only to modulation of the release of vasoactive substances from endothelial cells and fibroblasts (Rakugi *et al.*, 1990), but also through a direct action upon the underlying smooth muscle cells (Murray *et al.*, 1990; Dempsey *et al.*, 1991; Butler *et al.*, 1991). The molecular events underlying such an effect are unclear although the hydrolysis of phosphatidylinositol (4,5) bisphosphate [PtdIns(4,5)$P_2$], the mobilisation of both intra- and extracellular calcium and the activation of protein kinase C (PKC) have been implicated (Dempsey *et al.*, 1991; Maclean & Nally 1992; Jin *et al.*, 1992).

An intracellular signalling pathway which may also play a role in the regulation of smooth muscle cell growth and contraction involves the hydrolysis of phosphatidylcholine by phospholipase D (PLD) (Cook & Wakelam 1989; Billah & Anthes 1990; MacNulty *et al.*, 1990; Plevin *et al.*, 1992). The product from this reaction, phosphatidic acid, may have second messenger roles in agonist-stimulated smooth muscle contraction (Ohanian *et al.*, 1990). In addition, phosphatidic acid may be converted by phosphatidic acid phosphohydrolase to sn-1,2 diacylglycerol, the physiological activator of PKC (Nishizuka, 1984).

The aims of the present experiments were: (1) to characterize the effect of hypoxia upon agonist-stimulated PLD activity in primary cultures of pulmonary artery smooth muscle cells under oxygenated and hypoxic conditions and (2) to determine the role of PKC in modulating these effects. A preliminary account of these findings has been presented to the British Pharmacological Society (Plevin *et al.*, 1993).

## Methods

Sheep pulmonary artery smooth muscle cells were obtained by collagenase digestion and cultured in Dulbecco's Modified Eagles Medium (DMEM) containing 15% foetal calf serum, glutamine 2 mM, fungizone 25 $\mu$g ml$^{-1}$, and penicillin 250 i.u. ml$^{-1}$, streptomycin 250 $\mu$g ml$^{-1}$, at 37°C in an atmosphere of 95/5% air/$CO_2$. Passages 3–9 were used routinely for the experiments described below. The identity of the cells was confirmed by immunoblotting for smooth muscle $\alpha$-actin (results not shown).

Phospholipase D (PLD) activity was measured as the accumulation of [$^3$H]-phosphatidylbutanol. Sheep pulmonary artery smooth muscle cells were prelabelled for 40 h in serum-free DMEM containing [$^3$H]-palmitic acid (specific activity 12–20 kBq mmol$^{-1}$) at a concentration of 2 $\mu$Ci ml$^{-1}$. On the day of the experiment, the cells were preincubated in gassed Krebs solution composition (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11.7, pH 7.4 for 30 min at 37°C then incubated in Krebs solution containing 30 mM butanol for a further 5 min. The reaction was initiated by the addition of agonist in Krebs solution containing 30 mM butanol. However, during the low oxygen experiments, agonist and butanol were added simultaneously. The reaction then was terminated by rapid aspiration followed by the addition of

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0.5 ml ice cold methanol. After 10 min on ice, the cell wells were scraped and washed and the extracts transferred into glass vials. Labelled phospholipid products were extracted at room temperature for 60 min by the addition of 0.35 ml chloroform and aqueous and organic phases split by the further addition of chloroform (0.35 ml) and water (0.5 ml) followed by centrifugation. The aqueous phase was removed and the lower chloroform layer dried down under vacuum. The extract was redissolved in 50 μl chloroform and 25 μl applied to Whatman t.l.c. plates. The resolving solvent was the upper phase of ethylacetate/2,2 trimethylpentane/water and acetic acid (50/110/100/20 v/v). The peak corresponding to labelled phosphatidylbutanol ([$^3$H]-PtdBuOH) was excised and counted by liquid scintillation.

For measurement of [$^3$H]-inositol phosphate accumulation ([$^3$H]-IP), sheep pulmonary artery smooth muscle cells were prelabelled in serum-free media as outlined above but in the presence of [$^3$H]-inositol (specific activity 20.0 kBq mmol$^{-1}$). On the day of the experiments the cells were preincubated with Krebs for 20 min and then for a further 10 min in Krebs solution containing 10 mM lithium chloride before stimulation by the addition of agonist in a final volume of 0.5 ml. The reaction was terminated by aspiration and the addition of ice cold methanol (0.5 ml). Following scraping and transfer to plastic vials, the inositol polyphosphates were extracted by the addition of chloroform (0.5 ml) and methanol (0.5 ml) to give a final ratio of 2:1. The phases were then split by the addition of chlorofom/methanol/water to a final ratio of 1:1:0.8 and a sample of the aqueous phase was assayed for inositol phosphates by ion anion exchange chromatography on Dowex formate columns as previously described (Plevin *et al.*, 1992).

For the measurement of agonist-stimulated PKC activity, a modified version of the method used by Alexander *et al.* (1990) was employed. Sheep pulmonary artery smooth muscle cells, grown to confluency and quiescence on 6 cm$^2$ plates, were preincubated with Krebs solution at 37°C for 30 min. The cells were washed twice in a buffer containing (mM): KCl 150, $MgCl_2$ 5.16, PIPES 12.5, EGTA 12.5 and $CaCl_2$ 8.17, pH 7.4 and the reaction initiated in the same buffer containing agonist and 0.5 units ml$^{-1}$ of streptolysin-O, 200 μM ATP [γ-$^{32}$P]ATP, (300–450 c.p.m. pmol$^{-1}$, specific activity = 2200 Ci mmol$^{-1}$) and 200 μM of a PKC peptide pseudosubstrate in a final volume of 400 μl. Following a 10 min incubation period the reaction was terminated by the addition of 100 μl of 25% (w/v) trichloroacetic acid in 2 M acetic acid. After 10 min on ice, the cells were scraped and the extracts transferred to microfuge tubes and spun for 5 min for 14,000 *g* at 4°C. Aliquots of the supernatants were spotted onto 3 cm squares of P81 Whatman ion exchange chromatography paper. The squares were washed (× 3) in 100 ml of 75 mM phosphoric acid (10 min each wash) and then once in ethanol. The squares were dried and measured by scintillation counting. Non-specific phosphorylation (3–5% of total phosphorylation) in the absence of peptide was measured in each assay and subtracted from all samples. Two different PKC substrates were used in the experiments which yielded identical results (PKC 19-31ser$^{25}$ and GS-peptide).

For low oxygen experiments, sheep pulmonary artery smooth muscle cells were preincubated in Krebs solution degassed with nitrogen/$CO_2$ 95/5%. During preincubations and cell stimulations, a stream of nitrogen/$CO_2$ was passed over the cells. Under these conditions a $Po_2$ value of approximately 20 mmHg was achieved in the Krebs solution bathing the cells.

All dose-response data were analysed by an iterative curve fitting procedure (Delean *et al.*, 1980). Statistical comparisons were made with Student's unpaired *t* test.

The peptide pseudosubstrate, GS-peptide (Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys) was synthesized and purified by Dr E.G. Rowan of the Strathclyde Institute for Drug Research and Ro-31-8220 (3-{1-[3-(amidinothio)propyl]-3-indoyl}-4-(-methyl-3-indoyl)-1H-pyrrole 2,5-dione) was a kind gift from Dr G. Lawton of Roche Products Ltd., Welwyn Garden City, Herts. All radiochemicals were purchased from Amersham International. PKC 19-31ser$^{25}$ was purchased from Calbiochem. All other chemicals were of the highest quality commercially available.

## Results

Figure 1 shows the time course of [$^3$H]-PtdBuOH accumulation in sheep pulmonary artery smooth muscle cells in culture in response to endothelin-1 (ET-1), 5-hydroxytryptamine (5-HT) and the protein kinase C activator, tetradecanoylphorbol acetate (TPA). Endothelin-1 (100 nM) and 5-HT (30 μM) stimulated a rapid accumulation of [$^3$H]-PtdBuOH in pulmonary artery smooth muscle cells which reached a peak between 1–2 min, after which no further increase in accumulation was observed (Figure 1a). The maximum stimulation was different for the two agonists being 4.0 ± 1.5 fold in response to ET-1 ($n$ = 6), and 2.8 ± 0.7 fold in response to 5-HT ($n$ = 4). Following a lag time of approximately 30 s, TPA also stimulated the accumulation of [$^3$H]-

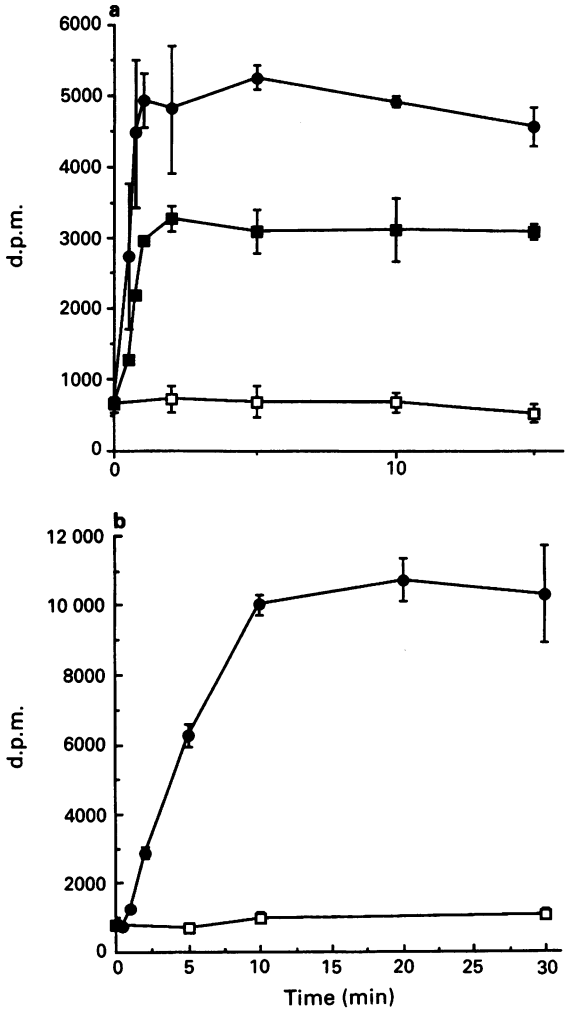


**Figure 1** Time course of endothelin-1 (ET-1)-, 5-hydroxytryptamine (5-HT)- and tetradecanoyl phorbol acetate (TPA)-stimulated [$^3$H]-phosphatidylbutanol accumulation in sheep pulmonary smooth muscle cells in culture. Cells prelabelled with [$^3$H]-palmitate, were incubated with maximum concentrations of ET-1 (100 nM) and 5-HT (30 μM) (a), and TPA (100 nM) (b), in the presence of 30 mM butanol. [$^3$H]-phosphatidylbutanol was assayed as outlined in the Methods section. Each point represents that mean ± s.d. of triplicate determinations from a single experiment representative of at least 3 others. (□) Control; (a) (■) 5-HT; (●) ET-1; (b) (●) TPA.

PtdBuOH in pulmonary artery smooth muscle cells (Figure 1b). However, in response to TPA, accumulation was sustained for a longer period reaching a peak at 10 min at approximately 8–10 fold the basal response (9.3 ± 2.0, $n = 7$).

ET-1, 5-HT and TPA stimulated [$^3$H]-PtdBuOH accumulation in a concentration-dependent manner. The ET-1 and TPA response was observed over the low nanomolar concentration range ($EC_{50}$ values: ET-1 = 1.9 ± 1.4 nM, TPA = 12.5 ± 4.9 nM, $n = 3$). 5-HT was less potent with an $EC_{50}$ value in the low micromolar range ($IC_{50}$ = 10.8 ± 3.7 μM, $n = 3$).

Figure 2 shows the effect of the specific PKC inhibitor Ro-318220 (Davis *et al.*, 1989) on both ET-1 and TPA stimulated PLD activity in pulmonary artery smooth muscle cells. Ro-318220 at concentrations of 10 μM or above virtually abolished the response to both compounds. However, there was a significant difference in the $IC_{50}$ value obtained for this effect against either the TPA or the ET-1 response ($IC_{50}$ for Ro-318220: TPA = 0.59 ± 0.09 μM, ET-1 7.3 ± 2.3 μM, $n = 4$; $P < 0.05$). Ro-318220 at 10 μM also abolished 5-HT-stimulated PLD activity (results not shown, $n = 1$).

Following 30 min incubation in low oxygen containing Krebs solution (20 mmHg), basal accumulation of [$^3$H]-phosphatidylbutanol increased by approximately 2 fold (2.01 ± 0.15, $n = 5$). The increase in basal accumulation observed in low oxygen containing buffer was abolished in the presence of the PKC inhibitor, Ro-318220 (Figure 3). In cells preincubated in low oxygen containing medium for 30 min the subsequent stimulation by TPA, ET-1 and 5-HT was reduced (Table 1). However, the decrease in stimulation in fold terms was greater for ET-1 and 5-HT than for TPA (Table 1). In contrast, when hypoxia was initiated simultaneously with agonist addition there was no change in the maximum accumulation of [$^3$H]-phosphatidylbutanol accumulation in response to ET-1 or TPA (results not shown).

TPA and ET-1 also stimulated PKC-mediated peptide pseudosubstrate phosphorylation in pulmonary artery smooth muscle cells (Figure 4). ET-1 (100 nM)-stimulated peptide pseudosubstrate phosphorylation reached a peak by 10 min (2.8 ± 0.5 fold, $n = 3$) and did not increase during the remainder of the time course. TPA (100 nM) also stimulated PKC activity which was sustained for up to 30 min, the longest time point studied (4.4 ± 1.2 fold, $n = 4$). In cells preincubated in low $O_2$ conditions for 30 min, basal PKC activity also increased by approximately 2 fold (Table 2). The maximum PKC activity stimulated by TPA remained the same resulting in an apparent reduction in the fold stimulation in response to the phorbol ester. However, the response to ET-1 was reduced to a greater extent than that observed for TPA (71 ± 12% vs 48 ± 8%, $n = 4$).

The effects of hypoxia upon ET-1-stimulated [$^3$H]-IP accumulation are shown in Figure 5 and Table 3. Preliminary experiments showed that ET-1 stimulated a linear increase in [$^3$H]-IP accumulation for up to 30 min. Following pretreatment in low oxygen containing medium, ET-1-stimulated accumulation of [$^3$H]-IP was reduced by 34 ± 9% ($n = 4$) whilst basal values were not affected. In control cells, pretreatment with 10 μM Ro-318220 enhanced ET-1 stimulated accumulation of [$^3$H]-IP by approximately 50% (52 ± 22% $n = 4$). Pretreatment of the cells with 10 μM Ro-318220 also prevented the hypoxia-induced reduction in ET-1-stimulated [$^3$H]-IP accumulation (Table 3).

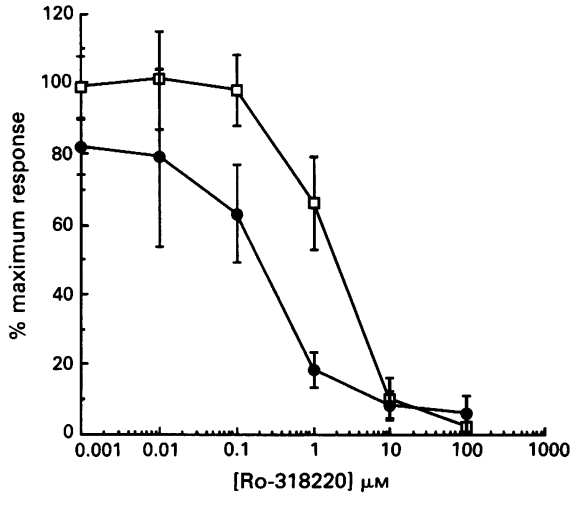


**Figure 2** The effect of Ro-318220 upon tetradecanoyl phorbol acetate (TPA)- and endothelin-1 (ET-1)-stimulated [$^3$H]-phosphatidylbutanol accumulation in sheep pulmonary smooth muscle cells in culture. Cells prelabelled with [$^3$H]-palmitate were preincubated with increasing concentrations of Ro-318220 for 20 min prior to stimulation with either ET-1 (100 nM) or TPA (100 nM) and assayed for [$^3$H]-phosphatidylbutanol accumulation as outlined in the Methods section. Each point represents the mean ± s.d. of triplicate determinations from a single experiment representative of at least 3 others. (●) TPA; (□) ET-1.

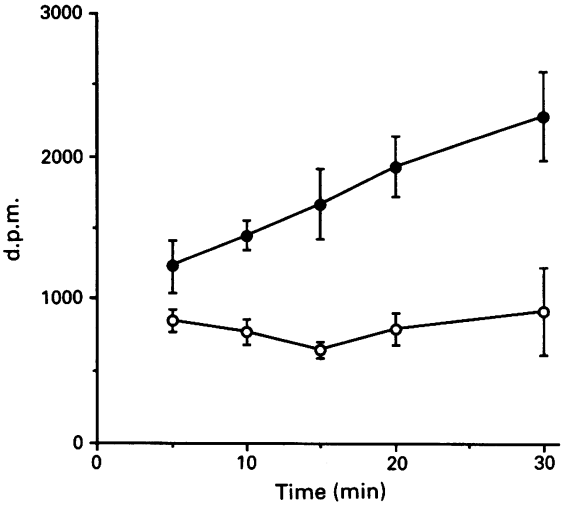


**Figure 3** The effect of Ro-318220 on hypoxia-induced [$^3$H]-phosphatidylbutanol accumulation in sheep pulmonary artery smooth muscle cells in culture. Cells prelabelled with [$^3$H]-palmitate were preincubated with 0.05% DMSO (●) or 10 μM Ro-318220 (○) for 20 min before incubation in low oxygen containing Krebs for the times indicated. [$^3$H]-phosphatidylbutanol accumulation was assayed as outlined in the Methods section. Each point represents the mean ± s.d. of triplicate determinations from a single experiment performed at least three times.

**Table 1** The effect of hypoxia upon agonist-stimulated [$^3$H]-phosphatidylbutanol accumulation in sheep pulmonary artery smooth muscle cells

| | *[$^3$H]-phosphatidylbutanol accumulation* (% basal) | |
|---|---|---|
| | *Control* | *Hypoxia* ($PO_2$ 20 mmHg) |
| TPA | 802 ± 196 | 347 ± 119 |
| ET-1 | 486 ± 134 | 157 ± 68 |
| 5-HT | 313 ± 87 | 108 ± 19 |

Cells prelabelled with [$^3$H]-palmitate were incubated in normal or low oxygen containing Krebs for 30 min then stimulated with either TPA (100 nM, 10 min) ET-1 (100 nM, 5 min) or 5-HT (30 μM, 5 min) in the presence of 30 mM butanol. Cell extracts were assayed for [$^3$H]-phosphatidylbutanol as outlined in the Methods section. Each value represents the mean ± s.d. from three experiments performed in triplicate. TPA = tetradecanoyl phorbol acetate; ET-1 = endothelin-1; 5-HT = 5-hydroxytryptamine.

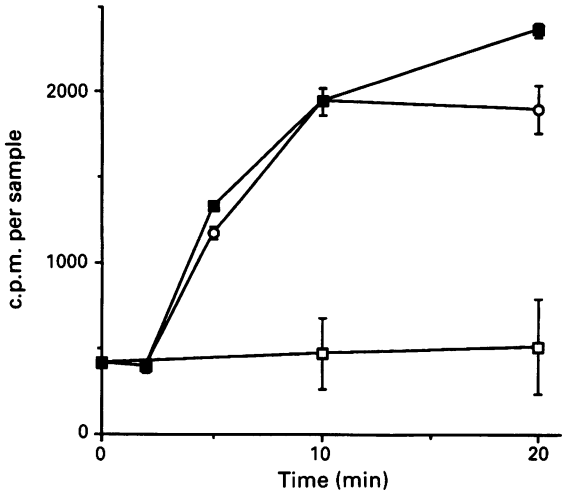


**Figure 4** Time course of tetradecanoyl phorbol acetate (TPA) and endothelin-1 (ET-1)-stimulated protein kinase C (PKC)-mediated peptide pseudosubstrate phosphorylation in sheep pulmonary artery smooth muscle cells in culture. Unlabelled cells were stimulated with either 0.05% DMSO (□), 100 nM TPA (■) or 100 nM ET-1 (○), for the times indicated then assayed for PKC activity as outlined in the Methods section. Each point represents the mean ± s.d. of triplicate determinations from a single experiment representative of three others.

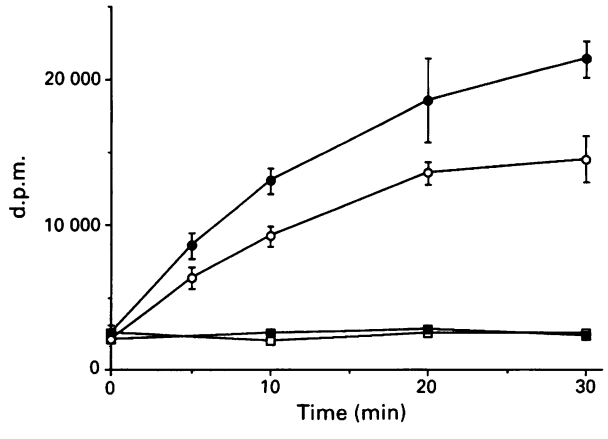


**Figure 5** The effect of hypoxia upon endothelin-1 (ET-1)-stimulated [$^3$H]-inositol phosphate accumulation in sheep pulmonary artery smooth muscle cells in culture. Cells prelabelled with [$^3$H]-inositol were preincubated in normal or low oxygen-containing Krebs for 30 min then stimulated with vehicle or 100 nM ET-1 for the times indicated. Cell extracts were assayed for total [$^3$H]-inositol phosphates as outlined in the Methods section. Each point represents the mean ± s.d. of triplicate determinations from a single representative experiment ($n = 4$). Control basal (□); low oxygen basal (■); control ET-1 (●); low oxygen ET-1 (○).

## Discussion

In sheep pulmonary artery cultured smooth muscle cells, both ET-1 and 5-HT produce a rapid and transient stimulation of PLD activity. This finding is consistent with results obtained in vasopressin-stimulated A10 smooth muscle cells and in bombesin-stimulated Swiss 3T3 fibroblasts (Plevin *et al.*, 1992; Plevin & Wakelam 1992; Briscoe *et al.*, 1993). We also observed that the response was dependent upon prior PKC activation since incubation with the PKC inhibitor, Ro-318220, abolished both TPA and agonist-stimulated PLD activity. However, it must be noted that the $IC_{50}$ values for Ro-318220 inhibition were different for TPA and ET-1 responses. We have previously suggested this difference to reflect

**Table 2** The effect of hypoxia upon tetradecanoyl phorbol acetate (TPA) and endothelin-1 (ET-1) stimulated protein kinase C (PKC)-mediated GS-peptide pseudosubstrate phosphorylation in sheep pulmonary artery smooth muscle cells in culture

| | *GS-peptide phosphorylation* (c.p.m.) | |
|---|---|---|
| | *Control* | *Low* $O_2$ (20 mmHg) |
| Basal | 478 ± 33 | 907 ± 99 |
| TPA | 1916 ± 89 | 2016 ± 127 |
| ET-1 | 1389 ± 79 | 1080 ± 78 |

Cells were incubated in normal or low oxygen containing Krebs for 30 min and then stimulated with vehicle, 100 nM TPA or 100 nM ET-1 for a further 10 min. GS-peptide phosphorylation was measured as outlined in the Methods section. Each value is the mean ± s.d. from a single experiment performed in triplicate which is representative of at least four independent experiments.

**Table 3** The effect of Ro-318220 on hypoxia-induced reduction of endothelin-1 (ET-1) stimulated [$^3$H]-IP accumulation in sheep pulmonary artery smooth muscle cells in culture

| | *Inositol phosphates* (d.p.m.) | |
|---|---|---|
| | *Control* | *Low* $O_2$ (20 mmHg) |
| Basal | 2627 ± 415 | 2727 ± 239 |
| Basal + Ro | 2887 ± 432 | 2562 ± 122 |
| ET-1 | 12320 ± 732 | 8512 ± 458 |
| ET-1 + Ro | 16555 ± 1257 | 15936 ± 963 |

Cells prelabelled with [$^3$H]-inositol were preincubated with 10 μM Ro-318220 or vehicle (0.05% DMSO) for 15 min before further incubation in normal or low oxygen containing Krebs for 30 min. Cells were incubated for a further 10 min with 100 nM ET-1. Each value represents the mean ± s.d. of results from a single experiment performed in triplicate and representative of 2 others.

a divergent effect of PKC isoforms upon the activation of PLD and initial receptor /G-protein/PLC coupling (Plevin *et al.*, 1992). These results are consistent with the presence of a sequential pathway in the agonist activation of PLD, involving initial hydrolysis of PtdIns(4,5)$P_2$ and the intermediate activation of PKC by sn-1,2 DAG (Cook & Wakelam, 1989; Cook *et al.*, 1990).

Hypoxia ($Po_2$ 20 mmHg for 30 min) introduced simultaneously with agonist did not affect the absolute maximum level of accumulation in response to ET-1. This is probably due to the fact that the agonist activation of PLD activity is rapidly desensitized and that changes in oxygen tension were not rapid enough to effect PLD activity. However, over a period of 30 min, [$^3$H]-phosphatidylbutanol accumulation increased to approximately twice the basal value. This increase was abolished by Ro-318220 suggesting that the effect was PKC mediated. In addition, 30 min by hypoxia also reduced the subsequent agonist activation of [$^3$H]-phosphatidylbutanol accumulation. For TPA this reduction reflected the increase in basal PLD activity but for ET-1 and 5-HT an additional decrease was observed. This suggested the involvement of another mechanism in the effect of hypoxia as well as an action upon basal PLD activity.

We further addressed the effect of hypoxia upon PKC activity by measuring PKC-mediated phosphorylation of a defined PKC pseudosubstrate in cells permeabilized with streptolysin-O. The onset of PKC pseudosubstrate phosphorylation was delayed in response to both ET-1 and TPA reflecting the time for permeabilisation to occur. In other systems agonist-stimulated pseudosubstrate phosphorylation has been shown to be abolished by either Ro-31822 or prolonged TPA pretreatment, suggesting that this assay is a

suitable marker for PKC activity (Saville *et al.*, unpublished observations). In cells preincubated in low $O_2$ containing media for 30 min we also observed that the maximum pseudosubstrate phosphorylation stimulated by TPA was not reduced suggesting that the level of maximum activity of PKC was not compromised. However, the basal PKC activity was increased by 2 fold and ET-1-stimulated PKC activity was reduced. The relative differences in the decreases in fold stimulation by TPA and ET-1 again suggested an additional effect of hypoxia upon receptor-mediated events upstream of PKC.

Further experiments suggested that the effect of hypoxia upon agonist-stimulated PLD and PKC activity may involve the additional regulation of the receptor/G-protein/PLC interaction since pretreatment of the cells in low oxygen containing Krebs reduced the subsequent ET-1-stimulated [$^3$H]-IP accumulation. This hypoxia-mediated effect may also involve the activation of PKC. Protein kinase C-mediated-negative feedback inhibition of agonist-stimulated inositol phosphate formation has been observed in a number of systems including bombesin-stimulated Swiss 3T3 fibroblasts and bradykinin-stimulated chromaffin cells in culture (Plevin *et al.*, 1990; Boarder & Challis 1992). We found that ET-1 stimulated [$^3$H]-inositol accumulation was reduced by pretreatment with the cells in low oxygen containing media and that this reduction was reversed by Ro-318220, results consistent with this proposal. Hypoxia-induced activation of PKC may therefore uncouple the receptor from the G-protein, reducing the subsequent stimulation of inositol phosphate accumulation and other second messenger formation. However, we cannot discount a PKC-mediated decrease in the number of ET-1 receptors, as observed in other vascular smooth muscle cells (Resink *et al.*, 1990), nor the possible inhibition of receptor-operated calcium channel activity. Since PLD activity in vascular smooth muscle cells is dependent on both PKC and external calcium (Lassegue *et al.*, 1991) one or both mechanisms could account for the subsequent reduction in agonist-activation of PLD and PKC.

In the context of these findings, it is clear that hypoxia-induced activation of PKC isoforms may have an important regulatory role on the subsequent activation of second messenger pathways such as PtdIns(4,5)$P_2$ hydrolysis in smooth muscle cells. This effect, whilst stimulating the cells to grow may not allow activation of pathways detrimental to the long term viability of the cell, for example sustained calcium mobilisation. Of additional importance in delineating the effects of hypoxia on pulmonary artery smooth muscle cell growth, may be a consideration of the temporal relationship between the exposure of the smooth muscle cell to vasoconstrictors and growth factors and the development of hypoxia.

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# The effect of calcium removal on the suppression by adenosine of epileptiform activity in the hippocampus: demonstration of desensitization

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**1** Previous work has suggested that presynaptic effects of adenosine may be dependent on divalent cations. The present study was undertaken to determine whether a similar requirement existed at postsynaptic sites.

**2** Extracellular recordings were made in the CA1 pyramidal cell layer of rat hippocampal slices following orthodromic stimulation of Schaffer collateral fibres in stratum radiatum or antidromic stimulation of the alveus. In antidromic stimulation experiments, $CaCl_2$ was omitted (calcium-free medium) or reduced to 0.24 mM (low calcium medium) and in some experiments $MgSO_4$ was increased to 2 mM. Kynurenic acid at concentrations of 1 and 5 mM in calcium-free medium and 1 mM in low calcium medium had no effect on secondary spike size.

**3** Adenosine and baclofen induced a concentration-dependent reduction in the amplitude of orthodromic potentials with maximum effects at 20 and 5 μM respectively.

**4** In nominally calcium-free medium, bursts of multiple population spikes were obtained in response to antidromic stimulation. Adenosine had little effect in reducing the secondary spike amplitude. At high concentration (2 mM) an initial depression was seen which declined within 3–5 min.

**5** Sensitivity to adenosine was restored in low calcium medium or by raising magnesium. Although raising the divalent cation concentration increased the inhibitory effect of adenosine, desensitization was still seen.

**6** 2-Chloroadenosine (100–500 μM) and **R**-PIA (50 μM), which are not substrates for either the nucleoside transporters or adenosine deaminase, were inactive in the absence of calcium. S-(2-hydroxy-5 nitrobenzyl)-6-thioinosine, an adenosine uptake blocker, at a concentration 100 μM had no effect on secondary potential size and did not restore adenosine sensitivity in calcium-free medium.

**7** Thapsigargin, which discharges intracellular calcium stores, had no significant effect at 1 μM on the bursts of action potentials and did not change the effect of 0.5 mM adenosine in calcium-free medium.

**8** Unlike adenosine, baclofen concentration-dependently reduced the secondary spike size in calcium-free medium and no sign of recovery was observed during maintained superfusion for up to 45 min. No cross-desensitization was seen between baclofen and adenosine.

**9** Applications of adenosine locally by pressure to neuronal somata or dendrites still resulted in desensitized responses in calcium-free medium.

**10** It is concluded that the postsynaptic sensitivity to adenosine is dependent on the concentration of divalent cations in the extracellular space implying an effect of cations on adenosine receptor activation or transduction processes.

**Keywords:** Adenosine; baclofen; calcium; hippocampus; thapsigargin; desensitization; epileptiform activity

## Introduction

Adenosine has a number of different actions in the CNS (Stone & Simmonds, 1991). Presynaptically it is able to act at $A_1$ receptors to modulate the release of several neurotransmitters, especially compounds with a predominantly excitatory action such as acetylcholine and glutamate (Corradetti *et al.*, 1984; Spignoli *et al.*, 1984; Fastbom & Fredholm, 1985; Scanziani *et al.*, 1992). This effect is consistent with early autoradiographic work which localized adenosine $A_1$ receptors to the terminals of excitatory neurones (Goodman *et al.*, 1983) and with more recent electrophysiological studies (Lambert & Teyler, 1991; Yoon & Rothman, 1991). Rather less inhibition is seen of the release of inhibitory transmitters such as noradrenaline (Jonzon & Fredholm, 1984) and γ-aminobutyric acid (GABA, Hollins & Stone, 1980; Lambert & Teyler, 1991; Yoon & Rothman, 1991).

In addition, adenosine has direct effects on the postjunctional cell. In several regions of brain, including the hippocampus and striatum, adenosine induced hyperpolarization by increasing potassium conductance (Greene & Haas, 1985; Trussel & Jackson, 1987; Gerber *et al.*, 1989), an effect which appears to involve a G-protein coupling since it is prevented by pertussis toxin (Trussel & Jackson, 1987). The suppression of low calcium bursting is similarly mediated (Fredholm *et al.*, 1989). Adenosine can also reduce calcium currents in some neurones (MacDonald *et al.*, 1986) and has recently been found to activate a chloride conductance on the dendrites of hippocampal pyramidal cells (Mager *et al.*, 1990).

It is not clear whether any or all of these mechanisms also contribute to the presynaptic effects of adenosine or, indeed, whether exactly the same receptor subtype mediates the pre- and postsynaptic effects. In recent work we have shown that the presynaptic activity of adenosine is dependent on the presence of magnesium ions, removal of the cation causing a large loss of potency of adenosine (Bartrup & Stone, 1990). This may be attributable to the enhanced activation of N-methyl-D-aspartate (NMDA) receptors which can reduce adenosine sensitivity (Bartrup & Stone, 1990), possibly as a result of a common action at neuronal N-type calcium chan-

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nels (Chernevskaya *et al.*, 1991). The present work was designed to assess the effects of divalent cations on the postsynaptic effects of adenosine; this paper focusses on calcium ions and the effect of adenosine on antidromically-induced epileptiform burst firing. The results have been presented briefly in a recent abstract (Hosseinzadeh & Stone 1993).

## Methods

Male Wistar rats (170–210 g) were anaesthetized with urethane (1.3 g $kg^{-1}$, i.p.) and decapitated. The brains were rapidly taken out and put in ice-cold artificial cerebrospinal fluid (ACSF) with the following composition (mM): NaCl 115, $KH_2PO_4$ 2.2, KCl 2.0, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 10. The hippocampi were cut transversely into 450 μm thick slices with a McIlwain tissue chopper. The slices were kept in the incubation chamber containing an ACSF-saturated atmosphere of 95% $O_2$ and 5% $CO_2$ at room temperature for at least one hour before using. Individual slices were then transferred to a 1 ml capacity recording chamber and superfused with ACSF at 30°C ± 0.5, gassed with the $O_2/CO_2$ mixture to yield a pH of 7.4. The slices were completely submerged in the medium which was perfused continuously at a rate of 4 ml $min^{-1}$.

In orthodromic stimulation experiments, cumulative concentration-response curves were constructed after applying adenosine or baclofen for 10 and 15 min respectively at each concentration.

In antidromic stimulation experiments, $CaCl_2$ was omitted or reduced to 0.24 mM and in some experiments $MgSO_4$ was raised from 1.2 to 2 mM.

In some experiments adenosine was applied locally to the somatic or dendritic region of pyramidal cells using pressure ejection from glass micropipettes (8–10 μm tip diameter), containing a solution of 20 μM adenosine in ACSF for orthodromic stimulation or 10 mM in calcium-free or low calcium for antidromic stimulation.

### *Recording of field potentials*

Recordings of orthodromic extracellular field potentials were made from the CA1 pyramidal cell layer following stimulation of the Schaffer collateral fibres in stratum radiatum near the border of CA2–CA3. Stimulation of the alveus was used for antidromic stimulation by a bipolar electrode. Pulses of 0.1 and 1 ms duration and 100–400 μA amplitude were delivered for antidromic and orthodromic stimulation respectively. Stimulus strength was reduced to submaximal levels so as to yield spike size of approximately 75% of maximum. Recordings were made with glass microelectrodes of tip diameters about 2 μm and containing 2 M NaCl.

### *Analysis of drug effects*

The sizes of the primary and secondary population spikes were normally measured as the peak to peak amplitude after perfusion for at least 10 min to allow the attainment of stable responses. Results are presented as mean ± s.e.mean for $n$ experiments and statistical significance was assessed by Student's $t$ test; differences were considered significant with $P<0.05$.

### *Materials*

Thapsigargin was dissolved in dimethylsulphoxide (DMSO) and other chemical agents were dissolved in ACSF. All agents were obtained from Sigma Chemical Co. Ltd.

## Results

### *Orthodromic stimulation*

Adenosine and baclofen induced a concentration-dependent reduction in the amplitude of orthodromic potentials. Maximum effects were seen at 20 and 5 μM respectively (Figure 1). The depressant effect of adenosine was readily reversible and washed out in less than 10 min (Figure 2). 2-Chloroadenosine 100 nM and R-phenylisopropyladenosine (R-PIA) 10 nM were also effective, reducing the population spike by 57.4 ± 7.96% ($n=5$, $P<0.01$) and 57.7 ± 11.3% ($n=5$, $P<0.01$) respectively. Kynurenic acid reduced potential size by 36 ± 6.7% ($n=6$, $P<0.01$) at 0.2 mM and by 81.9 ± 5.9% ($n=6$, $P<0.01$) at 0.5 mM.

When applied locally, adenosine was effective in reducing orthodromic potentials either at the soma region, 70.6 ± 16.4% inhibition ($n=3$, $P<0.05$) or in the dendritic tree, 58.2 ± 10.1% inhibition ($n=5$, $P<0.01$).

The population spike was completely abolished by changing from ACSF to nominally calcium-free or low calcium (0.24 mM) medium.

### *Antidromic stimulation*

Within 5–15 min of superfusion with calcium-free medium, a secondary spike developed in response to the antidromic stimulus and, by 45–90 min, 2–3 afterdischarges became stable. In low calcium medium (0.24 mM) the appearance of a secondary spike was much slower and could take up to 30 min to stabilize.

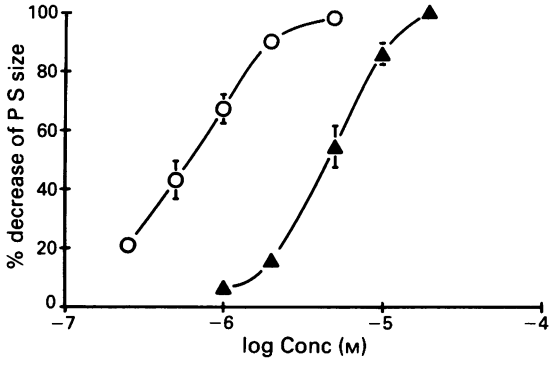


**Figure 1** Cumulative concentration-response curves for the depression of orthodromically evoked CA1 population spikes (PS) by baclofen (O) and adenosine (▲). Each point represent the mean ± s.e.mean for $n=6$ experiments.

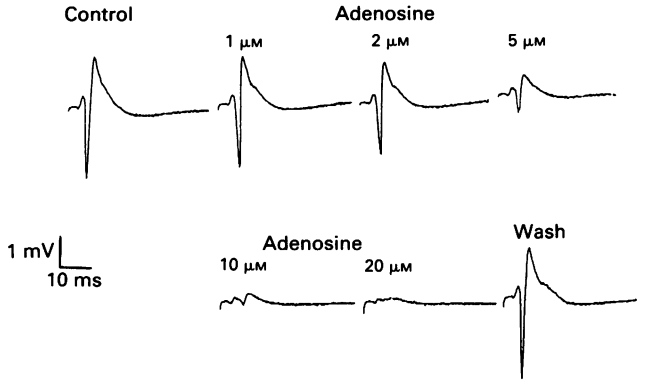


**Figure 2** Sample records of orthodromic potentials in CA1 before, during and 10 min following the cumulative addition of adenosine, 1–20 μM. Calibrations 1 mV, 10 ms.

Kynurenic acid at concentrations of 1 mM ($n = 3$) and 5 mM ($n = 2$) in calcium-free medium and 1 mM ($n = 3$) in low calcium medium had no effect on secondary spike size.

In nominally calcium-free medium, adenosine had little effect on the secondary spike amplitude, reducing it by 8.96 ± 4.25% (mean ± s.e.mean, NS, $n = 11$) at 200 μM, 21.88 ± 3.52% ($P < 0.001$, $n = 8$) at 500 μM and 10.46 ± 3.94% (NS, $n = 5$) at 1 mM (Figure 3). In calcium-free medium, adenosine 200 μM still had no significant effect even when stimulus strength was reduced so that the amplitude of the control secondary spike was less than 50% of its maximum. At a concentration of 2 mM an initial depression (72.90 ± 9.22%, $n = 3$) of secondary spike size was seen but this effect desensitized, completely disappearing within 3–5 min (Figure 4).

Raising the concentration of divalent cations, by adding 0.24 mM calcium or 0.8 mM magnesium (total of 2 mM magnesium) respectively, increased sensitivity to adenosine measured after 10 min. For example, adenosine at 200 μM reduced the secondary population spike size by 74.60 ± 7.26% ($P < 0.01$, $n = 3$) in 0.24 mM calcium and by 90.42 ± 5.42% ($P < 0.01$, $n = 4$) in calcium-free medium with 0.8 mM added magnesium. A concentration-response curve for reduction by adenosine of the secondary spike in 0.24 mM calcium is plotted in Figure 5a and the restoration of sensitivity to adenosine in calcium-free medium containing 2 mM magnesium is illustrated by sample records in Figure 5b. Changing calcium-free medium to low calcium or medium with 0.8 mM added magnesium had no significant effect itself on the secondary spike size.

Although raising the divalent cation concentrations substantially increased (Figure 5a) the inhibitory effect of adenosine, desensitization was still seen. It was normally apparent after the previous exposure of slices to several applications of adenosine, although exposure to low concentrations was sufficient to enable subsequent desensitization to higher concentrations. For example, exposure of naive slices to 100 μM adenosine caused inhibition of the secondary antidromic spike by 55.02 ± 3.17% ($n = 6$) after 10 min. If slices were previously exposed for periods of 10 min to 20 μM and 50 μM adenosine with washing between each period to regain control response size, then the inhibition by 100 μM adenosine was reduced to 34.9 ± 1.63% ($P < 0.001$, $n = 4$).

Similarly, responses to 2 mM adenosine were reduced from 92.19 ± 3.62% in naive slices to 33.75 ± 2.30% following pretreatment with lower concentrations of adenosine ($P < 0.01$, $n = 3$). In the presence of low calcium or raised magnesium, desensitization was less apparent than in calcium-free solutions. Indeed desensitization was often then incomplete after 10 min, whereas it was virtually complete in less than 5 min in calcium-free medium (compare Figures 4 and 6). Prolonging the perfusion of adenosine for up to 15 min allowed full desensitization in the low calcium medium (Figure 6).

When applied locally from micropipettes containing 10 mM adenosine, the purine was not able to depress antidromically induced bursts in calcium-free medium, whether applied into the somatic or dendritic regions. However, in low calcium media adenosine did depress the secondary spikes when applied either to the cell bodies or dendrites (Table 1).

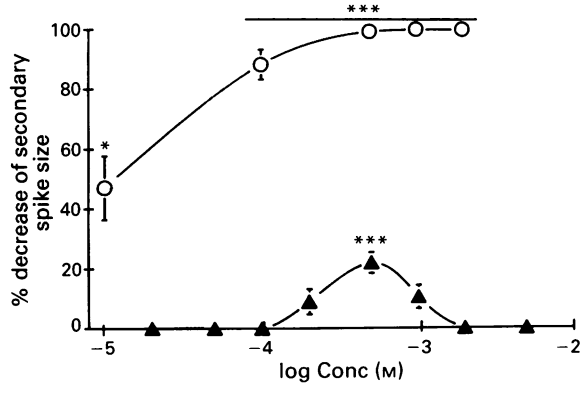


**Figure 3** Concentration-response curves for the depression of antidromically evoked CA1 secondary spikes by baclofen (○) and adenosine (▲) in calcium-free medium. Each point represents the mean ± s.e.mean ($n \geq 5$ for adenosine, $n \geq 3$ for baclofen). Student's paired $t$ test was employed to determine the significance level ($^*P < 0.05$; $^{***}P < 0.001$).

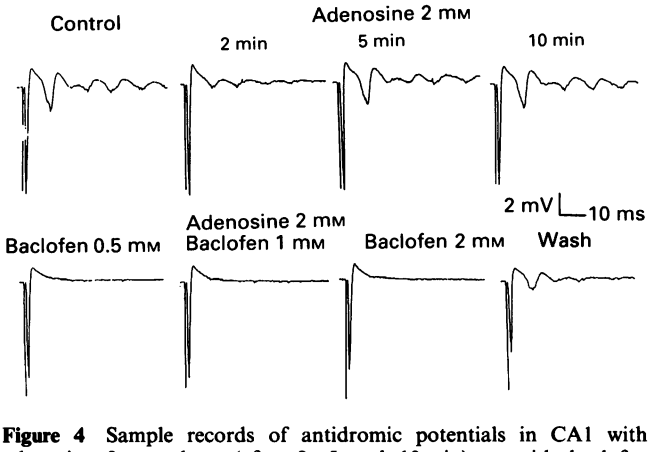


**Figure 4** Sample records of antidromic potentials in CA1 with adenosine 2 mM alone (after 2, 5 and 10 min) or with baclofen 0.5 mM (30 min), 1 mM (30 min) and 2 mM (45 min) in calcium-free medium. Note that adenosine lost its effect after 2 min but no cross-desensitization is seen with baclofen. Calibrations 2 mV and 10 ms.

### *Purine analogues*

Because responses to adenosine were lost at concentrations greater than 1 mM (Figure 3), the possibility was considered

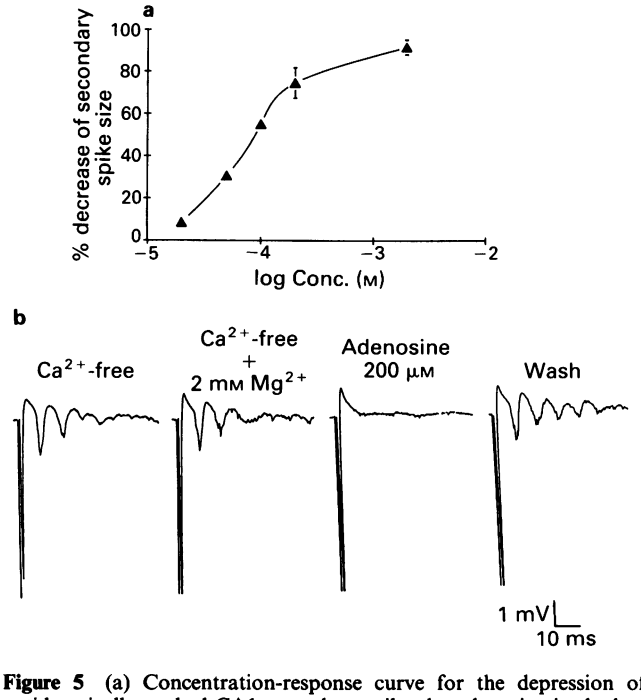


**Figure 5** (a) Concentration-response curve for the depression of antidromically evoked CA1 secondary spikes by adenosine in the low calcium medium. Each point represents the mean ± s.e.mean for $n \geq 3$. (b) Sample records of CA1 antidromic potentials in calcium-free medium with switching to calcium-free medium plus 2 mM magnesium (30 min) before, during and 15 min following perfusion of 200 μM adenosine. Calibrations 1 mV and 10 ms.

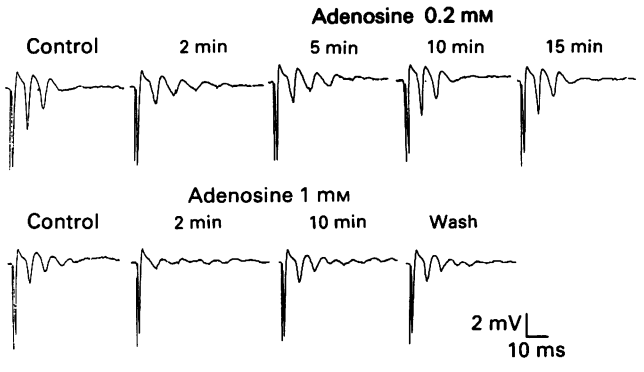


**Figure 6** Records of evoked burst activity showing desensitization to the depressant effect of adenosine at 0.2 mM and 1 mM in 2 different slices perfused with low calcium medium. Several applications of adenosine were made prior to the records illustrated. After each application, slices were washed for 30 min with low calcium medium (0.24 mM) to resore control potential size. Calibrations 2 mV, 10 ms.

**Table 1** Effect of somatic or dendritic adenosine on antidromic secondary spikes in low calcium media

| | *Somatic* | *Dendritic* |
|---|---|---|
| First application | 30.60 ± 5.27 | 40.22 ± 7.03 |
| Second application | 32.35 ± 4.79 | 24.52 ± 7.58* |
| Third application | 30.27 ± 5.25 | 20.50 ± 9.11 |
| $n$ = | (6) | (6) |

*Significantly different from first application, $P<0.05$

that uptake of the nucleoside into the postsynaptic cell might at these concentrations initiate biochemical changes which suppressed receptor-mediated responses. Experiments were, therefore, performed with non-permeable analogues of adenosine and with the transport inhibitor NBT1.

Superfusion of two $A_1$ adenosine agonists, which are not degradable or subject to uptake processes (2-chloroadenosine 100 μM and 500 μM for 15 min ($n = 3$) and (R-)-$N^6$-phenylisopropyladenosine (R-PIA) 50 μM ($n = 3$) for 30 min) had no significant effect on bursts of multiple population spikes in calcium-free medium.

S-(2-hydroxy-5-nitrobenzyl)-6-thioinosine (HNBTI), an adenosine transport inhibitor, had no effect on the bursts when superfused alone for 30 min at 100 μM. Similarly HNBTI proved unable to elicit a response when applied together with adenosine (2 mM) in calcium-free medium ($n = 3$).

### *Thapsigargin*

Thapsigargin, which discharges intracellular calcium stores by inhibition of the endoplasmic reticulum calcium ATPase, had no significant effect on the bursts when applied alone at a concentration of 1 μM, or when superfused with adenosine 0.5 mM for 10 min in calcium-free medium ($n = 3$).

### *Baclofen*

Unlike adenosine, baclofen continued to reduce secondary spike size in a concentration-dependent fashion even in calcium-free medium although its potency was reduced (Figure 3). Inhibition by baclofen was also desensitized with continuous superfusion, though the phenomenon was much slower to develop. For example, the inhibitory effect of continuous superfusion with baclofen 100 μM was reduced from 87.96 ± 5.10% ($n = 4$) at 10 min to 42.92 ± 2.92% ($n = 2$) if perfusion was maintained for 30 min. However, at just maximal concentrations of 500 μM or above, the secondary spike potential was abolished and no sign of recovery was observed during maintained superfusion for up to 45 min ($n = 4$). If slices were superfused in calcium-free medium with adenosine at 2 mM for 10 min, by which time its inhibitory effect had disappeared, the addition of a just-maximal concentration of baclofen (500 μM) was still able to abolish the secondary evoked potential with no recovery during 30 min superfusion (Figure 4).

## Discussion

In normal ACSF, recurrent, feedforward inhibition and calcium-dependent potassium-mediated afterhyperpolarizations oppose sustained neuronal discharges. Lowering extracellular calcium reduces these normal processes and bursts of action potentials appear (Haas & Jefferys, 1984; Taylor & Dudek, 1984; Agopyan & Avoli, 1988). Measurement with ion-selective electrodes during the field bursts showed that extracellular potassium increased (Yaari *et al.*, 1983; Haas & Jefferys, 1984) to an extent which may contribute to the induction of epileptiform bursting. There is also an increase in the excitability of neurones due to the fact that lowering calcium of the medium removes the stabilizing effect of this ion on membrane surface charges (Begenisich, 1988; Agopyan & Avoli, 1988).

The sensitivity of postsynaptic neurones to adenosine, as reflected in suppression of these epileptiform burst discharges, seems to be dependent on cation concentrations. Sensitivity is almost abolished in calcium-free media except for a variable, small response at high micromolar concentrations. Some sensitivity is restored either by the addition of low levels of calcium, or by raising magnesium concentrations. Other studies (e.g. Lee *et al.*, 1984) have reported a greater sensitivity to adenosine of secondary spikes induced by antidromic stimulation. The reason for this difference from the present results may lie in the use of 4 mM magnesium in the medium. While the potency of baclofen is also reduced in calcium-free media, this effect is much less marked than for adenosine: the $ED_{50}$ concentration of baclofen increases from 0.8 μM to 10 μM (Figures 1 and 3), whereas adenosine is unable to produce 50% inhibition in the absence of calcium. The differences may imply some specificity in the importance of calcium for adenosine receptor function. The loss of adenosine sensitivity in calcium-free media is not likely to be due to a change of uptake or metabolism of adenosine because 2-chloroadenosine and R-PIA, which are not substrates for either the nucleoside transporters or adenosine deaminase were equally inactive in the absence of calcium.

The loss of efficacy of adenosine in calcium-free medium is probably not due to the neuronal hyperexcitability itself because increasing the applied concentration up to as much as 5 mM proved ineffective. Equally, reducing secondary spike size to a clearly submaximal level (less than 50% of maximum) did not restore any adenosine effect. It should also be noted that another agonist, baclofen, at low concentrations (10 μM) could still reduce the secondary spike size even in the absence of calcium.

Another possible explanation which was considered, was that some release of transmitters might continue in the absence of calcium, as concluded by Scholz & Miller (1992) and Huang (1993). The release of glutamate might then activate the population of receptors sensitive to N-methyl-D-aspartate (NMDA), an action which has been found to diminish responses to adenosine (Bartrup & Stone, 1990; Bartrup *et al.*, 1991). Such an explanation might also account for the ability of raised magnesium concentrations to increase adenosine responsiveness. However, addition of the excitatory amino acid antagonist, kynurenic acid (Perkins & Stone,

1982; Stone, 1993) had no effect on bursts induced by calcium-free or low calcium media.

Two possible explanations remain for the dependence of adenosine sensitivity on calcium. Firstly, calcium or other divalent cations may be needed for binding of the agonist to its receptors. Such an explanation would be consistent with the studies of Goodman *et al.* (1981), Yeung *et al.* (1985) and Johanssen *et al.* (1992) who have demonstrated the enhancement of adenosine binding to neuronal membranes by divalent cations including calcium and magnesium. This idea would also be consistent with the recognised cation dependency of ligand binding to G-protein coupled receptors.

The reservation which must be attached to this explanation is that the cation requirement of ligand binding is generally assumed to occur at the inner, cytoplasmic face of the cell membrane. The present results may indicate that a similar requirement exists at the extracellular face of the receptor. Alternatively, it may be necessary to postulate that the removal of extracellular calcium also results in a depletion of intracellular calcium associated with membrane function. It was to test this hypothesis that thapsigargin was used. Thapsigargin inhibits the calcium-activated ATPase responsible for the uptake and storage of calcium in the endoplasmic reticulum (Inesi & Sagara, 1992); it should, therefore, even in the absence of extracellular calcium, produce a rise in free intracellular calcium levels at least transiently, which might be expected to restore a temporary sensitivity to adenosine. This did not occur. It seems possible, therefore that the calcium requirement for the action of adenosine is at the external face of the membrane. This in turn would be consistent with the report of Greene & Haas (1985) that the intracellular injection of EGTA to buffer internal calcium did not change the hyperpolarization produced by adenosine.

The second possible explanation is that calcium removal results in the loss of ionic conductances needed for the effects of adenosine. For example, adenosine is known to enhance the activation of calcium-activated potassium conductances yet, clearly, these will not be operating in the absence of external calcium.

### *Desensitization*

A second observation in the present work is of the emergence of desensitization to the inhibitory effect of adenosine.

Early attempts to show adenosine desensitization in brain slices were unsuccessful (Sattin & Rall, 1970). The first report about desensitization to adenosine was in neuroblastoma cells in tissue culture (Green, 1977), although a clear demonstration of this phenomenon was also reported by McNeal *et al.* (1980) in guinea-pig cerebral cortex. Desensitization to adenosine has been reported in different tissues such as hippocampus (Lee *et al.*, 1986; Porter *et al.*, 1988), striatum (Porter *et al.*, 1988; Abbracchio *et al.*, 1992; 1993), neocortex (McNeal *et al.*, 1980; Porter *et al.*, 1988; Abbracchio *et al.*, 1993), locus coeruleus (Regenold & Illes, 1990), neuroblastoma (Kenimer & Nirenberg, 1981; Kelly *et al.*, 1990), liver (Buxton *et al.*, 1987), kidney (Newman & Levitzki, 1983), smooth muscle (Hayashi *et al.*, 1985; Ramkumar *et al.*, 1991), vascular smooth muscle (Anand-Strivastava *et al.*, 1989; Hussain & Mustafa, 1993), heart or myocytes (Shryock *et al.*, 1989; Liang & Donovan, 1990), endothelial cells (Luty *et al.*, 1989), mast cells (Marquardt & Walker, 1987), adipocyte cells (Parsons & Stiles, 1987; Hoffman *et al.*, 1989; Longabaugh *et al.*, 1989; Stoneham, 1989). Adenosine desensitization was seen both *in vivo* (Lee *et al.*, 1986) and *in vitro* (Abbracchio *et al.*, 1992). Abbracchio *et al.* (1992) have recently reported an apparent desensitization to $A_1$ agonists of adenylate cyclase activity in rat striatum without any accompanying change of $A_1$ receptor binding and Porter *et al.* (1988) demonstrated a decrease of $A_2$ receptor density and cyclase stimulation in the striatum *in vivo*.

Most studies of adenosine desensitization have been focussed on adenylate cyclase activity (e.g. Kenimer & Nirenberg, 1981; Anand-Srivastava *et al.*, 1989; Luty *et al.*, 1989), although functional effects of adenosine such as depressed effects in electrophysiological studies (Regenold & Illes, 1990), glycogenolysis (Buxton *et al.*, 1987), relaxation of smooth muscle (Hayashi *et al.*, 1985), and cardiac myocyte contractility (Shryock *et al.*, 1989) have also been found to exhibit desensitization. In rat locus coeruleus neurones, intracellular recording showed that adenosine reduced the firing rate and caused hyperpolarization. Both effects were transient and fading occurred during contact with the drug. When adenosine was added twice at an interval of 10 min, the second application had no or only a slight effect (Regenold & Illes, 1990).

Lee *et al.* (1986) showed that a brief period of global CNS anoxia resulted in a persistent down-regulation of [$^3$H]-CHA binding sites in the CA1 hippocampal pyramidal cells but not in the neocortex or striatum of gerbils. This was later shown however, to be preceded by an up-regulation of adenosine receptors (Kato *et al.*, 1991) within 1 h of recirculation. The relationship between this work and the present study is unclear, but ischaemia may have profoundly different effects from the removal of calcium. In addition, the receptors studied here are restricted to the postsynaptic neuronal surface, whereas the changes occurring in anoxia may be far more diffusely distributed.

Despite these various studies, no desensitization to adenosine has previously been reported in electrophysiological investigations in the hippocampus. Indeed, Thompson *et al.* (1992) specifically emphasised that with intracellular recordings from hippocampal pyramidal cells (in a normal bathing medium) no decline of sensitivity could be detected following the repeated application of 50 $\mu$M adenosine over several hours. In this as in most earlier studies, however, the use of intrasomatic recordings would detect primarily actions of adenosine exerted directly on the cell body. Dendritic responses would be less apparent, yet it is now recognised that dendritic receptors of adenosine do exist (Tetzlaff *et al.*, 1987), linked to potassium and chloride channels. The use of extracellular recordings of epileptiform activity in the present study should allow any contribution of dendritic receptors to overall neuronal excitability to be observed. However, a direct examination of somatic and dendritic sensitivity revealed that adenosine lost its activity in calcium-free medium, when applied locally to either site. It is therefore concluded that adenosine receptors are similarly affected by calcium-free media along the length of the pyramidal neurone. This also strengthens the view that it is the loss of divalent cations which induces desensitization, and not the manner of recording (i.e. extracellular recording of secondary spikes rather than intracellular recording as in the work of Thompson *et al.*, 1992). It should be noted, however, that in the presence of calcium, locally applied adenosine responses showed a greater tendency to desensitize in the dendrites compared with the cell-body. This may indicate slight differences in the receptors or associated ion channels in the two regions.

The emergence of desensitization is difficult to explain, but may reflect a time-dependent change of receptor state (or G-protein coupling) to an inactive form in the absence of calcium. A change of this type has been characterized for GABA, intracellular calcium promoting a decrease of receptor affinity for GABA in bullfrog sensory neurones (Inoue *et al.*, 1986). It is of some interest, however, that desensitization to baclofen was not observed to the same degree, since it has been reported that some occlusion occurs between adenosine and baclofen responses which might indicate the involvement of a common potassium channel or G-protein in hippocampal (Nicoll, 1988) or neocortical neurones (McCormick & Williamson, 1989). The specificity of desensitization to adenosine here would imply that either adenosine and baclofen are not acting via common potassium channels in the hippocampal CA1 cells, or that desensitization is independent of the ion channels and is confined to adenosine receptors or G-protein coupling.

Since extracellular concentrations of calcium can fall dramatically during seizures or ischaemia, a loss of neuronal sensitivity to adenosine may occur in these situations, removing a possible important stabilizing influence on neuronal excitability and raising further the risk of seizure spread or of neuronal damage. It may also mean that purine agonists may be of limited value in the treatment of seizure disorders.

In conclusion the present work indicates that the postsynaptic sensitivity to adenosine of hippocampal pyramidal cells is dependent on the extracellular concentrations of calcium and magnesium. This dependence is reflected both in an absolute loss of potency and in the appearance of desensitization.

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# Further characterization of 5-hydroxytryptamine receptors (putative 5-$HT_{2B}$) in rat stomach fundus longitudinal muscle

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**1** The present study was undertaken to isolate and characterize pharmacologically homogeneous populations of 5-hydroxytryptamine (5-HT) receptors from a possible mixed receptor population mediating contraction of the longitudinal muscle of rat stomach fundus. Our aim was to extend the pharmacological characterization of the 5-$HT_{2B}$ receptor which is reported to be expressed in this preparation.

**2** To minimize spontaneous activity and any influence of circular muscle on the contractile response, narrow (1–1.5 × 20 mm) segments of mucosa-denuded longitudinal muscle were used. Under these conditions, blockade of monoamine oxidase with pargyline (100 μM for 15 min) caused a leftward displacement of concentration-effect curves for both 5-methoxytryptamine (5-MeO-T) and tryptamine. Neither pargyline nor a number of uptake inhibitors affected responses to 5-HT.

**3** In pargyline pretreated preparations, the order of potency of a number of tryptamine analogues was as follows: 5-MeO-T ⩾ α-Me-5-HT ⩾ 5-HT > 5-carboxamidotryptamine (5-CT) > tryptamine > 2-Me-5-HT. In addition several ligands known to act as agonists at either 5-$HT_{2A}$ or 5-$HT_{2C}$ receptors including 1-*m*-chlorophenylpiperazine (*m*-CPP), Ru 24969, MK 212 and SCH 23390 were also agonists in rat fundus whilst sumatriptan, renzapride and 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) were very weak or inactive. With the exception of 2-Me-5-HT and *m*-CPP, most agonists produced monophasic concentration-effect curves consistent with an interaction at a single site. High concentrations of 2-Me-5-HT evoked relaxations which were blocked by phentolamine (1 μM) suggesting an interaction with α-adrenoceptors. *m*-CPP often evoked biphasic concentration-effect curves with a second contractile phase which was insensitive to yohimbine at concentrations higher than required for antagonism of responses to 5-HT.

**4** LY 53857, methiothepin, methysergide, ritanserin and ICI 170809 were potent but non-surmountable antagonists of 5-HT in rat fundus. In contrast, several ligands behaved as surmountable antagonists with the following order of potency: rauwolscine > yohimbine = mesulergine ⩾ mianserin = SB 204070 ⩾ WY 26703 ⩾ SB 200646 > pirenpirone ⩾ renzapride. DAU 6285, granisetron, spiperone, ketanserin, phentolamine and GR 127935 did not affect responses to 5-HT at concentrations up to 1 μM. The agonist and concentration independent profile of antagonism supported a single site interaction for both agonists and antagonists.

**5** We conclude that despite small differences concerning the enantiomeric selectivity and affinity of rauwolscine and yohimbine, the close pharmacological identity of 5-HT receptors in rat stomach fundus and the recently cloned 5-$HT_{2B}$ receptor is maintained. SB 200646, which demonstrates some selectivity for 5-HT receptors in rat stomach fundus, should provide a useful ligand for confirmation of this view and allow discrimination of 5-$HT_{2B}$ function both *in vitro* and *in vivo*.

**Keywords:** Rat stomach fundus; 5-$HT_{2B}$-receptors; contraction, SB 200646, SB 204070

## Introduction

5-$HT_2$ receptors have recently been sub-classified into 5-$HT_{2A}$, 5-$HT_{2B}$ and 5-$HT_{2C}$ receptor subtypes (Humphrey *et al.*, 1993). The 5-$HT_{2B}$ mRNA transcript has been identified in rat stomach fundus (Foguet *et al.*, 1992a,b; Kursar *et al.*, 1992; Wainscott *et al.*, 1993) and in the small intestine, kidney, heart and cerebellum of the mouse (Loric *et al.*, 1992). Although no functional correlate of the mouse 5-$HT_{2B}$ receptor has yet been reported, it has been proposed that the rat homologue equates with an 'orphan' receptor which mediates a contractile response to 5-HT in stomach fundus (Foguet *et al.*, 1992a,b; Kursar *et al.*, 1992; Wainscott *et al.*, 1993). The receptor in rat fundus has proved difficult to classify using only pharmacological criteria and whilst some groups have maintained that it represents a novel receptor subtype (Cohen & Wittenauer, 1985; Cohen & Colbert, 1986; Cohen & Fludzinski, 1987; Blackburn *et al.*, 1988; Baez *et al.*, 1991), it has also been proposed that a variant of 5-$HT_{1D}$ (Kalkman & Fozard, 1991), 5-$HT_{2C}$ (Buchheit *et al.*, 1986), 5-$HT_{2A}$ (Gregg & Osborne, 1985) or a mixture of receptors (Barlow & Khan, 1959; Winter & Gessner, 1968; Buchheit *et al.*, 1986) may mediate the contractile response to 5-HT receptor agonists in this tissue. Indeed, a multiple site hypothesis may explain the occurrence of shallow (Cline-schmidt *et al.*, 1985) or overtly biphasic (Buchheit *et al.*,1986) agonist concentration-effect curves and the existence of pharmacological differences between the cloned 5-$HT_{2B}$ receptor and the putative 5-$HT_{2B}$ receptor which is naturally expressed in rat stomach (Foguet *et al.*, 1992b). Comparisons of cloned 5-$HT_{2B}$ receptors and naturally expressed 5-HT receptors in rat fundus may be further complicated by a failure to account for uptake and metabolism. Although agonist $pEC_{50}$ values determined in rat stomach fundus correlate well with affinity values for cloned 5-$HT_{2B}$ receptors (Wainscott *et al.*, 1993), the inclusion of agonists which are known to be substrates for monoamine oxidase in rat stomach fundus may cast doubt on the validity of the comparison. In this regard it has been reported that unlike 5-HT or α-Me-5-HT, the less polar indoleamines including tryptamine and 5-methoxytryptamine (5-MeO-T) readily cross the cell membrane and are metabolized by monoamine oxidase resulting in up to a 40 fold under-estimation of potency (Vane, 1959).

Questions remain as to whether the pharmacological

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profile of the 5-HT receptor in the rat stomach fundus reflects either the presence of multiple receptor subtypes or the influence of endogenous metabolic processes which may limit the actions of some but not all ligands. The present study was designed to isolate homogeneous populations of receptors from a possible mixed population and to define the pharmacological characteristics of those which contribute to the contractile response to 5-HT. Care has been taken to work as closely as possible to equilibrium conditions in order to obtain an explicit pharmacological characterization of putative contractile 5-$HT_{2B}$ receptors in rat stomach fundus. To extend the pharmacological characterization of this receptor we have evaluated several recently described antagonists including GR 127935, SB 204070 and SB 200646 which demonstrate high selectivity for 5-$HT_{1D}$, 5-$HT_4$ and 5-$HT_{2C}$ receptors respectively (Skingle *et al.*, 1993; Wardle *et al.*, 1993; Forbes *et al.*, 1993).

## Methods

### *Tissue preparation*

Whole stomachs were obtained from male Sprague Dawley rats (250–350 g) and strips of longitudinal muscle (1.0–1.5 × 20 mm) were dissected from the greater curvature of the fundus and mounted between stainless steel hooks in 10 ml tissue baths containing oxygenated (95% $O_2$/5% $CO_2$) Tyrode solution at 37°C. The composition of Tyrode solution was as follows (mM): NaCl 136.9, KCl 2.7, $NaH_2PO_4$ 0.4, $MgCl_2$ 1.0, glucose 5.6, $NaHCO_3$ 11.9, $CaCl_2$ 1.8 and indomethacin 0.003. After 15 min, tissues were 'primed' with KCl (50 mM) and over the following 45 min period were exposed to an $EC_{80}$ concentration of 5-HT (10 nM) at intervals of not less than 10 min until a consistent contractile response (± 5% of previous response) was obtained. Responses were recorded with Dynamometer UH1 isometric transducers coupled either to a Letromed MT8-channel chart recorder or a MACLAB/8 (AD instruments) recording system linked to an Apple Macintosh II Ci computer.

Several experiments were performed to determine final experimental conditions. The findings are given in Results and led to the adoption of the following protocol. All experiments were conducted after removal of the mucosae and after tissues had been exposed to the irreversible monamine oxidase (MAO) inhibitor pargyline (100 μM) for 15 min. For studies with 2-Me-5-HT, phentolamine (1 μM) was included in the Tyrode solution. To remove the mucosae, strips were pinned out with sufficient tension to allow the mucosal layer to be pulled slightly away from underlying muscle. The mucosae could then be easily removed with fine dissecting scissors.

*Agonist potency* Agonist concentration-effect curves were fitted using the following three parameter equation using Kaleidagraph (Synergy Software) on an Apple Macintosh II Ci computer.

$$E = \frac{\alpha}{1 + (EC_{50}/[A])^n} \quad (1)$$

α, [A] and n represent the maximum response, agonist concentration and curve mid point slope factor respectively. The $EC_{50}$ is the concentration of agonist that produces 50% of the maximal response.

For comparison of agonist potency, two concentration-effect curves were constructed in each preparation, the first to the standard agonist 5-HT and the second, 1 h later, to either 5-HT again (in time control experiments) or to a test agonist. Agonist responses were expressed as a percentage of the maximum response determined during construction of the first concentration-effect curve. Agonist potenty was expressed both in terms of absolute potency as $pEC_{50}$ estimates, relative to their individual maxima, and as concentration-ratios (concentration-ratio = $EC_{50}$ test agonist ÷ $EC_{50}$ 5-HT) and intrinsic activities relative to $E_{max}$ parameters obtained from the preceding control concentration-effect curve to 5-HT. On repetition, curves to 5-HT were highly reproducible and $EC_{50}$ location parameters differed by no more than 2 fold. For this reason, correction factors were not routinely applied to account for changes in sensitivity between first and second concentration-effect curves.

*Antagonist affinity* Affinity estimates for antagonists were expressed as $pA_2$ values, calculated according to the method of Arunlakshana & Schild (1959) and by single point analysis using the following equation (2).

$$pA_2 = -\log_{10}[B] + \log_{10}[CR-1] \quad (2)$$

[B] represents the concentration of antagonist and CR represents the ratio of $EC_{50}$ location parameters for 2nd and 1st agonist concentration-effect curves in the presence and absence of antagonist respectively. Equation 2 was used only if curve maxima for the 1st and 2nd concentration-effect curves differed by no more than 5%.

### *Compounds used*

5-Hydroxytryptamine (5-HT) creatinine sulphate, 5-methoxytryptamine (5-Me-O-T) hydrochloride, tryptamine HCl, U-46619 (9,11 dideoxy-11a, 9α epoxy-methanoprostaglandin $F_{2\alpha}$), carbachol chloride, PCPA (DL-*p*-chlorophenylalanine methyl ester hydrochloride), pargyline hydrochloride, cocaine HCl, yohimbine HCl, corticosterone acetate, indomethacin, phentolamine HCl, *m*-CPP (1-*m*-chlorophenylpiperazine di HCl), spiperone HCl and reserpine HCl were obtained from Sigma Chemical Company (Dorset). 5-Carboxamido tryptamine (5-CT) maleate, rauwolscine HCL, ketanserin tartrate, ritanserin, methiothepin mesylate, methysergide maleate, phenoxybenzamine HCl and SCH 23390 HCl ((+)-8-chloro-2,3,4,5,tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol) were obtained from RBI (Semat Technical Ltd, Hatfield Herts). 2-Methyl-5-hydroxytryptamine (2-Me-5-HT) HCl and α-methyl-5-hydroxytryptamine (α-Me-5-HT) were obtained from Cookson Chemicals. WY 26703 (N-isobutyl-suphonyl-*N*-methyl-1,3,4,6,7,11bα-hexahydro-2H-benzo-[A]-quinolizin-2β-amine. HCl) was suppled by Wyeth-Ayerst (U.K.). ICI 170 809 (2-(2-dimethylamino-2-methylpropylthio)-3-phenylquinoline) was obtained from ICI (U.K.). LY 53857 (4-isopropyl-7-methyl-9-(2-hydroxy-1-methylpropoxycarbonyl)-4, 6, 6A, 7, 8, 9, 10, 10A-octahydroindolo (4,3-FG) quinoline maleate) was supplied by Eli-Lilly (U.S.A.). Pirenpirone was supplied by Janssen (Belgium). DAU 6285 (1-H-benzimidazole-1-carboxylic acid, 2,3 dihydro-6-methoxy-2-oxo 8-methyl-8-azabicylco (3,2,1) oct-3-yl ester) was supplied by Dr C.A. Rizzi, Boehringer Ingelheim Italia. SB 200646 (N-1-methyl-5-indolyl)-*N'*-(3-pyridyl) urea HCl), granisetron (endo-1-methyl-*N*-(9-methyl-9-azabicyclo[3.3.1]non-3-yl-1*H*-indazole-3-carboxamide HCl), renzapride ([(±)-(endo)]-4-amino-5-chloro-2-methoxy-*N*-(1-azabicyclo-[3.3.1]-non-4-yl)] benzamide HCl), mianserin, sumatriptan, RU 24969 (5-methoxy-3-(1,2,5,6-tetrahydropyridyl) indole), 8-OH-DPAT ((±)-8-hydroxy-2-(di-n-propylamino) tetralin), GR 127935 (N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl) [1,1,-biphenyl]-4-carboxamide) and SB 204070A ([1-butyl-4-piperidinylmethyl-)-8-amino-7-chloro-1, 4-benzodioxan-5-carboxylate]) were synthesized at SmithKline Beecham Pharmaceuticals, Harlow, Essex.

## Results

### *Preliminary experiments*

5-HT (1 nM–10 μM) did not cause relaxation of muscle strips (2–2.5 mm × 20 mm) pre-contracted with either U46619, carbachol or KCl. In the absence of pre-contraction the

non-cumulative addition of 5-HT evoked a concentration-dependent contractile response. Accurate measurements of responses, particularly to threshold concentrations of 5-HT, were confounded by baseline instability which consisted of periodic phasic and tonic contractions. Concentration-response curves for 5-HT derived using these preparations were not overtly biphasic but were shallow and often spanned 4 to 4.5 log units. After dissecting thinner muscle strips (1–1.5 mm × 20 mm) to minimize the potential contribution of circular muscle to the 5-HT induced contractile response (Offermeier & Ariëns, 1966) and after removal of the mucosal layer, almost all spontaneous activity was abolished. These modifications yielded concentration-response curves that were steeper than those obtained using larger intact preparations (Figure 1).

The maximum response to 5-HT was obtained at concentrations of 0.1–0.3 μM and higher concentrations did not evoke contractions of greater magnitude but induced a marked phasic contractile activity which was not completely reversed even upon washing preparations over a 2 h period.

Several experiments were conducted to determine whether 5-HT receptors are located on the muscularis mucosa of the stomach fundus. Mucosal strips were carefully dissected away from the underlying muscle and set up under the same conditions as described for longitudinal muscle. Although all displayed marked spontaneous activity and all contracted on exposure to KCl, none of the twelve strips tested responded either to 5-HT or to carbachol (0.1 nM–100 μM).

Pretreatment of animals with reserpine (10 mg kg$^{-1}$ 16 h prior to experiment) or PCPA (150–300 mg kg$^{-1}$ for 3–5 days) had no effect on tissue responses to 5-HT, $n \geq 4$ for each treatment (data not presented). Likewise, responses to 5-HT were not affected by tetrodotoxin (0.1 μM) nor by the 5-HT re-uptake inhibitors cocaine (10 nM–30 μM), paroxetine (0.1 μM) and fluoxetine (0.1 μM), $n \geq 4$ (data not presented). Corticosterone (30 μM) caused non-surmountable antagonism of responses to 5-HT (Table 2). These data indicated the use of either tetrodotoxin or uptake inhibitors was unnecessary. On exposure to pargyline (100 μM), many tissues responded with a slowly developing increase in basal tension which returned to pre-exposure levels over the following 1 h equilibration period. The elevation in tension was reversed on addition of yohimbine (0.3 μM) or methiothepin (0.1 μM) but was unaffected by pretreatment of animals with PCPA (150–300 mg kg$^{-1}$, i.p. for up to 5 days). Location parameters of concentration-effect curves for 5-HT were not influenced by prior exposure of tissues to pargyline. However, concentration-effect curves to 5-MeO-T and tryptamine were displaced 3.2 ± 0.9 fold ($n = 4$) and 9.2 ± 0.2 ($n = 4$) to the left respectively. This sensitizing effect on 5-MeO-T and tryptamine reflects the propensity of the more lipophilic indoleamines to passive uptake and deamination by monamine oxidase and therefore pargyline was used routinely in all subsequent experiments.

Changing from a non-cumulative to a cumulative dosing protocol did not influence location parameters of concentration-effect curves to 5-HT (curve maxima ± s.e.mean and $pEC_{50}$ (95% CL) were 98.8 ± 0.86, 8.56 (8.22–8.91) and 100.5 ± 2.4, 8.51 (8.04–8.99), $n = 4$, for non-cumulative versus cumulative concentration-effect curves respectively). This suggested that the receptor did not desensitize appreciably over the time course of the curve and a cumulative dosing procedure was adopted for all subsequent experiments.

### *Agonist potency*

Under the conditions defined in preliminary experiments, 5-HT acted as a potent contractile agonist in longitudinal muscle strips of rat fundus [mean $pEC_{50}$ (95% CL) of 8.64 (8.53–8.76), $n = 57$] and produced well defined and monophasic concentration-effect curves. At lower concentrations, responses to 5-HT were primarily phasic in nature and often faded back to baseline, whereas higher concentrations evoked responses comprising an initial phasic contraction followed by a tonic component that showed limited fade (Figure 2a).

5-MeO-T, α-Me-5-HT, 5-CT, and tryptamine were full agonists with respect to 5-HT and produced qualitatively similar responses (Table 1, Figure 3). 2-Me-5-HT possessed bimodal activity causing contraction at lower concentrations

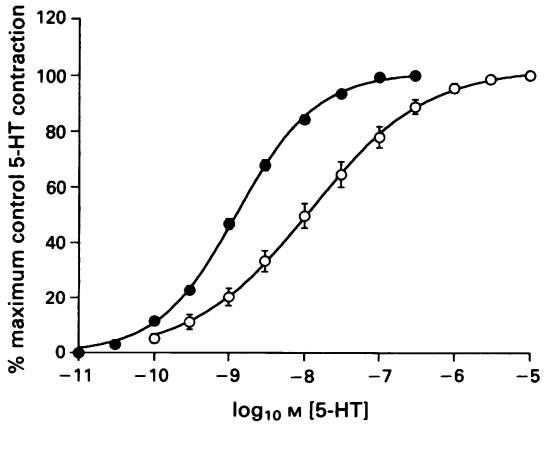


**Figure 1** Concentration-effect curves for 5-HT in intact (○) and mucosa-denuded (●) preparations of rat stomach fundus longitudinal muscle. Each point represents the arithmetic mean ± s.e.mean of $n = 10$ experimental determinations.

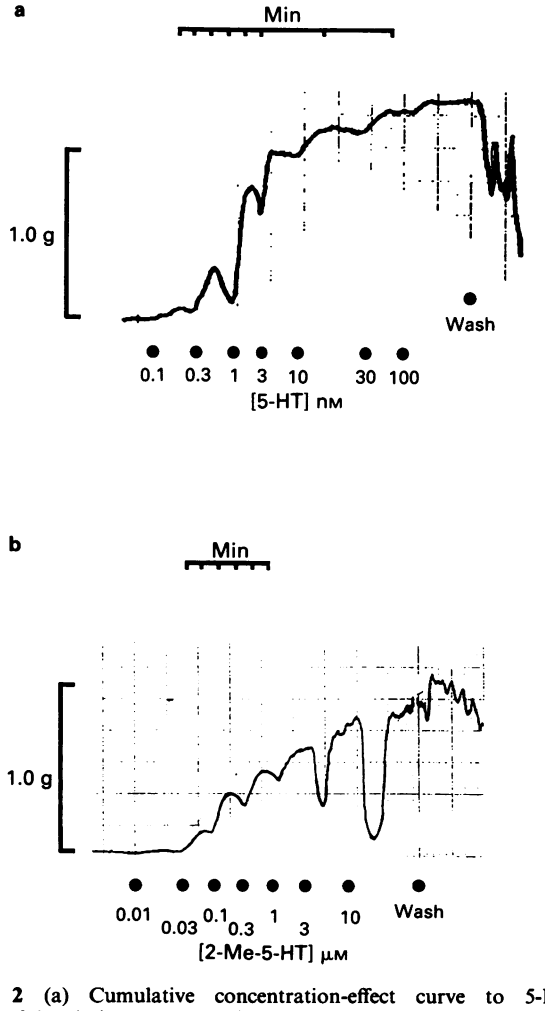


**Figure 2** (a) Cumulative concentration-effect curve to 5-HT (0.1 nM–0.1 μM) in rat stomach fundus longitudinal muscle. (b) Cumulative concentration-effect curve to 2-Me-5-HT (0.01–10 μM) in rat stomach fundus longitudinal muscle.

**Table 1** Agonist potency at putative 5-$HT_{2B}$ receptors in rat stomach fundus

| *Agonist* | $pEC_{50}$ | (95% CL) | *ECR*[a] ± s.e.mean | *IA*[b] ± s.e.mean | n |
|---|---|---|---|---|---|
| α-Me-5-HT | 8.42 | (8.18–8.67) | 0.9 ± 0.08 | 0.97 ± 0.10 | 13 |
| 5-MeO-T | 8.79 | (8.63–8.96) | 0.9 ± 0.10 | 1.01 ± 0.02 | 18 |
| 5-HT | 8.64 | (8.53–8.76) | 1.0 | 1.00 | 57 |
| *m*-CPP | 7.68 | (7.16–8.20) | 2.8 ± 1.15 | 0.38 ± 0.05 | 4 |
| 5-CT | 7.98 | (7.74–8.23) | 7.8 ± 0.87 | 1.00 ± 0.02 | 12 |
| Tryptamine | 7.23 | (7.02–7.46) | 41.7 ± 10.0 | 0.97 ± 0.03 | 6 |
| RU 24969 | 7.02 | (6.81–7.25) | 49.8 ± 11.0 | 0.62 ± 0.05 | 10 |
| MK 212 | 6.43 | (6.25–6.61) | 97.4 ± 12.0 | 1.04 ± 0.04 | 8 |
| SCH 23390 | 6.58 | (6.15–6.98) | 137.9 ± 44.8 | 0.66 ± 0.08 | 4 |
| 2-Me-5-HT | 6.61 | (6.40–6.84) | 150.9 ± 16.7 | 0.79 ± 0.04 | 8 |
| 8-OH-DPAT | 5.52 | (5.10–5.87) | 1264.0 ± 398.0 | 0.71 ± 0.09 | 5 |
| Sumatriptan | <4.52 | | >2000.0 | | 4 |
| Renzapride | <5.00 | | >2000.0 | | 4 |

Each $pEC_{50}$ value represents the arithmetic mean with 95% confidence limits in parentheses.
[a]ECR (equipotent concentration ratio) = $EC_{50}$ test agonist ÷ $EC_{50}$ 5-HT.
[b]IA (intrinsic activity) = α test agonist ÷ α 5-HT. ECR and IA values were determined in experiments in which concentration-effect curves for both agonists were obtained in the same preparation (for further details see Methods).

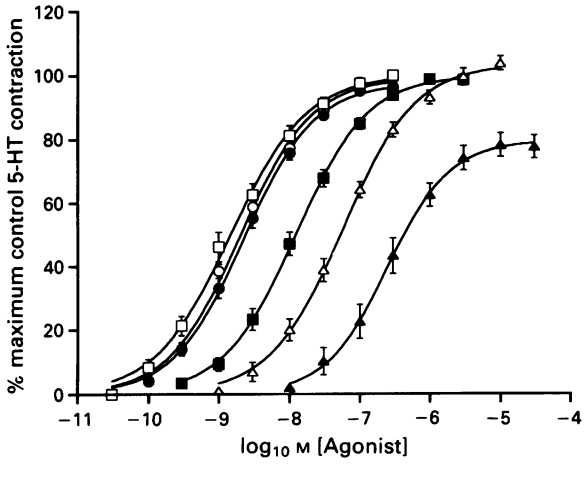


**Figure 3** Cumulative concentration-effect curves to 5-HT (○), α-Me-5-HT (●), 5-MeO-T (□), 5-CT (■), tryptamine (△) and 2-Me-5-HT (▲) in rat stomach fundus longitudinal muscle. Each point represents the arithmetic mean ± s.e.mean (see Table 1 for number of estimates). Data for 2-Me-5-HT were obtained in the presence of phentolamine (1 μM) to block α-adrenoceptors. For abbreviations, see text.

and phasic relaxations which were superimposed upon the contractions as concentrations were increased (Figure 2b). The relaxant responses were abolished by phentolamine (1 μM) suggesting that α-adrenoceptors mediate the relaxant response to this agonist. Phentolamine (1 μM) had no effect on responses to 5-HT. After blockade of α-adrenoceptors, 2-Me-5-HT produced a monophasic concentration-response curve and was a partial agonist with respect to 5-HT (Table 1, Figure 3).

Several other compounds which are known to act as agonists at either 5-$HT_{2A}$ or 5-$HT_{2C}$ receptors including RU 24969, SCH 23390 (Hoyer *et al.*, 1989) and MK 212 (Conn & Sanders-Bush, 1987) were moderately potent agonists in rat stomach fundus, whereas other compounds known to be only weakly active at 5-$HT_{2A}$ and 5-$HT_{2C}$ receptors including 8-OH-DPAT, sumatriptan and renzapride were either weak or inactive (Table 1). The putative 5-$HT_{2C}$ selective agonist, *m*-CPP (see Kennett, 1993) also possesses agonist properties in rat fundus (Clineschmidt *et al.*, 1985). In the present study, biphasic curves were often observed. Where biphasic curves were evident, the best fit (using equation 1) to the higher potency phase yielded a mean $EC_{50}$ (95% CL) of 7.68 (7.62–7.94) and a variable intrinsic activity (relative to the 5-HT maximum response) which ranged from 0.2–0.5. The lower potency phase of the curve for *m*-CPP occurred over the range (1.0 μM – 100 μM), was poorly defined and difficult to quantify. The low potency phase of the *m*-CPP concentration-effect curve was resistant to antagonism by yohimbine (0.3 μM).

**Table 2** Non-surmountable antagonists of 5-HT in rat stomach fundus longitudinal muscle

| *Antagonist* | *Conc.* (nM) | **CR* ± s.e.mean | Δα ± s.e.mean |
|---|---|---|---|
| LY 53857 | 10 | 38.0 ± 2.9 | 0.55 ± 0.11 |
| Methiothepin | 10 | 12.01 ± 3.31 | 0.67 ± 0.03 |
| Methysergide | 1 | 1.66 ± 0.27 | 0.87 ± 0.09 |
| | 3 | 3.48 ± 0.32 | 0.49 ± 0.02 |
| | 10 | – | 0.00 |
| ICI 170 809 | 10 | 4.65 ± 1.5 | 0.84 ± 0.10 |
| | 100 | 76.5 ± 15.5 | 0.76 ± 0.12 |
| Ritanserin | 100 | 8.67 ± 3.60 | 0.74 ± 0.10 |
| Corticosterone | 30,000 | 2.59 ± 1.2 | 0.82 ± 0.2 |

*CR = Ratio of $EC_{50}$ estimates determined in the same preparation in the absence and presence of antagonists ($n \geq 4$).
Δα = Maximum response obtained with 5-HT in the presence of antagonist ÷ control 5-HT maximum response obtained in the same preparation.

## *Antagonist studies*

Several antagonists displayed complex behaviour in rat fundus. Thus, LY 53857, methiothepin, ritanserin and ICI 170809 were non-surmountable antagonists and produced rightward displacement of 5-HT concentration-response curves and a pronounced depression of the maximum response. Methysergide also caused a concentration-dependent depression in the maximum response to 5-HT but this was not associated with any significant rightward displacement of concentration-effect curves for 5-HT. The effects of these compounds on location parameters of 5-HT concentration-effect curves are shown in Table 2. In contrast, rauwolscine, yohimbine, mesulergine, mianserin, WY 26703, SB 200646, SB 204070, pirenpirone and renzapride acted as surmountable antagonists evoking a concentration-dependent rightward displacement of concentration-effect curves for 5-HT (Table 3). Full Schild regression analysis was performed for both yohimbine (Figure 4a) and SB 200646 (Figure 5a) and yielded $pA_2$ estimates of 7.9 and 7.5 with slopes of 1.06 and 0.85 respectively (Figures 4a and b). Yohimbine also antagonized responses to several other agonists yielding an agonist independent $pA_2$ of approximately 7.8 (Table 4).

In a few instances, antagonists, in addition to causing displacement of agonist concentration-effect curves also caused a significant elevation in the maximum response. This phenomenon was independent of both the agonist and antagonist used but correlated with the occurrence of a low level of spontaneous activity prior to and during the construction of control concentration-effect curves and a reduction of this activity after exposure to the antagonist.

As *m*-CPP was a weak partial agonist in rat fundus it was also examined as an antagonist of 5-HT. In preparations in which *m*-CPP (0.1 μM) produced only a small response or failed to evoke a response, a subsequent concentration-effect

**Table 3** Antagonist affinity estimates versus 5-HT in rat stomach fundus longitudinal muscle

| *Antagonist* | *Conc.* (μM) | $pA_2$ | (95% CL) | Δα ± s.e.mean | n |
|---|---|---|---|---|---|
| Rauwolscine | 0.1 | 8.5 | (8.4–8.6) | 0.97 ± 0.04 | 15 |
| Yohimbine | 0.01–1 | 7.9† | | | ⩾8 |
| Mesulergine | 0.1 | 7.9 | (7.7–8.1) | 0.99 ± 0.06 | 4 |
| Mianserin | 1 | 7.6 | (7.4–7.9) | 0.95 ± 0.02 | 4 |
| SB 204070 | 1 | 7.6 | (7.5–7.7) | 0.97 ± 0.01 | 4 |
| WY 26703 | 1 | 7.4 | (7.2–7.6) | 1.05 ± 0.03 | 12 |
| SB 200646 | 0.1–3 | 7.5* | | | ⩾7 |
| Pirenpirone | 10 | 6.5 | (6.3–6.7) | 1.00 ± 0.02 | 6 |
| Renzapride | 3 | 6.3 | (6.1–6.7) | 0.98 ± 0.02 | 4 |
| DAU 6285 | 1 | <6.0 | | 0.99 ± 0.01 | 4 |
| Granisetron | 1 | <6.0 | | 1.02 ± 0.03 | 4 |
| Spiperone | 1 | <6.0 | | 1.05 ± 0.04 | 4 |
| Ketanserin | 1 | <6.0 | | 0.96 ± 0.02 | 4 |
| Phentolamine | 1 | <6.0 | | 0.98 ± 0.02 | 8 |
| GR 127935 | 1 | <6.0 | | 0.99 ± 0.02 | 3 |

† and *$pA_2$ determined by Schild regression analysis, slopes = 1.06 and 0.85 respectively.
Δα = maximum response obtained with 5-HT in the presence of antagonist ÷ control 5-HT maximum response obtained in the same preparation.

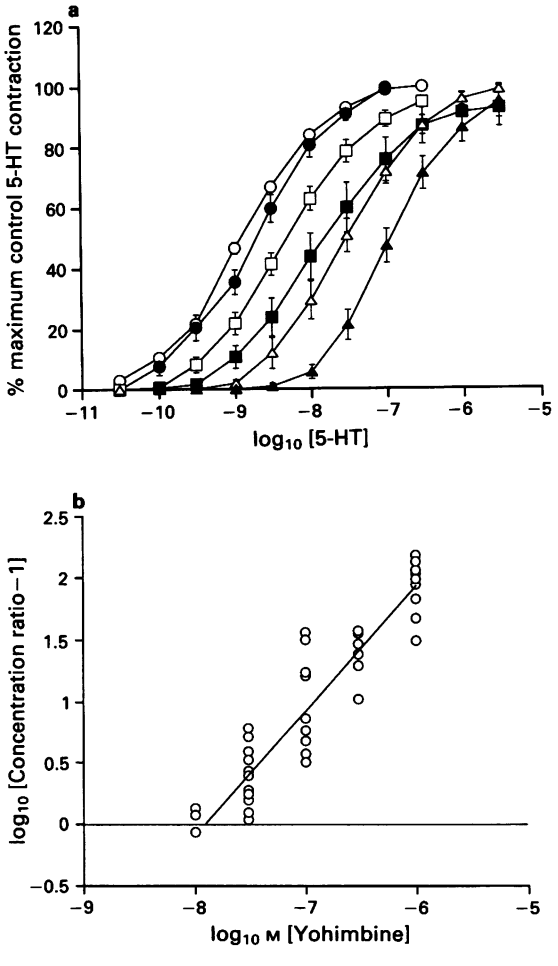


**Figure 4** (a) Cumulative concentration-effect curve to 5-HT in the absence (○), and presence of 10 nM (●), 30 nM (□), 100 nM (■), 300 nM (△) and 1 μM (▲) yohimbine in rat stomach fundus longitudinal muscle. Each point represents the arithmetic mean ± s.e.mean ($n \geqslant 8$ experimental determinations. (b) Schild regression analysis derived from agonist concentration-ratios with 10, 30, 100, 300 nM and 1 μM yohimbine.

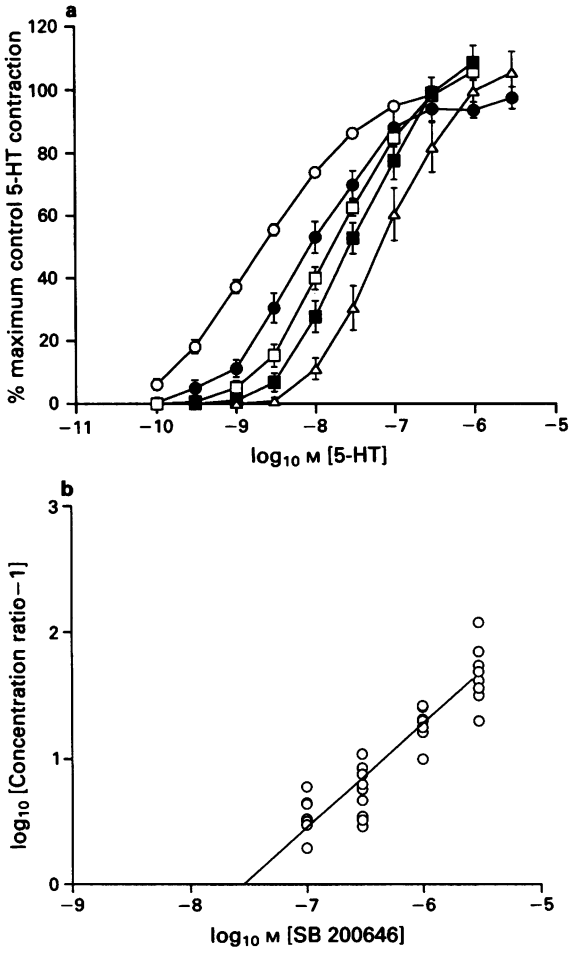


**Figure 5** (a) Cumulative concentration-effect curve to 5-HT in the absence (○), and presence of 0.1 μM (●), 0.3 μM (□), 1 μM (■) and 3 μM (△) SB 200646 in rat stomach fundus longitudinal muscle. Each point represents the arithmetic mean ± s.e.mean ($n \geqslant 7$ experimental determinations). (b) Schild regression analysis derived from agonist concentration-ratios with 0.1, 0.3, 1 and 3 μM SB 200646.

**Table 4** $pA_2$ estimates for yohimbine determined versus tryptamine analogues in rat stomach fundus longitudinal muscle

| *Agonist* | *$pA_2$* | *Slope* | n |
|---|---|---|---|
| 5-HT | 7.9 | 1.06 | 8–12 |
| 5-MeO-T | 7.9 | 1.05 | 4 |
| α-Me-5-HT | 7.7 | 1.03 | 4 |
| 5-CT | 7.8 | 1.13 | 6 |
| Tryptamine | *7.8 (7.4–8.3) | | 4 |
| 2-Me-5-HT | *7.8 (7.7–7.9) | | 4 |

*Single point analysis using 0.1 μM yohimbine (95% CL).

curve to 5-HT was shifted approximately 10 fold to the right yielding a mean $pA_2$ (95% CL) of 7.9 (7.6–8.3), $n = 4$.

Spiperone, ketanserin, granisetron, the 5-$HT_4$ receptor selective antagonist DAU 6285 (Turconi *et al.*, 1992) and the 5-$HT_{1D}$ receptor selective antagonist, GR 127935 (Skingle *et al.*, 1993) did not affect responses to 5-HT at concentrations up to and including 1.0 μM.

Several experiments were conducted to address the existence of a phenoxybenzamine-resistant tryptamine receptor (Winter & Gessner, 1968). Responses to 5-HT were abolished in tissues which had been exposed to phenoxybenzamine (10 μM for 30 min). In the same tissues tryptamine (up to 100 μM) did not evoke a contractile response.

## Discussion

The aim of the present study was to isolate single populations of receptors which contribute to the contractile response to 5-HT receptor agonists in the rat stomach fundus isolated longitudinal muscle preparation in order to obtain an explicit pharmacological profile of the contractile 5-HT receptors in this tissue. This may then allow a more definitive comparison with the cloned 5-$HT_{2B}$ receptor. To achieve this aim it was necessary to account for endogenous factors that may complicate the interpretation of both agonist and antagonist data and steps were taken to ensure that experiments were conducted as closely as possible to equilibrium conditions.

In preliminary studies it was found that intact strips of stomach fundus possessed considerable spontaneous contractile activity which prevented accurate measurement of responses at threshold concentrations of agonists. This activity appeared to be generated by the muscularis mucosa as, when separated, the mucosal layer still displayed marked activity whilst the muscularis externa did not.

Shallow concentration-effect curves for tryptamines are commonly observed in rat stomach fundus (Clineschmidt *et al.*, 1985; Buchheit *et al.*, 1985) and have been cited as evidence for multiple 5-HT receptor subtypes (Foguet *et al.*,1992b). However, in preparations which were dissected as thinly as practical and from which the mucosae had been removed, concentration-effect curves for 5-HT were found to be steeper when compared to the larger, intact muscle strips. It is possible that the process of stripping away the mucosal layer has removed a second site which contributes to 5-HT-induced contraction. Indeed, 5-HT has been shown to evoke a contractile response in muscularis mucosae taken from the fundus region of rat stomach (Horn & Zweifach, 1963). Unfortunately these findings could not be reproduced in the present study and the character of a putative mucosal receptor could not be evaluated. An alternative possibility is that a small component of circular muscle layer which lies perpendicular to the longitudinal muscle, contributes to the shallow profile of concentration-effect curves (Offermeir & Ariëns, 1966). In this regard, 5-HT is reported to induce contraction in circular muscle which is of lower potency relative to that observed in longitudinal muscle preparations (Vane, 1959). A summation of effects mediated by multiple units of tissues has been proposed to explain a similar transition from shallow to steep curves for CCK-8 after dissection of smaller preparations of guinea-pig gall bladder (Bishop *et al.*, 1992). It is perhaps relevant to point out that sensitivities of preparations to 5-HT and other tryptamines with similar intrinsic activity were approximately 0.5 log units greater than those observed by other investigators (Clineschmidt *et al.*,1985; Kalkman & Fozard, 1991; Wainscott *et al.*, 1993). Whether this increase in sensitivity can be explained entirely by the steepening of concentration-effect curves (i.e. preventing an overestimation of $EC_{50}$ parameters) or by the presence of additional metabolic processes in intact muscle strips is not known.

In the present study cocaine, paroxetine, fluoxetine and corticosterone caused no sensitization to 5-HT and thus failed to provide evidence for the existence of either neuronal or extra-neuronal uptake processes for 5-HT in rat stomach fundus. These findings are supported by earlier studies which demonstrated very little tissue accumulation of [$^3$H]-5-HT (Handschumacher & Vane, 1963; 1967).

As responses to 5-HT were unaffected by both indomethacin and tetrodotoxin, an involvement of endogenous prostaglandins or neurotransmitters is unlikely. Likewise, responses to 5-HT were unaffected by pretreatment of animals with either reserpine or PCPA suggesting little role for either catecholamines or a PCPA-sensitive pool of 5-HT. However, several observations were perhaps compatible with the presence of a PCPA-resistant pool which could release biologically active levels of 5-HT. Thus PCPA pretreatment (150 mg $kg^{-1}$ for up to 5 days) failed to prevent an infrequently observed, methiothepin- and yohimbine-sensitive increase in baseline tension after exposure of tissues to pargyline.

It is important to note that the pargyline-induced elevation in baseline tension was absent in the majority of preparations but where present, it was reversed after repeated washout over the equilibration period. Whilst it is unlikely that the presence of low levels of endogenous 5-HT had any profound influence on the profile of most pharmacological agents tested in rat fundus, the possibility of some minor influence cannot be discounted. Thus, blockade of an elevation in baseline tension or some other adaptive change (i.e. desensitization) mediated by endogenous 5-HT may account for the observed elevation in maximum response to agonists after subsequent exposure of preparations to antagonists. Such a mechanism has been considered in detail to explain an elevation in maximum response to 5-HT at 5-$HT_4$ receptors in rat oesophagus after exposure to the antagonist, DAU 6285 (Waikar *et al.*, 1992). The effects of pargyline on baseline tissue state indicates that MAO plays an important role in modulating the actions of endogenously synthesized 5-HT. It has been shown previously that MAO may also inactivate exogenously administered tryptamines and lead to a significant underestimation of potency (Vane, 1959). This illustrates the influence of endogenous metabolic processes on the observed pharmacological profile of a receptor system. These findings were supported in the present study by the observation that inhibition of MAO resulted in potentiation of responses to both tryptamine and 5-MeO-T, whereas responses to 5-HT were unaffected. For this reason, and despite the influence of pargyline on endogenous 5-HT levels, it was considered important to conduct all experiments after inhibition of MAO. Although the effect of MAO inhibition on other agonists was not examined directly, it is highly probable that these findings could be extended to other lipophilic tryptamine analogues which may penetrate the cell via passive diffusion (Vane, 1959).

Reports relating to a putative 'phenoxybenzamine resistant tryptamine receptor' (Winter & Gessner, 1968) and the occurrence of biphasic concentration-effect curves for RU24969 and certain tryptamines (Buchheit *et al.*, 1985)

have also led to speculation that 5-HT receptor agonists interact with multiple sites in rat stomach fundus. In the present study, no compelling evidence was found to support this view. Responses to tryptamine were abolished after exposure of tissues to phenoxybenzamine and biphasic curves to tryptamines and RU 24969 were not observed. The reason for the discrepancy is not known although as already discussed, it is possible that these agonists possess additional properties in intact preparations. Only 2-Me-5-HT displayed complex behaviour in rat fundus, evoking mixed contractile/relaxant responses. Sensitivity to phentolamine suggests that the relaxant phase was mediated via activation of $\alpha_1$-adrenoceptors which are reported to be present in rat fundus (Kelly & MacDonald, 1990). In the presence of phentolamine, 2-Me-5-HT, like 5-HT, α-Me-5-HT, 5-CT, 5-MeO-T and tryptamine evoked well defined and monophasic concentration-effect curves with a profile consistent with that predicted for an agonist acting at a single class of receptors. Furthermore, exposure of tissues to antagonists of varied structural classes and with varied selectivity for 5-HT receptors, failed to expose a second site. Indeed, studies with yohimbine yielded an agonist independent $pA_2$ of 7.8, indicating a common site of action for each agonist.

Under the conditions defined in preliminary experiments, the following tryptamine 'fingerprint' was obtained; 5-MeO-T $\geqslant$ α-Me-5-HT $\geqslant$ 5-HT $>$ 5-CT $>$ tryptamine $>$ 2-Me-5-HT. The low potency of both 5-CT and 2-Me-5-HT relative to 5-HT, indicated that 5-$HT_1$-like, 5.$HT_{1A}$, or 5-$HT_3$ receptors did not play a significant role. This view is supported by the poor activity of the selective 5-$HT_{1A}$ and 5-$HT_{1B/1D}$ agonists 8-OH-DPAT and sumatriptan and by a low affinity for granisetron, ICS 205930, spiperone (Hoyer, 1989) and the recently described antagonist, GR 127935, which possesses high affinity for 5-$HT_{1D\alpha}$ 5-$HT_{1D\beta}$ and 5-$HT_1$-like receptors in dog basilar artery (Skingle *et al.*, 1993). The low affinity of GR 127935 presents compelling evidence against a role for a variant of the 5-$HT_{1D}$ receptor in rat stomach fundus as originally proposed by Kalkman & Fozard (1991).

Whilst modifications to the assay have resulted in a significantly increased sensitivity to 5-HT and several related agonists, the tryptamine fingerprint agrees well with that determined previously in rat stomach fundus (Kalkman & Fozard, 1991). It is similar to that expected for activation of either 5-$HT_{2A}$ or 5-$HT_{2C}$ subtypes (Hoyer *et al.*, 1989; Leff & Martin, 1988) and support its inclusion in the 5-$HT_2$ receptor family. The tryptamine profile is also somewhat similar to that observed at 5-$HT_4$ receptors (Baxter *et al.*, 1991) but a role for 5-$HT_4$ receptors is unlikely, as DAU 6285 was not an antagonist at concentrations at least 10 fold in excess of its equilibrium dissociation constant for 5-$HT_4$ receptors (Turconi *et al.*,1991). Furthermore, the $pA_2$ for the 5-$HT_4$ antagonist SB 204070 in rat fundus (Table 3) was approximately 1000 fold higher than its apparent $pA_2$ at 5-$HT_4$ receptors (Wardle *et al.*, 1993). The absence of 5-$HT_4$ receptors is further supported by the lack of intrinsic efficacy of the 5-$HT_4$ receptor agonist, renzapride (Baxter *et al.*, 1991), although it is interesting to note that renzapride was a weak antagonist of 5-HT in rat fundus as it also acts as an antagonist at putative 5-$HT_{1P}$ receptors in the enteric nervous system (Mawe *et al.*, 1989). However, a role for 5-$HT_{1P}$ receptors is also unlikely in that 5-MeO-T, which was amongst the most potent of the agonists tested in rat fundus, is reported to possess neither affinity nor efficacy at 5-$HT_{1P}$ receptors (Takaki *et al.*, 1985; Branchek *et al.*, 1988).

On the basis of antagonist affinity, the low affinity of pirenpirone, spiperone and ketanserin indicate clear pharmacological differentiation between the contractile 5-HT receptors in rat fundus and 5-$HT_{2A}$ receptors but do not allow discrimination from the 5-$HT_{2C}$ or 5-$HT_{2B}$ subtypes. A close pharmacological affiliation of contractile 5-HT receptors in rat stomach fundus and 5-$HT_{2C}$ receptors has been recognized for some time (Buchheit *et al.*, 1985; and see Wainscott *et al.*, 1993). This relationship is further supported by the observation that SB 200646, which shows some 50 fold selectivity for rat and human 5-$HT_{2C}$ receptors over 5-$HT_{1A}$, 5-$HT_{1D}$, 5-$HT_{2A}$, 5-$HT_3$ and 5-$HT_4$ receptors (Forbes *et al.*, 1993; Wood *et al.*, 1993) also acts as a moderately potent antagonist of 5-HT in rat fundus and possesses a similar affinity to that observed at 5-$HT_{2C}$ receptors. Furthermore, the potent and selective 5-$HT_4$ antagonist, SB 204070, which possesses a moderate affinity for 5-$HT_{2C}$ receptors ($pK_i$ = 6.9, see Wardle *et al.*, 1993) also displayed a similar, albeit slightly higher affinity versus 5-HT in rat fundus

Of all the ligands tested in the present study only yohimbine and rauwolscine will permit clear discrimination of cloned 5-$HT_{2B}$ receptors and 5-HT receptors in rat fundus from both 5-$HT_{2A}$ and 5-$HT_{2C}$ (Clineschmidt *et al.*, 1985; Hoyer, 1989). In earlier studies, Clineschmidt *et al.* (1985) tested several compounds on the basis of their structural similarity to yohimbine. One of these, WY 26703 (Lattimer *et al.*, 1984) was a moderately potent antagonist in rat fundus, a finding supported in the present study. Like yohimbine and rauwolscine, WY 26703 possesses low affinity ($pK_i < 6.0$) for 5-$HT_{2C}$ receptors in piglet choroid plexus (unpublished observation) and all three compounds may therefore represent key ligands for the identification of 5-$HT_{2B}$ receptors *in vitro*. Unfortunately, these compounds also demonstrate significant affinity for other 5-HT and non-5-HT receptors (Lattimer *et al.*, 1984; Hoyer, 1989) and are therefore not likely to yield useful information relating to 5-$HT_{2B}$ mediated function in more complex *in vivo* systems. In this regard, SB 200646, whilst possessing significant affinity for the 5-$HT_{2C}$ receptor, is otherwise a reasonably selective antagonist for the putative 5-$HT_{2B}$ receptor in rat fundus and may prove useful for characterization of 5-$HT_{2B}$ receptor function *in vivo*. SB 200646 is reported to attenuate *m*-CPP induced anxiogenic-like activity in rats (Kennett *et al.*, 1993). Although it is likely that these effects are mediated by blockade of 5-$HT_{2C}$ receptors, the observations that both *m*-CPP (Clineschmidt *et al.*, 1985 and present study) and SB 200646 interact with putative 5-$HT_{2B}$ receptors in rat fundus, may lead to speculation that these effects may be partly mediated via modulation of 5-$HT_{2B}$ receptor function. It is important to note however that although 5-$HT_{2B}$ mRNA has been identified in mouse cerebellum (Loric *et al.*, 1992), it has so far not been detected in rat brain (Foguet *et al.*, 1992b).

The affinity and enantiomeric selectivity of yohimbine and rauwolscine for putative 5-$HT_{2B}$ receptors in rat stomach fundus support the earlier observations of Clineschmidt *et al.* (1985) and Kalkman & Fozard (1991) but differ somewhat from values obtained at cloned 5-$HT_{2B}$ receptors. Rauwolscine is consistently 10 fold more potent in rat fundus than at the cloned 5-$HT_{2B}$ receptor (compare Clineschmidt *et al.*, 1985; Kalkman & Fozard, 1991; Foguet *et al.*, 1992b; Wainscott *et al.*, 1993; present study). Furthermore, rauwolscine possesses a 3 to 10 fold higher affinity than yohimbine in rat fundus but approximately equal affinities at the cloned receptor. It has been proposed that these and other differences may be explained by the presence of multiple 5-HT receptor subtypes in rat stomach fundus (Foguet *et al.*, 1992b). The data obtained in the present study are not consistent with that view. It is also possible that the differences are intrinsic to the unnatural expression of the cloned 5-$HT_{2B}$ receptor (Foguet *et al.*, 1992a,b), but an alternative explanation, that the stomach fundus receptor is closely related but not identical to the cloned 5-$HT_{2B}$ receptor cannot be ruled out. Indeed, the close pharmacological identity of the structurally distinct 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptor subtypes may be viewed as a precedent (Hartig *et al.*, 1992). Other 'orphan' receptors have been identified in the rat which show pharmacological similarities to both 5-$HT_{2B}$ and 5-$HT_{2C}$ receptor subtypes (Bodelsson *et al.*, 1993). The possibility that closely related receptors are also present in the rat stomach fundus, either on blood vessels or other tissue elements, cannot be discounted. It is also worth re-iterating that the character of the receptor which mediates a contractile response to 5-HT in

the circular muscle of the stomach fundus (Vane, 1959) is still unknown.

In conclusion, we have isolated what appears to be a single class of receptors mediating a contractile response to 5-HT in longitudinal muscle of rat stomach fundus. Data obtained with both agonist and antagonist probes, each with varied activity at the established 5-HT receptor subtypes, failed to indicate the presence of multiple 5-HT receptor subtypes. The pharmacological profile of the receptor in rat fundus suggests the closest affiliation to the cloned 5-$HT_{2B}$ receptor and supports its original definition as a novel 5-HT receptor subtype (Cohen & Wittenauer, 1985; Cohen & Colbert, 1986; Cohen & Fludzinski, 1987; Blackburn *et al.*, 1988; Baez *et al.*, 1991). In this regard our finding that SB 200646 possesses high selectivity for putative 5-$HT_{2B}$ receptors in rat stomach fundus should permit confirmation of the relationship between this site and cloned 5-$HT_{2B}$ receptors and a greater understanding of the relevance of the 5-$HT_{2B}$ receptor to physiological and pathophysiological processes.

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# Effects of phosphodiesterase isoenzyme inhibitors on cutaneous inflammation in the guinea-pig

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**1** Inflammation is central to the pathophysiology of asthma. The recent findings that different inflammatory cells may express different phosphodiesterase (PDE) isoenzymes have centred attention on inhibitors of these isoenzymes as new drugs for the treatment of asthma. In this study, we investigated the effect of different PDE isoenzyme inhibitors on the accumulation of $^{111}$In-labelled eosinophils and local oedema formation at sites of allergic- and mediator-induced inflammation in guinea-pig skin.

**2** Systemic treatment with SK&F 94120, a type III PDE inhibitor, or zaprinast, a type V PDE inhibitor, had no effect on the $^{111}$In-eosinophil accumulation and oedema formation induced by i.d. injection of zymosan-activated plasma (ZAP), PAF, histamine or in a passive cutaneous anaphylaxis (PCA) reaction.

**3** Systemic treatment with rolipram, a type IV PDE inhibitor, effectively inhibited $^{111}$In-eosinophil accumulation induced by ZAP, PAF, histamine and in a PCA reaction. However, oedema formation measured in the same sites was not affected. Systemic administration of higher doses of theophylline produced similar results. In contrast, $^{111}$In-neutrophil accumulation induced by ZAP or in a PCA reaction was not altered by systemic treatment with rolipram.

**4** Locally-injected rolipram had little effect on $^{111}$In-eosinophil accumulation and oedema formation induced by histamine, PAF and in a PCA reaction.

**5** These data show that systemic, but not local, treatment with rolipram effectively inhibits allergic- and mediator-induced $^{111}$In-eosinophil accumulation but not oedema formation or $^{111}$In-neutrophil accumulation. This, taken together with the potent inhibitory effects of PDE type IV inhibitors on eosinophil function *in vitro*, suggest that this class of drugs may be beneficial in disease states such as asthma where eosinophils are thought to play a major pathophysiological role.

**Keywords:** Eosinophil; phosphodiesterase inhibitors; rolipram; inflammation; allergy; passive cutaneous anaphylaxis reaction

## Introduction

Eosinophils are inflammatory cells thought to have important effector function in allergic diseases such as asthma (Venge, 1990), rhinitis (Klementsson, 1992), dermatitis (Bruijnzeel-Koomen *et al.*, 1992) and conjunctivitis (Foster *et al.*, 1991). These cells are capable of secreting several lipid and protein mediators which, in the airways, can alter bronchial smooth muscle tonus, cause oedema formation and affect the function of other cells (Djukanovic *et al.*, 1990; Venge, 1990). Eosinophils possess cationic proteins in their granules (e.g. major basic protein and eosinophil-derived neurotoxin) which can be released upon activation and inflict damage to epithelial cells (Wardlaw *et al.*, 1988; Montefort *et al.*, 1992) which, in the lung, may be an important pathological mechanism in allergic diseases like asthma. For example, accumulation of eosinophils and their activation appears to correlate with disease severity (Bentley *et al.*, 1992) and eosinophil secretory products, for example major basic protein, reproduce some of the asthmatic symptoms in experimental animals (Djukanovic *et al.*, 1990; Gundel *et al.*, 1991).

Despite the new findings in the understanding of asthma pathophysiology, there is evidence suggesting an increase in the prevalence and severity of the disease (Lebowitz & Spinaci, 1993). This increase has led to the search for new drug treatments for asthma. One strategy to develop drugs has been based on the use of theophylline which has bronchodilator and anti-inflammatory activities and may therefore be useful in the treatment of asthma (Persson, 1986). Thus, theophylline has been shown to inhibit polymorphonuclear function at therapeutic levels (Nielson *et al.*, 1988) and late airway responses to allergen challenge, even at sub-therapeutic doses (Ward *et al.*, 1993). Interestingly, some chronic steroid-dependent asthmatic subjects may find an improvement in their symptoms when using theophylline (Nassif *et al.*, 1981). However, the narrow therapeutic index, potential toxicity and need for plasma monitoring of theophylline make this drug difficult to use (Johnston, 1990).

The mechanism of action of theophylline is as yet unclear, but inhibition of phosphodiesterase (PDE) enzymes is a possibility which has gained strong support (Kuehl *et al.*, 1987). PDEs are enzymes responsible for the breakdown of cyclic nucleotides (adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and guanosine 3′:5′-cyclic monophosphate (cyclic GMP)) within cells (Beavo & Reifsnyder, 1990; Nicholson *et al.*, 1991). Five families of PDE isoenzymes (PDE I–V) have been identified and these are differentially distributed in cells (Beavo & Reifsnyder, 1990; Nicholson *et al.*, 1991). Inflammatory cells contain mainly a PDE type IV isoenzyme (cyclic AMP-specific) which accounts for most of the metabolism of cyclic AMP in these cells (Torphy & Undem, 1991; Giembycz & Dent, 1992). Inhibition of this isoenzyme (PDE IV) in both neutrophils and eosinophils leads to an effective inhibition of cell function (Nielson *et al.*, 1990; Dent *et al.*, 1991). This is consistent with the widespread anti-inflammatory effects induced by elevating intracellular levels of cyclic nucleotides in various cell types (Torphy & Undem, 1991). Also relevant to the treatment of asthma is the observation that inhibition of PDE types III and IV effectively suppress contraction of human bronchial smooth muscle *in vitro* and bronchoconstriction induced by different stimuli or antigen in sensitized animals (Torphy & Undem, 1991; de Boer *et al.*, 1992; Howell *et al.*, 1993).

In guinea-pig skin, intradermal injection of different known mediators of inflammation leads to a dose-dependent accumulation of radiolabelled eosinophils and local oedema formation (Faccioli *et al.*, 1991; Teixeira *et al.*, 1993a). The

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injection of antigen in sites previously sensitized with an antigen-specific (BGG) $IgG_1$-rich anti-serum (passive cutaneous anaphylaxis (PCA) reaction) also leads to the accumulation of radiolabelled eosinophils and oedema formation (Weg *et al.*, 1992; Teixeira *et al.*, 1993a). In this PCA reaction, oedema formation is dependent on the release of histamine and newly formed lipid mediators, PAF and leukotriene $D_4$ ($LTD_4$) (Weg *et al.*, 1991). The mediators responsible for cell accumulation have not been fully characterized, but a 5-lipoxygenase product, probably $LTB_4$, appears to play an important role (Teixeira & Hellewell, 1994). The mechanism by which radiolabelled eosinophils accumulate in guinea-pig skin has been recently demonstrated to be dependent on both CD18 and VLA-4 integrin adhesion molecules on the eosinophil (Weg *et al.*, 1993; Teixeira *et al.*, 1994). The aim of the present study was to assess the effects of selective inhibitors of PDE types III, IV and V isoenzymes on the accumulation of $^{111}$In-eosinophils and oedema formation in response to various known mediators of inflammation and in a type I allergic (PCA) reaction in guinea-pig skin. The effect of a type IV PDE inhibitor on $^{111}$In-neutrophil accumulation was also studied. The following selective PDE isoenzyme inhibitors were used: SK&F 94120 (type III inhibitor), rolipram (type IV inhibitor) and zaprinast (type V inhibitor) (Beavo & Reifsnyder, 1990; Dent *et al.*, 1991). The findings with these PDE inhibitors in this model of eosinophil accumulation may have a bearing on not only asthma but also on other allergic diseases such as rhinitis (Klementsson, 1992), dermatitis (Bruijnzeel-Koomen *et al.*, 1992) and allergic conjunctivitis (Foster *et al.*, 1991) where eosinophils are thought to play a role.

## Methods

### *Preparation of zymosan-activated plasma*

Zymosan-activated plasma (ZAP) was used as a source of guinea-pig C5a des Arg. Guinea-pig heparinized (10 iu $ml^{-1}$) plasma was incubated with zymosan (5 mg $ml^{-1}$) at 37°C. After 30 min, zymosan was removed by centrifugation (2 × 10 min at 3000 *g*). ZAP was then desalted on a PD-10 Sephadex G-25M column and stored in aliquots at − 20°C.

### *Preparation of passive cutaneous anaphylaxis sera and reactions*

Details of the preparation of sera and doses of antigen are described elsewhere (Weg *et al.*, 1991). Briefly, male guinea-pigs (Harlan Porcellus, Oxon; 350–400 g) were immunized with bovine gamma-globulin (BGG) in Freund's complete adjuvant followed by a boost on day 21 and serum collected on day 30. Recipient animals received an i.d. injection of 50 µl of a 1/50 dilution of the anti-serum followed, 16–20 h later, by the injection of antigen (BGG, 0.01–1 µg per site). Most of the tissue fixing antibody was of the $IgG_1$ isotype (Weg *et al.*, 1991).

### *Induction, purification and radiolabelling of guinea-pig eosinophils*

The method is described in detail elsewhere (Faccioli *et al.*, 1991; Teixeira *et al.*, 1993a). Briefly, ex-breeder female guinea-pigs (Harlan Porcellus; 700–800 g) were treated with neat horse serum (1 ml, i.p.) every other day for two weeks and the cells collected by peritoneal lavage with heparinized saline (10 iu $ml^{-1}$) 2 days after the last injection. The cells obtained were layered onto a discontinuous Percoll-HBSS (calcium- and magnesium-free) gradient followed by centrifugation (1500 *g*, 25 min at 20°C). Eosinophils (> 95% pure, > 98% viable) were collected from the 1.090/1.095 and 1.095/1.100 g $ml^{-1}$ density interfaces. The purified eosinophils were radiolabelled by incubation with $^{111}InCl_3$ (100 µCi in 10 µl) chelated to 2-mercaptopyridine-N-oxide (Merc, 40 µg in 0.1 ml of 50 mM PBS, pH 7.4) for 15 min at room temperature. The cells were then washed twice in HBSS (calcium- and magnesium-free) containing 10% guinea-pig platelet-poor plasma and resuspended at a final concentration of $10^7$ cells $ml^{-1}$ prior to injection.

### *Induction, purification and radiolabelling of guinea-pig neutrophils*

Neutrophils were elicited in the peritoneal cavity of the naive ex-breeder guinea-pigs by the i.p. injection of 15 ml of a 5% (w/v) solution of casein as previously described (Teixeira *et al.*, 1993b). After 12 h, the animals were killed and the peritoneal cavity washed with heparinized saline (10 iu $ml^{-1}$). The rest of the procedure was as described for the eosinophils. The cells were also collected from the 1.090/1.095 and 1.095/1.100 g $ml^{-1}$ interfaces. The purity of the preparation was greater than 98% and the rare contaminants were eosinophils and occasional mononuclear cells. Viability, tested by trypan blue exclusion, was greater than 98%. Neutrophils were also radiolabelled with $^{111}$In-Merc and resuspended at a concentration of $10^7$ cells $ml^{-1}$.

### *Measurement of local oedema formation and leukocyte accumulation in guinea-pig skin*

Radiolabelled leukocyte infiltration and oedema formation were measured simultaneously in the skin. $^{125}$I-labelled human serum albumin, ([$^{125}$I]-HSA, 5 µCi) was added to the $^{111}$In-labelled eosinophils or neutrophils prior to i.v. injection ($2.5 \times 10^6$ cells per animal), into recipient guinea-pigs (Harlan Porcellus; 350–400 g) anaesthetized with Hypnorm (0.2 ml, i.m.). PDE inhibitors were given either systemically or locally. For systemic treatment, drugs were given at the dose of 5 mg $kg^{-1}$ i.p. 30 min and 0.5 mg $kg^{-1}$ i.v. 10 min prior to the injection of radiolabelled cells and [$^{125}$I]-HSA. Similar doses of rolipram have been shown to inhibit antigen- or mediator-induced bronchoconstriction effectively in the guinea-pig (Howell *et al.*, 1993; Underwood *et al.*, 1993). Experiments were conducted in pairs and control animals received vehicle in the same volume as treated animals. For the local treatment, rolipram (0.1 to 10.0 µg per site) was mixed with the mediators or antigen prior to the i.d. injections. Five minutes after injection of cells, inflammatory mediators or antigen were injected i.d. in 0.1 ml volumes into the dorsal skin of the shaved animals. Each animal received a duplicate of each treatment following a randomized injection plan and the inflammatory response ($^{111}$In-labelled cell accumulation and oedema formation) was assessed after 2 h. At this time, blood was obtained by cardiac puncture and the animals were killed by an overdose of sodium pentobarbitone. The dorsal skin was removed, cleaned free of excess of blood and the skin sites punched out with a 17 mm punch. The samples were counted in an automatic 5-head gamma-counter (Canberra Packard Ltd, Pangbourne, Berks) and the counts were cross-channel corrected for the two isotopes.

The number of leukocytes accumulating in each site is expressed as $^{111}$In-labelled cells per skin site and oedema formation as the ratio of $^{125}$I counts of the skin sample divided by the $^{125}$I counts in 1 µl of plasma.

### *Reagents*

The following compounds were purchased from Sigma Chemical Company (Poole, Dorset): bradykinin, dimethylsulphoxide (DMSO), histamine, casein, bovine gamma globulin (BGG), theophylline and zymosan. Hanks solutions, HEPES and horse serum were purchased from Life Technologies Limited (Paisley, Scotland). Percoll was from Pharmacia (Milton Keynes, Bucks) and C16 PAF from Bachem (Saffron Walden, Essex). [$^{125}$I]-human serum albumin ([$^{125}$I]-HSA) and $^{111}InCl_3$ were purchased from Amersham International plc,

Amersham. The following selective PDE isoenzyme inhibitors were used: SK&F 94120 (type III inhibitor), rolipram (type IV inhibitor) and zaprinast (type V inhibitor) (see Beavo & Reifsnyder, 1990, for review on PDE inhibitors). SK&F 94120 and zaprinast were dissolved in saline with 0.01 M sodium hydroxide while rolipram was dissolved in DMSO and diluted further in saline. These drugs were a gift from Sandoz, Basle, Switzerland.

### *Statistical analysis*

Comparisons between control and untreated groups were carried out using Student's paired $t$ test. For the local treatment, two-way analysis of variance (ANOVA) was used. Percentage inhibition was calculated after subtracting background (saline) values. Results were presented as the mean ± s.e.mean for the number of animals given and were considered significant when $P<0.05$.

## Results

Two h after i.v. injection of the radiolabelled cells, 11.5 ± 1.9% ($n = 14$) and 4.7 ± 1.3% ($n = 5$) of infused $^{111}$In-eosinophils and $^{111}$In-neutrophils, respectively, were circulating. None of the drug treatments used in this study significantly altered the number of circulating radiolabelled cells (data not shown). Our previous experiments have shown that the majority of radiolabelled cell accumulation and oedema formation induced by known mediators of inflammation or in a PCA reaction occurs over the first 2 h (Weg *et al.*, 1992).

### *Effects of SK&F 94120 and zaprinast on $^{111}$In-eosinophil accumulation and oedema formation*

At the dose used (5 mg kg$^{-1}$, i.p. and 0.5 mg kg$^{-1}$, i.v.), SK&F 94120, a type III PDE inhibitor (Beavo & Reifsnyder, 1990), had no effect on the $^{111}$In-eosinophil accumulation or oedema formation in a PCA reaction (0.1 to 1.0 μg of BGG per site, Figure 1). Similarly, SK&F 94120 had no effect on the $^{111}$In-eosinophil accumulation (Table 1) and oedema formation (data not shown) in response to i.d. ZAP (10 to 100% in saline) and PAF ($10^{-10}$ and $10^{-9}$ mol per site). Responses elicited by i.d. histamine ($2.5 \times 10^{-9}$ and $2.5 \times 10^{-8}$ mol per site) or bradykinin ($10^{-10}$ and $10^{-9}$ mol per site) were also unaffected (data not shown). The same doses of inflammatory stimuli were used in all subsequent experiments unless stated otherwise.

Zaprinast (5 mg kg$^{-1}$, i.p. and 0.5 mg kg$^{-1}$, i.v.), a type V PDE inhibitor (Beavo & Reifsnyder, 1990), also had no significant effect on the $^{111}$In-eosinophil accumulation and oedema formation in a PCA reaction (Figure 2) or in response to i.d. injection of ZAP, PAF, bradykinin or histamine (Table 1 and data not shown).

Preliminary experiments using theophylline at similar doses (5 mg kg$^{-1}$, i.p. and 0.5 mg kg$^{-1}$, i.v.) showed that $^{111}$In-eosinophil accumulation or oedema formation induced by the same stimuli were unaltered (data not shown). However, higher doses of theophylline (50 mg kg$^{-1}$, i.p. and 5 mg kg$^{-1}$, i.v.) effectively inhibited $^{111}$In-eosinophil accumulation

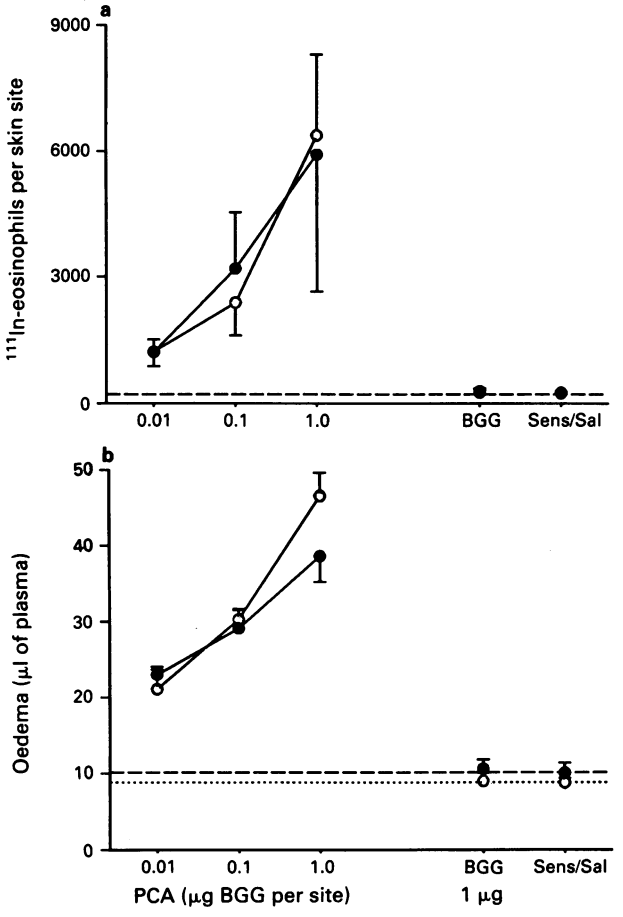


**Figure 1** Effect of systemic treatment with SK&F 94120 (5 mg kg$^{-1}$, i.p. and 0.5 mg kg$^{-1}$, i.v.) on $^{111}$In-eosinophil accumulation (a) and oedema formation (b) in a passive cutaneous anaphylaxis (PCA) reaction in guinea-pig skin. Control animals are shown by (●) and treated animals by (○). Inflammatory responses were assessed 2 h after i.d. injection of antigen (bovine gamma-globulin, BGG) in saline-treated sites (shown as BGG) or sites previously sensitized with an $IgG_1$-rich anti-sera (shown as PCA). The lines across the graphs represent the background values in sensitized skin sites injected with saline (Sens/Sal) in control (dashed line) and SK&F 94120-treated (dotted line) animals. Results are mean ± s.e.mean of 5 pairs of animals.

**Table 1** Effect of systemic treatment with zaprinast or SK&F 94120 on the $^{111}$In-eosinophil accumulation induced by PAF and zymosan activated plasma in guinea-pig skin

| | | $^{111}$*In-eosinophils per site* | | | |
|---|---|---|---|---|---|
| | | *SK&F 94120* | | *Zaprinast* | |
| | | *Control* | *Treated* | *Control* | *Treated* |
| Saline | | 202 ± 38 | 189 ± 8 | 202 ± 39 | 214 ± 36 |
| ZAP | 10% | 2939 ± 859 | 2963 ± 771 | 2986 ± 907 | 2324 ± 584 |
| | 30% | 7351 ± 2303 | 6148 ± 1173 | 8319 ± 2412 | 5677 ± 1386 |
| | 100% | 12615 ± 3148 | 12379 ± 2732 | 14089 ± 3215 | 10026 ± 2339 |
| PAF | $10^{-10}$ | 1423 ± 666 | 1634 ± 413 | 1434 ± 275 | 1259 ± 268 |
| | $10^{-9}$ | 1939 ± 642 | 2898 ± 848 | 2997 ± 919 | 2341 ± 413 |

Zaprinast or SK&F 94120 were given at a dose of 5 mg kg$^{-1}$, i.p. and 0.5 mg kg$^{-1}$, i.v. 30 and 10 min, respectively, before the i.v. injection of $^{111}$In-eosinophils. PAF ($10^{-10}$ amd $10^{-9}$ mol per site) and zymosan-activated plasma (ZAP, 10%, 30% and 100% in saline) were injected i.d. and $^{111}$In-eosinophil accumulation assessed 2 h later. Results are mean ± s.e.mean of 5 pairs of animals.

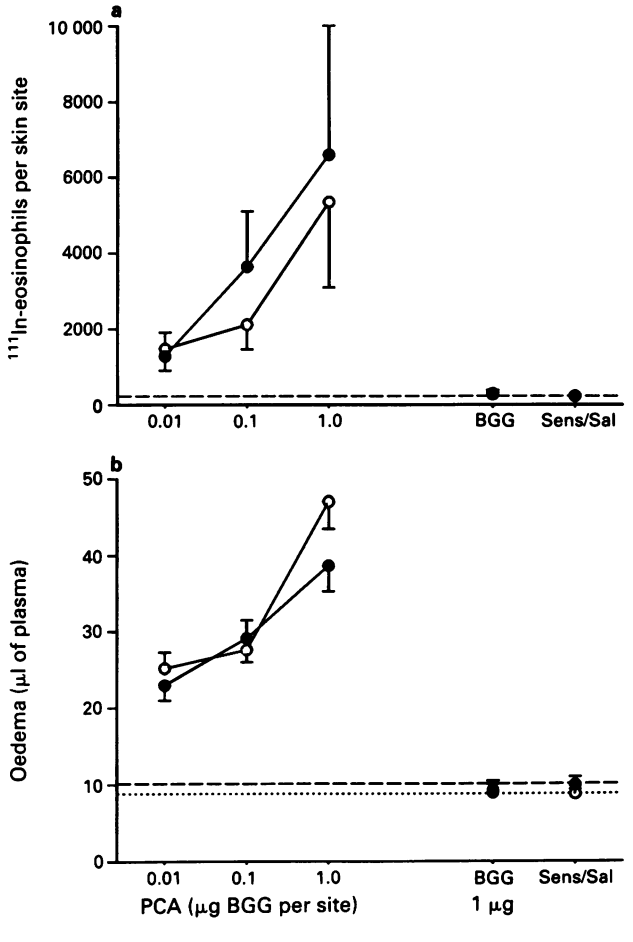


**Figure 2** Effect of systemic treatment with zaprinast (5 mg kg$^{-1}$, i.p. and 0.5 mg kg$^{-1}$, i.v.) on $^{111}$In-eosinophil accumulation (a) and oedema formation (b) in a passive cutaneous anaphylaxis (PCA) reaction in guinea-pig skin. Control animals are shown by (●) and treated animals by (○). Inflammatory responses were assessed 2 h after i.d. injection of antigen (bovine gamma-globulin, BGG) in saline-treated sites (shown as BGG) or sites previously sensitized with an $IgG_1$-rich anti-sera (shown as PCA). The lines across the graphs represent the background values in sensitized skin sites injected with saline (Sens/Sal) in control (dashed line) and zaprinast-treated (dotted line) animals. Results are mean ± s.e.mean of 5 pairs of animals.

**Table 2** Effects of systemic treatment with theophylline on $^{111}$In-eosinophil accumulation induced by PAF, zymosan-activated plasma and in a PCA reaction in guinea-pig skin

| | | $^{111}$*In-eosinophils per site* | |
|---|---|---|---|
| | | *Control* | *Theophylline* |
| Saline | | 134 ± 5 | 166 ± 24 |
| ZAP | 10% | 1510 ± 563 | 506 ± 63** |
| | 30% | 3154 ± 375 | 1476 ± 117* |
| | 100% | 9582 ± 1327 | 4793 ± 826* |
| PAF | $10^{-10}$ | 1263 ± 253 | 407 ± 40** |
| | $10^{-9}$ | 2025 ± 186 | 394 ± 56** |
| PCA | 0.01 | 811 ± 152 | 342 ± 97** |
| | 0.1 | 2428 ± 180 | 506 ± 169** |
| | 1.0 | 4430 ± 1154 | 654 ± 180** |

Theophylline was given at the dose of 50 mg kg$^{-1}$, i.p. and 5.0 mg kg$^{-1}$, i.v. 30 and 10 min respectively, before the i.v. injection of $^{111}$In-eosinophils. Zymosan-activated plasma (ZAP, 10%, 30% and 100% in saline), PAF ($10^{-10}$ and $10^{-9}$ mol per site) and antigen (BGG, 0.01 to 1.0 μg per site) in sites pre-sensitized with $IgG_1$-rich sera were injected i.d. and $^{111}$In-eosinophil accumulation assessed 2 h later. Results are mean ± s.e.mean of 3 pairs of animals. *$P<0.05$ and **$P<0.01$.

induced by different meadiators or in a PCA reaction (Table 2). For example, $^{111}$In-eosinophil accumulation in the PCA reaction (0.1 μg of BGG per site) was inhibited by 85% ($n = 3$, $P<0.01$). Oedema formation measured in the same sites was not significantly affected (data not shown).

### *Effects of systemic rolipram on $^{111}$In-eosinophil accumulation and oedema formation*

The type IV PDE inhibitor, rolipram (5 mg kg$^{-1}$, i.p. and 0.5 mg kg$^{-1}$, i.v.) virtually abolished the $^{111}$In-eosinopil accumulation in the PCA reaction, but did not affect oedema formation measured in the same sites (Figure 3). For example, rolipram inhibited by 97% the $^{111}$In-eosinophil accumulation in sites injected with 0.1 μg of BGG. Similarly, ZAP-induced $^{111}$In-eosinophil accumulation, but not oedema formation, was inhibited by up to 89% by systemic rolipram (Figure 4). Table 3 depicts the effects of rolipram on the $^{111}$In-eosinophil accumulation and oedema formation induced by i.d. injection of PAF, histamine and bradykinin. Histamine induced small, but significant, $^{111}$In-eosinophil accumulation. Both histamine- and PAF-induced $^{111}$In-eosinophil accumulation, but not oedema formation, were

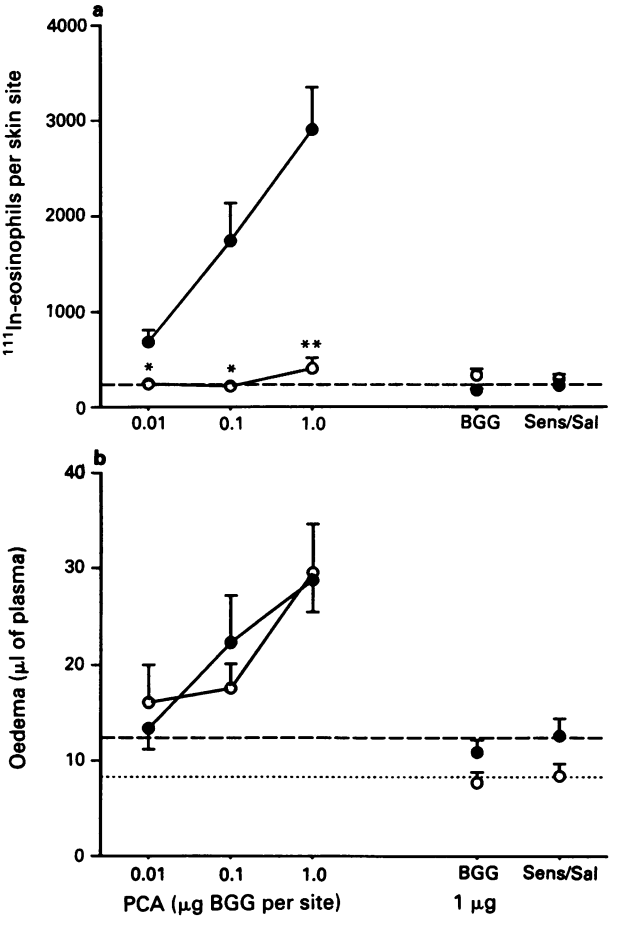


**Figure 3** Effects of systemic treatment with rolipram (5 mg kg$^{-1}$, i.p. and 0.5 mg kg$^{-1}$, i.v.) on $^{111}$In-eosinophil accumulation (a) and oedema formation (b) in a passive cutaneous anaphylaxis (PCA) reaction in guinea-pig skin. Control animals are shown by (●) and treated animals by (○). Inflammatory responses were assessed 2 h after i.d. injection of antigen (bovine gamma-globulin, BGG) in saline-treated sites (shown as BGG) or sites previously sensitized with an $IgG_1$-rich anti-sera (shown as PCA). The lines across the graphs represent the background values in sensitized skin sites injected with saline (Sens/Sal) in control (dashed line) and rolipram-treated (dotted line) animals. Results are mean ± s.e.mean of 5 pairs of animals where *$P<0.05$ and **$P<0.01$, respectively, when compared to control animals.

significantly inhibited by systemic rolipram (Table 3). Bradykinin did not induce significant influx of $^{111}$In-eosinophils when compared to saline (Table 3).

### *Effects of systemic rolipram on $^{111}$In-neutrophil accumulation*

Both neutrophils and eosinophils have been shown to possess a PDE type IV isoenzyme which seems to account for most of the cyclic AMP hydrolytic activity in these cells (Nielson *et al.*, 1990; Dent *et al.*, 1991). Inhibitors of this isoenzyme (such as rolipram) have been shown to inhibit both eosinophil and neutrophil function effectively *in vitro* (Nielson *et al.*, 1990; Dent *et al.*, 1991; Souness *et al.*, 1991). Since

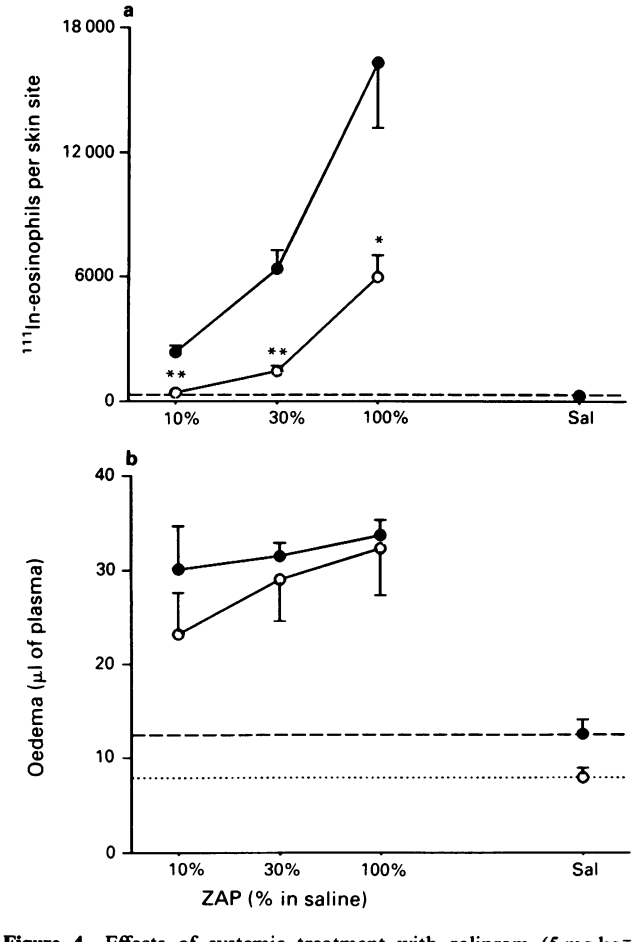


**Figure 4** Effects of systemic treatment with rolipram (5 mg kg$^{-1}$, i.p. and 0.5 mg kg$^{-1}$, i.v.) on zymosan-activated plasma (ZAP)-induced $^{111}$In-eosinophil accumulation (a) and oedema formation (b) in guinea-pig skin. Control animals are shown by (●) and treated animals by (○). Inflammatory responses were assessed 2 h after i.d. injection of ZAP or saline (Sal). The lines across the graphs represent the background values in response to i.d. injection of saline in control (dashed line) and rolipram-treated (dotted line) animals. Results are mean ± s.e.mean of 5 pairs of animals where $*P<0.05$ and $**P<0.01$, respectively, when compared to control animals.

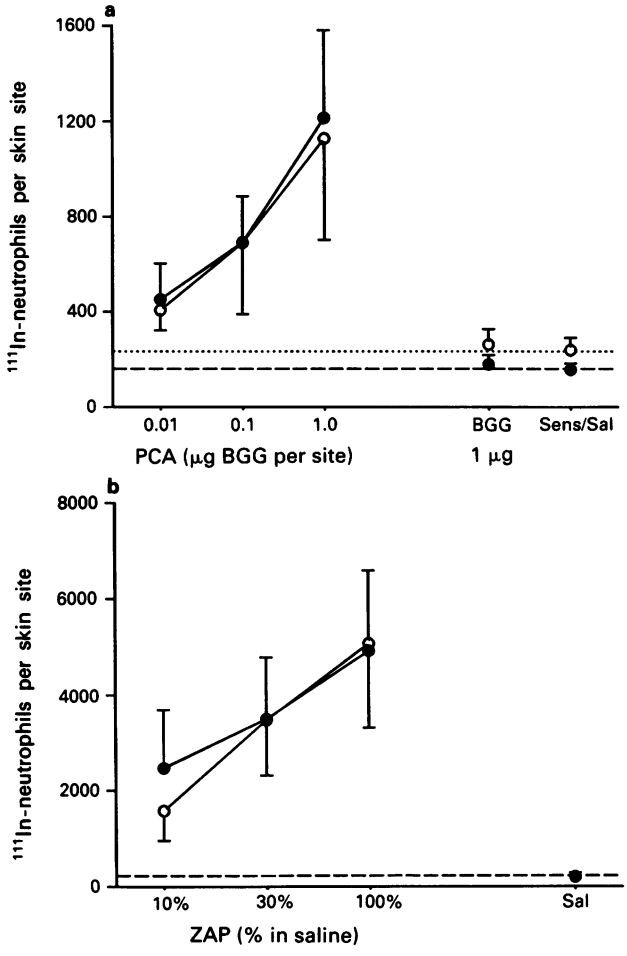


**Figure 5** Effect of systemic treatment with rolipram (5 mg kg$^{-1}$, i.p. and 0.5 mg kg$^{-1}$, i.v.) on $^{111}$In-neutrophil accumulation in (a) a passive cutaneous anaphylaxis (PCA) reaction and (b) in response to i.d. injection of zymosan-activated plasma (ZAP) in guinea-pig skin. Control animals are shown by (●) and treated animals by (○). Inflammatory responses were assessed 2 h after i.d. injection of antigen (bovine gamma-globulin, BGG) in saline-treated sites (shown as BGG), sites previously sensitized with an $IgG_1$-rich anti-sera (shown as PCA) or in sites injected with ZAP or saline (Sal). The lines across the graphs represent the background values in saline sites or sensitized skin sites injected with saline (Sens/Sal) in control (dashed line) and rolipram-treated (dotted line) animals. Results are mean ± s.e.mean of 5 pairs of animals.

**Table 3** Effect of systemic treatment with rolipram on the $^{111}$In-eosinophil accumulation and oedema formation in guinea-pig skin

| *Stimuli* (mol per site) | *$^{111}$In-eosinophils per site* Control | Rolipram | *Oedema (μl of plasma)* Control | Rolipram |
|---|---|---|---|---|
| Saline | 277 ± 46 | 173 ± 13 | 7.9 ± 1.0 | 12.5 ± 1.5 |
| BK $10^{-10}$ | 401 ± 77 | 196 ± 13 | 37.3 ± 2.9 | 36.6 ± 3.9 |
| $10^{-9}$ | 448 ± 99 | 210 ± 19 | 44.5 ± 2.9 | 42.4 ± 3.1 |
| Hist $2.5 \times 10^{-9}$ | 702 ± 192 | 207 ± 98 | 36.6 ± 3.7 | 25.5 ± 5.8 |
| $2.5 \times 10^{-8}$ | 766 ± 152 | 248 ± 26* | 51.5 ± 6.7 | 51.8 ± 4.6 |
| PAF $10^{-10}$ | 1434 ± 275 | 358 ± 31* | 34.1 ± 2.5 | 34.7 ± 3.3 |
| $10^{-9}$ | 2997 ± 919 | 890 ± 353* | 55.4 ± 4.0 | 53.8 ± 6.8 |

Rolipram was given at a dose of 5 mg kg$^{-1}$, i.p. and 0.5 mg kg$^{-1}$, i.v. 30 and 10 min, respectively, before the i.v. injection of $^{111}$In-eosinophils and [$^{125}$I]-HSA. The inflammatory stimuli were injected i.d. and $^{111}$In-eosinophil accumulation and oedema formation assessed 2 h later. The following stimuli were used: bradykinin (BK), histamine (Hist) and platelet-activating factor (PAF) which were administered at the doses shown. Results are mean ± s.e.mean of 5 pairs of animals. $*P<0.05$.

rolipram suppressed $^{111}$In-eosinophil accumulation in our model, we decided to test if accumulation of $^{111}$In-neutrophils was also inhibited. Figure 5 shows the effects of systemic rolipram on the $^{111}$In-neutrophil accumulation in a PCA reaction (Figure 5a) and in response to i.d. injection of ZAP (Figure 5b). There was no inhibition of $^{111}$In-neutrophil accumulation and oedema formation measured in the same sites was also unaltered (data not shown). PAF-induced $^{111}$In-neutrophil accumulation was also not affected by systemic administration of rolipram (data not shown).

### *Effects of intradermal administration of rolipram on $^{111}$In-eosinophil accumulation and oedema formation*

In order to assess whether rolipram could also inhibit $^{111}$In-eosinophil accumulation when given locally, increasing concentrations of rolipram were mixed with PAF ($10^{-9}$ mol per site), histamine ($2.5 \times 10^{-8}$ mol per site) or antigen (1 μg of BGG) prior to their i.d. injection. At the doses used (0.1 to 10.0 μg per site), rolipram had little effect on $^{111}$In-eosinophil accumulation and oedema formation induced by the inflammatory stimuli or in the PCA reaction (Figure 6). Only PAF-induced $^{111}$In-eosinophil accumulation was partially suppressed by rolipram at 10 μg per site. Higher doses of rolipram could not be used because of limited solubility.

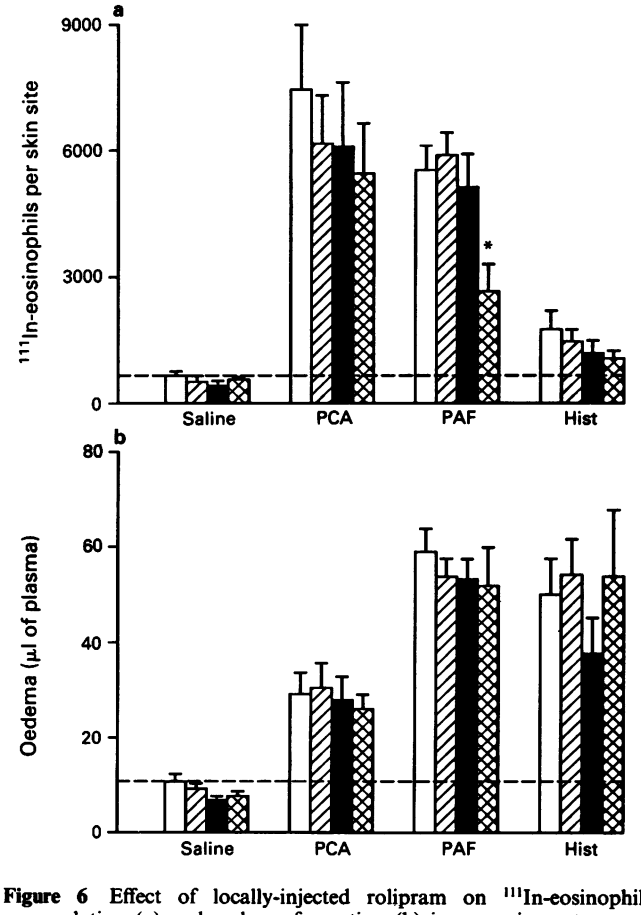


**Figure 6** Effect of locally-injected rolipram on $^{111}$In-eosinophil accumulation (a) and oedema formation (b) in a passive cutaneous anaphylaxis (PCA, 1 μg of antigen per site) reaction and induced by PAF ($10^{-9}$ mol per site) or histamine (Hist, $2.5 \times 10^{-8}$ mol per site). Inflammatory responses were assessed 2 h after i.d. injection of mediators and antigen either alone (open columns) or with rolipram at 0.1 μg per site (hatched columns), 1.0 μg per site (solid columns) or 10.0 μg per site (cross-hatched columns). The line across the graphs represent the background values in response to i.d. injection of saline. Results are mean ± s.e.mean of 5 pairs of animals where *represents $P < 0.05$ when compared to i.d. injection of mediators or antigen alone.

## Discussion

We have studied the effects of selective inhibitors of PDE isoenzymes III, IV and V on the local accumulation of radiolabelled eosinophils, neutrophils and plasma protein induced by antigen or different known mediators of inflammation in guinea-pig skin. Our findings can be summarized as follows: (1) Systemic treatment with a type III (SK&F 94120) or a type V (zaprinast) PDE inhibitor had no significant effect on the $^{111}$In-eosinophil accumulation and oedema formation induced by the different inflammatory stimuli; (2) Systemic treatment with theophylline significantly inhibited $^{111}$In-eosinophil accumulation but not oedema formation induced by ZAP, PAF and in the PCA reaction; (3) Systemic treatment with rolipram, a type IV PDE inhibitor, effectively suppressed the accumulation of $^{111}$In-eosinophils, but had no effect on the accumulation of $^{111}$In-neutrophils or oedema formation in response to the i.d. inflammatory stimuli; (4) Co-injection of rolipram with PAF, histamine or antigen had little effect on the accumulation of $^{111}$In-eosinophils or oedema formation induced by these stimuli. These results show that systemic, but not local, PDE type IV inhibition was associated with strong inhibition of allergic and mediator-induced $^{111}$In-eosinophil accumulation in guinea-pig skin.

Theophylline has been previously shown to inhibit the influx of eosinophils into the airways of different animal models of allergic inflammation (Spicer *et al.*, 1990; Sturm *et al.*, 1990; Gristwood *et al.*, 1991). In our studies in guinea-pig skin, theophylline also effectively inhibited the accumulation of $^{111}$In-eosinophils induced by different mediators and in a PCA reaction when given at 50 mg kg$^{-1}$, but not at 5 mg kg$^{-1}$. The need for higher doses of theophylline to achieve effective inhibition of eosinophil accumulation is in agreement with previously published data (Spicer *et al.*, 1990; Sturm *et al.*, 1990; Gristwood *et al.*, 1991).

Griswold *et al.* (1993) recently reported that oral administration of rolipram inhibited neutrophil accumulation and oedema formation in the inflamed mouse ear and peritoneum with an $ED_{50}$ of 1.7 mg kg$^{-1}$ and 2.5 mg kg$^{-1}$, respectively. Zaprinast, up to 10 mg kg$^{-1}$, had no inhibitory effect. In the guinea-pig, oral administration of rolipram has been reported to dose-dependently inhibit eosinophil accumulation in the conjunctiva induced by topical application of leukotrienes (Newsholm & Schwartz, 1993). Maximum inhibition was observed at 10 mg kg$^{-1}$ but greater than 80% inhibition was found with as little as 0.1 mg kg$^{-1}$. In our studies we found that administration of rolipram at 5 mg kg$^{-1}$, i.p. and 0.5 mg kg$^{-1}$, i.v. gave maximal inhibition of eosinophil accumulation although we did not conduct full dose-response analysis. Preliminary studies with rolipram at 0.5 mg kg$^{-1}$ i.v. gave inconsistent inhibition of eosinophil accumulation and therefore the compound was also administered i.p. at the higher dose.

The effects of PDE inhibition on guinea-pig pulmonary function and cellular influx after antigen challenge has also been reported in recent publications (Howell *et al.*, 1993; Underwood *et al.*, 1993). Rolipram inhibited antigen-induced bronchoconstriction both *in vitro* and *in vivo*, but it had no effect on bronchoconstriction induced by $LTC_4$ or histamine (Howell *et al.*, 1993; Underwood *et al.*, 1993). In contrast, zaprinast at oral doses up to 200 mg kg$^{-1}$ failed to inhibit antigen-induced bronchoconstriction (Howell *et al.*, 1993). However, airway hyperreponsiveness (AHR) and eosinophil influx measured 24 h after antigen challenge were also inhibited by rolipram. The observation that rolipram inhibited antigen-induced but not mediator-induced responses led the authors to suggest that inhibition of mast cells could account for some of the inhibitory effects of rolipram (Underwood *et al.*, 1993). However, rolipram may inhibit eosinophil influx even when given 12 h after antigen challenge mitigating against an early inhibitory effect only on the mast cell (Sturm *et al.*, 1990).

In guinea-pig skin, rolipram inhibited both antigen- and mediator-induced $^{111}$In-eosinophil accumulation. We cannot rule out a role for mast cells as the cellular target for the inhibitory actions of rolipram, but several pieces of evidence suggest that these cells may not be the main site of action. Firstly, we have no evidence for a mast cell-dependent component to responses to PAF, but these were effectively inhibited by rolipram. Additionally, one would expect that, if rolipram were acting on the mast cell to inhibit mediator release, both oedema formation and $^{111}$In-neutrophil accumulation would also be suppressed accordingly. Finally, i.d. injection of rolipram with PAF or antigen had little effect on the $^{111}$In-eosinophil accumulation induced by these stimuli favouring an effect on circulating cells, rather than skin tissue cells.

If the mast cell is not the main cellular target for rolipram-induced inhibition of $^{111}$In-eosinophil accumulation, what are other possible sites of action? Guinea-pig eosinophils have been previously shown to possess a membrane bound, cyclic AMP-specific, cyclic GMP-and calmodulin-insensitive, Ro-20-1724-inhibitable isoenzyme (Dent *et al.*, 1991; Souness *et al.*, 1991). These characteristics are of a type IV PDE isoenyzme which accounts for most of the cyclic AMP hydrolytic activity in these cells (Dent *et al.*, 1991). Systemic rolipram treatment may lead to an inhibition of the PDE type IV in circulating $^{111}$In-eosinophils, thus inhibiting their accumulation. If that is the case, PDE IV inhibitors are capable of inhibiting not only eosinophil function *in vitro*, but also their accumulation *in vivo*. This may be of potential benefit in diseases where eosinophils play a major pathophysiological role.

Another putative site for the inhibitory action of rolipram is the endothelial cell. Analysis of the PDE isoenzyme profile of endothelial cells from different species has shown that these cells possess mainly types II, III and IV isoenzymes (Suttorp *et al.*, 1993). Whether inhibition of PDE type IV isoenzymes in endothelial cells can inhibit the transmigration of leukocytes *in vitro* or into inflamed tissues *in vivo* is presently unknown, but deserves further investigation. Nevertheless, it has been shown previously that increased levels of cyclic AMP in endothelial cells may differentially affect the expression of adhesion molecules (Pober *et al.*, 1993) which could be translated into decreased or preferential accumulation of leukocytes within a given tissue. We are at present investigating this hypothesis.

Human neutrophils possess a PDE type IV isoenzyme which accounts for most of the nucleotide hydrolytic activity in these cells (Nielson *et al.*, 1990; Schudt *et al.*, 1991). As found with eosinophils, human neutrophil activity is also suppressed *in vitro* by inhibitors of PDE type IV such as rolipram (Nielson *et al.*, 1990). However, in the present study systemic rolipram had no effect on the accumulation of $^{111}$In-neutrophils induced by different inflammatory stimuli in guinea-pig skin. This is in accordance with a recent abstract by Boucheron *et al.* (1991) who showed that guinea-pig neutrophil function is less inhibited by PDE inhibitors than the human neutrophil. In the same studies however, rolipram effectively inhibited cyclic nucleotide hydrolytic activity in cell lysates (Boucheron *et al.*, 1991). Thus, it is possible that rolipram does not have access to neutrophil PDE enzymes or that cyclic AMP turnover in these cells is low and a stimulus which activates adenylate cyclase is also necessary (Boucheron *et al.*, 1991). The lack of effect of rolipram on $^{111}$In-neutrophil accumulation does not exclude the endothelial cell as its main cellular target. In fact, agents which increase cyclic AMP levels in endothelial cells have been shown to inhibit preferentially the expression of VCAM-1 (the ligand for VLA-4, an integrin present on eosinophils but not neutrophils) but not ICAM-1 *in vitro* (Pober *et al.*, 1993). Interestingly, we have shown that other agents such as prostaglandins of the E series and isoprenaline which may enhance cyclic AMP *in vivo* inhibit $^{111}$In-eosinophil accumulation but not $^{111}$In-neutrophil accumulation (Teixeira *et al.*, 1993a).

Microvascular leakage of plasma protein in the hamster cheek pouch and in the guinea-pig lung was attenuated by inhibition of PDE types III or IV and PDE type IV (Raeburn *et al.*, 1991; Ortiz *et al.*, 1992; Svensjo *et al.*, 1992). In contrast, in the present study we found no inhibitory effect on local oedema formation in guinea-pig skin with any of the PDE inhibitors used. This discrepancy may relate, in part, to methodological differences since in the guinea-pig lung studies, leakage of plasma protein was measured either 5 or 10 min after PAF challenge whereas we measured local oedema formation after 2 h. Experiments *in vitro* using porcine endothelial cells have established a role for PDE types III and IV in protecting against $H_2O_2$-induced increased vascular permeability (Suttorp *et al.*, 1993). Endothelial cells also possess a PDE type II isoenzyme (Suttorp *et al.*, 1993). However, there is no inhibitor of this enzyme available at the moment. We are not aware of studies on the PDE isoenzyme profile of guinea-pig endothelial cells, but it is possible that a type II isoenzyme may account for a significant portion of cyclic nucleotide hydrolytic activity as it does in porcine endothelial cells (Suttorp *et al.*, 1993). Alternatively, inhibition of only PDE type III or IV separately may be insufficient to inhibit increases in vascular permeability and addition of both inhibitors may be necessary (as occurs in bronchial smooth muscle; Torphy *et al.*, 1993).

The importance of the eosinophil for the pathophysiology of allergic diseases such as asthma is well recognized (Djukanovic *et al.*, 1990). However, it is not known if drugs which inhibit eosinophil accumulation and/or function will be useful in the treatment of these diseases. Also unknown is the relevance of PDE inhibition for asthma treatment, even though at least some of the useful effects of theophylline may be mediated in this way (Kuehl *et al.*, 1987). The observation that rolipram can mimic the inhibitory effects of theophylline in this model is further evidence that at least some of the anti-inflammatory properties of theophylline are mediated via PDE inhibition. In addition, it suggests that PDE type IV inhibitors may also mimic the useful effects of theophylline in allergic diseases such as asthma. Interestingly, atopic subjects may have higher levels of PDE activity in their inflammatory cells (Hanifin & Chan, 1988; Townley, 1993) and this may contribute to altered inflammatory cell function in asthma (Morley, 1993). The suppressive activity of PDE type IV inhibitors on eosinophil accumulation shown in this and other recent studies (Griswold *et al.*, 1993; Newsholme & Schwartz, 1993; Underwood *et al.*, 1993) and on eosinophil function (Souness *et al.*, 1991; Dent *et al.*, 1991) warrant a search for better and safer drugs which could be tested in human diseases. Furthermore, $^{111}$In-eosinophil accumulation in the guinea-pig skin is a useful model for testing the effects of putative PDE type IV inhibitors *in vivo*.

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# Effect of potassium channel blockade and $\alpha_2$-adrenoceptor activation on the release of nitric oxide from non-adrenergic non-cholinergic nerves

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**1** Using a superfusion bioassay cascade, we studied the effect of $K^+$ channel blockers and $\alpha_2$-adrenoceptor agents on the release of a transferable factor, previously characterized as nitric oxide (NO) or a nitric oxide-related substance (NO-R), in response to non-adrenergic non-cholinergic (NANC) nerve stimulation in the canine ileocolonic junction (ICJ).

**2** The non-selective $K^+$ channel blockers, 4-aminopyridine (4-AP, 50 μM) and tetraethylammonium (TEA, 1 mM) and the more selective blocker of $Ca^{2+}$-activated $K^+$ channels, charybdotoxin (*Leiurus quinquestriatus* venom (LQV), 0.4 μg $ml^{-1}$), significantly enhanced the release of NO-R induced by low frequency stimulation (2–4 Hz). In the presence of 4-AP and TEA, the release of NO-R was nearly abolished by tetrodotoxin (2 μM), and by L-$N^G$-nitroarginine (L-NOARG, 0.1 mM). Relaxations induced by direct injection of exogenous NO (5–50 pmol) or nitroglycerin (GTN, 10–30 pmol) onto the rabbit aortic detector ring were not affected.

**3** The $\alpha_2$-adrenoceptor agonist, UK-14,304 (0.3 μM) inhibited the release of NO-R induced by low (2–4 Hz), but not that induced by high (16 Hz), frequency stimulation. This inhibitory effect was completely reversed by the $\alpha_2$-adrenoceptor antagonist, yohimbine (0.3 μM). Neither UK-14,304 nor yohimbine affected the relaxations induced by exogenous NO (5 pmol) or GTN (10 pmol) on the aortic detector ring.

**4** On the other hand, in the presence of the $K^+$ channel blockers 4-AP (50 μM) or charybdotoxin (LQV, 0.4 μg $ml^{-1}$), UK-14,304 (0.3 μM) failed to inhibit the electrically-induced release of NO-R.

**5** From these results, we conclude that the electrically-induced release of NO-R from NANC nerves of the canine ICJ is enhanced by $K^+$ channel blockers but inhibited by $\alpha_2$-adrenoceptor activation. In addition, these results suggest that the prejunctional modulation of NO-R release by $\alpha_2$-adrenoceptors may involve neuronal $K^+$ channels.

**Keywords:** $\alpha_2$-adrenoceptors; bioassay; ileocolonic junction; $K^+$ channels; NANC neurotransmission; nitric oxide release; prejunctional modulation

## Introduction

Since nitric oxide (NO), or a NO related substance (NO-R), has been proposed to be involved in inhibitory non-adrenergic non-cholinergic (NANC) neurotransmission in the gastrointestinal tract (Boeckxstaens *et al.*, 1990a; Bult *et al.*, 1990), evidence has been accumulating demonstrating the importance of this nitrergic innervation in the control of gastrointestinal motility and its role in gastrointestinal diseases (for review; see Sanders & Ward, 1992; Stark & Szurszewski, 1992). As motility patterns represent the final outcome of a coordinated interaction between contractile and relaxant events, communication between excitatory and inhibitory neurones is of fundamental importance. One such mechanism of neuronal interaction is represented by prejunctional modulation of neurotransmitter release, which is, for example, responsible for the negative feedback of noradrenaline release via prejunctional $\alpha_2$-adrenoceptors (Langer, 1981).

Although the involvement of $\alpha_2$-adrenoceptors in the prejunctional modulation of NANC nerves has been reported (Fontaine *et al.*, 1984; Kojima *et al.*, 1988; MacDonald *et al.*, 1990), evidence that such a mechanism also modulates the nitrergic innervation is still sparse (Lefebvre & Smits, 1992; Boeckxstaens *et al.*, 1993). We previously demonstrated that the canine ileocolonic junction (ICJ) is a good model to study the nitrergic innervation and eliminated adenosine 5'-triphosphate and vasoactive intestinal polypeptide as mediators of the NANC relaxations in this tissue (Boeckxstaens *et al.*, 1990b,d) indicating that in the canine ICJ, NO or NO-R represents the final inhibitory NANC neurotransmitter (Boeckxstaens *et al.*, 1990a,c; Bult *et al.*, 1990). Recently, we demonstrated that activation of $\alpha_2$-adrenoceptors by UK-14,304 or clonidine reduced the nitrergic relaxations induced by NANC nerve stimulation. This inhibitory effect was antagonized by the specific $\alpha_2$-adrenoceptor antagonist, yohimbine, suggesting a possible prejunctional adrenergic modulation of NO-R release (Boeckxstaens *et al.*, 1993). In addition, it has been suggested that activation of prejunctional $\alpha_2$-adrenoceptors increases specific $K^+$ currents in the nerve terminal, thereby decreasing the duration of the action potential and consequently reducing the amount of neurotransmitter release (Illes, 1986; Miller, 1990). Based on this knowledge, we also studied the effect of $K^+$ channel blockers on the nitrergic relaxations and provided pharmacological evidence for the presence of prejunctional $K^+$ channels modulating the nitrergic innervation of the canine ICJ (De Man *et al.*, 1993); blockade of $K^+$ channels by 4-aminopyridine (4-AP), tetraethylammonium (TEA) or charybdotoxin significantly enhanced the nerve-mediated nitrergic relaxations without affecting the postjunctional response to NO or nitroglycerin. Similar to the prejunctional effect of $\alpha_2$-adrenoceptor activation, these results indirectly suggested a prejunctional effect of the blockers of $K^+$ channels on the NO-R release. In order to confirm these findings, the present study was designed to investigate the effect of both $\alpha_2$-adrenoceptor activation and

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$K^+$ channel blockade on the actual release of NO-R, measured by bioassay. Furthermore, we studied the possible involvement of the $K^+$ channels in the $\alpha_2$-adrenoceptor-mediated modulation of the NO-R release.

## Methods

### *Tissue preparation*

*Donor tissue* Mongrel dogs of either sex (weight 10–30 kg) were anaesthetized with sodium pentobarbitone (30 mg $kg^{-1}$, i.v.) and bled to death. A laparotomy was performed and the ileum was resected 5 cm above and 2 cm below the ileocolonic junction. After removal of the mucosa and the submucosa, a circular muscle strip was mounted in a superfusion chamber with a volume of about 0.5 ml and perfused at 3 ml $min^{-1}$ with a modified Krebs-Ringer solution (composition in mM: NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, CaEDTA 0.026 and glucose 11.1), containing superoxide dismutase (20 u $ml^{-1}$), guanethidine (3 μM) and L-arginine (50 μM). The solution was aerated with a mixture of 80% $N_2$, 15% $O_2$ and 5% $CO_2$. The perfusion chamber contained two platinum ring electrodes through which the muscle strip was pulled (Boeckxstaens *et al.*, 1991). Electrical pulses (rectangular waves, 2–16 Hz, 2 ms) were delivered by a Grass stimulator and a direct current amplifier in pulse trains of 20 s with an interval of at least 10 min, resulting in reproducible responses.

*Detector tissue* The effluent of the superfusion tube then superfused a New Zealand rabbit abdominal aortic ring (2 mm wide) which was denuded of its endothelium by gentle rubbing and contracted by infusion of noradrenaline (0.1 μM). The rabbit aortic ring was connected to a strain gauge transducer (Statham UC2) for continuous measurement of isometric tension. A bolus of acetylcholine (3 nmol) was injected directly onto the rabbit aortic ring to verify the absence of endothelium. Subsequently, atropine (0.3 μM) was introduced into the Krebs-Ringer solution. The sensitivity of the aortic ring was standardized by bolus injections of nitroglycerin (GTN, 10 pmol). The muscle strip was then allowed to equilibrate for at least 30 min before experimentation.

### *Experimental protocols*

In a first series of experiments, the effect of the $K^+$ channel blockers 4-aminopyridine (4-AP, 50 μM), tetraethylammonium (TEA, 1 mM) and charybdotoxin (*Leiurus quinquestriatus* venom (LQV), 0.4 μg $ml^{-1}$) was investigated on the electrically (2–16 Hz, 2 ms)-induced release of the nitric oxide related substance (NO-R) and on the relaxations to a bolus injection of exogenous NO (5–50 pmol) and GTN (10–30 pmol) directly onto the aortic ring. These experiments were repeated in the presence of the blocker of NO biosynthesis, L-$N^G$-nitro-arginine (L-NOARG, 0.1 mM) and of the blocker of neuronal conductance, tetrodotoxin (TTX, 2 μM).

In a second series of experiments, we evaluated the effect of the $\alpha_2$-adrenoceptor-agonist UK-14,304 (0.3 μM), in the presence and absence of the $\alpha_2$-adrenoceptor antagonist, yohimbine (0.3 μM), on the electrically (4–16 Hz, 2 ms)-induced release of NO-R and on the relaxations to a bolus injection of NO (5 pmol) and GTN (10 pmol).

Finally, the effect of UK-14,304 (0.3 μM) was re-evaluated in the presence of the $K^+$ channel blocker 4-AP (50 μM) or charybdotoxin (LQV, 0.4 μg $ml^{-1}$).

### *Drugs used*

The following drugs were used: acetylcholine chloride, 4-aminopyridine, L-arginine, charybdotoxin (*Leiurus quinquestriatus* venom, LQV), L-$N^G$-nitro-arginine, tetraethylammonium chloride, yohimbine hydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.); atropine sulphate (Federa, Brussels, Belgium); guanethidine monosulphate (Ciba-Geigy, Switzerland); tetrodotoxin (Janssen Chimica, Beerse, Belgium); nitroglycerin (Merck, Darmstadt, Germany); noradrenaline hydrogentartrate (Fluka AG, Buchs, SG, Switzerland). UK-14,304 tartrate (5-bromo-6-[2 imidazolin-2-ylamino]-quinoxaline) was a gift of Pfizer Central Research (Sandwich, England) and bovine recombinant superoxide dismutase was a gift of Grünenthal GmbH (Aachen, Germany).

All drugs were administered as aqueous solutions. Noradrenaline was dissolved in 0.57 mM ascorbic acid. All solutions were prepared on the day of experimentation. TTX and charybdotoxin were stored in small amounts in stock-vials at −20°C. Charybdotoxin was used as the whole venom of *Leiurus quinquestriatus* (Miller *et al.*, 1985). Solutions of NO were prepared as described (Kelm *et al.*, 1988). All drugs were allowed to incubate at least 15 min prior to each stimulation.

### *Presentation of results and statistical analysis*

The results are expressed as percentage decrease of the noradrenaline-induced contraction of the rabbit aortic ring. Values are shown as mean ± s.e.mean for the number of dogs indicated. For statistical analysis, a Student's two tailed *t* test for paired observations was used. *P* values of less than 0.05 were considered to be significant.

## Results

### *NO-R release in response to electrical stimulation*

In the presence of atropine (0.3 μM), guanethidine (3 μM), L-arginine (50 μM) and superoxide dismutase (20 u $ml^{-1}$), electrical stimulation (2–16 Hz, 2 ms) of the canine ICJ induced the release of a labile transferable factor, previously characterized as NO-R (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1991), that relaxed the rabbit aorta (2 Hz: 0.7 ± 0.4%, 4 Hz: 4.8 ± 1.4% and 16 Hz: 28.7 ± 4.4% of the noradrenaline-induced contraction) (Figure 1). Direct injection of exogenous NO (5 pmol) or GTN (10 pmol) on the rabbit aortic detector tissue induced similar relaxations (NO: 6.9 ± 1.4%; GTN: 7.0 ± 1.5% of the noradrenaline-induced contraction).

### *Effect of $K^+$ channel blockers on NO-R release*

The release of NO-R, induced by low (2 and 4 Hz), but not by high (16 Hz) frequency stimulation of the canine ICJ was significantly enhanced by the non-selective $K^+$ channel blockers 4-AP (50 μM), TEA (1 mM) and by the more selective large conductance $Ca^{2+}$-activated $K^+$ channel blocker, charybdotoxin (LQV, 0.4 μg $ml^{-1}$). The relaxations to exogenous NO (5 pmol) or GTN (10 pmol), with amplitudes comparable to that induced by 4 Hz stimulation of the ICJ, were not affected by the $K^+$ channel blockers (Figure 1).

### *Effect of TTX and L-NOARG on NO-R release in the presence of $K^+$ channel blockers*

In the presence of 4-AP (50 μM) or TEA (1 mM), the release of NO-R, induced by electrical stimulation (2–16 Hz, 2 ms) of the canine ICJ, was almost completely abolished by TTX (2 μM) or L-NOARG (0.1 mM) (Figure 2). The relaxations of comparable amplitude induced by direct injection of NO (50 pmol) or GTN (30 pmol) on the rabbit aortic ring were not affected by TTX or L-NOARG (Figure 2).

### *Effect of UK-14,304 and yohimbine on NO-R release*

Infusion of UK-14,304 (0.3 μM) inhibited the release of NO-R from the canine ICJ: the relaxation of the rabbit aorta in response to the electrically-induced release of NO-R, was

inhibited from 4.4 ± 0.5% to 0% at 4 Hz ($n = 4$) and from 15.4 ± 1.3% to 6.1 ± 1.1% at 8 Hz ($n = 4$) (Figure 3). The inhibitory effect of UK-14,304 was completely reversed by yohimbine (0.3 μM) (Figure 3), whereas infusion of yohimbine (0.3 μM) alone had no effect on these relaxations (results not shown). Relaxations of the same amplitude induced by direct injection of NO (5 pmol) or GTN (10 pmol) on the rabbit aortic ring and that induced by 16 Hz stimulation of the ICJ were not affected by UK-14,304 (0.3 μM) or by UK-14,304 (0.3 μM) plus yohimbine (0.3 μM) (Figure 3).

### *Effect of $K^+$ channel blockers on the inhibition of NO-R release by UK-14,304*

As described above, the release of NO-R was significantly increased by 4-AP (50 μM) and charybdotoxin (LQV, 0.4 μg $ml^{-1}$), especially the release induced by low frequency stimulation. In the presence of these $K^+$ channel blockers, UK-14,304 now failed to inhibit the NO-R release, even at low frequency stimulation (2–4 Hz, 2 ms) (Figure 4), whereas none of these drugs affected the sensitivity of the rabbit aortic ring, as illustrated by the lack of effect on the response to NO (5 pmol) and GTN (10 pmol) (Figure 4).

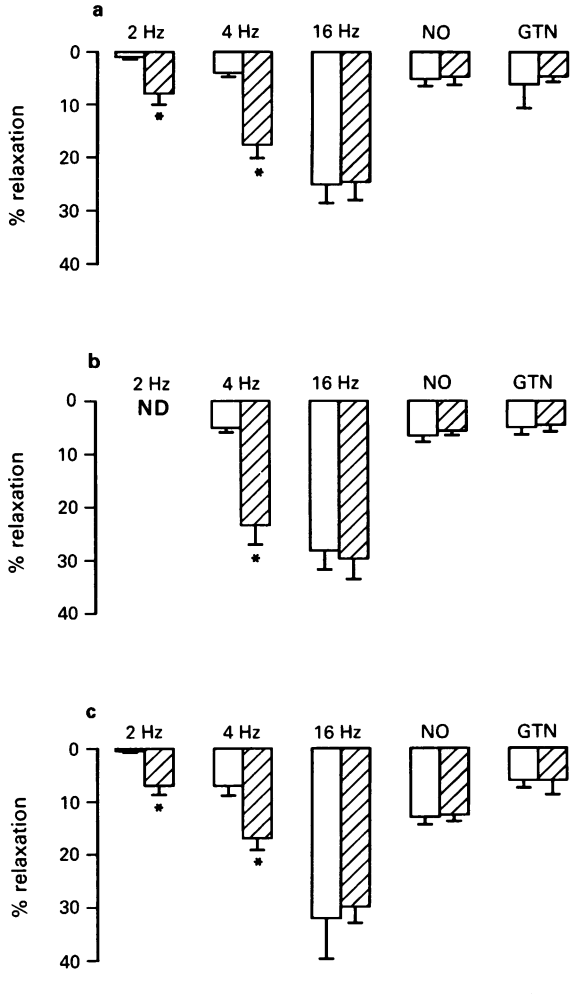


**Figure 1** Effect of (a) 4-aminopyridine (4-AP, 50 μM), (b) tetroethylammonium (TEA, 1 mM) and (c) charybdotoxin (LQV, 0.4 μg $ml^{-1}$) on the relaxations of the rabbit aorta in response to the release of NO-R induced by electrical stimulation (2–16 Hz, 2 ms) of the canine ICJ and to bolus injections of NO (5 pmol) and nitroglycerin (GTN, 10 pmol) directly onto the rabbit aorta. The open columns represent the results obtained in control conditions, the hatched columns represent those obtained in the presence of the respective drugs. Results are expressed as percentage decrease of the noradrenaline (0.1 μM)-induced contraction and shown as mean ± s.e.mean for $n = 4–11$ experiments. $*P < 0.05$, significantly different from control, Student's $t$ test for paired observations. ND = not done.

## Discussion

Prejunctional receptors, located on adrenergic, cholinergic and NANC axon terminals, are involved in the prejunctional modulation of neurotransmitter release (for references, see Introduction). In addition, there is growing evidence that these receptors are coupled to neuronal ion channels, indicating that these ion channels may be an important target for prejunctional modulation of neurotransmitter release (Illes, 1986; Belardetti & Siegelbaum, 1988; Miller, 1990). In the present study, we demonstrated that the nitrergic innervation of the canine ICJ is subject to both mechanisms of prejunctional modulation: $\alpha_2$-adrenoceptor activation reduced the NO-R release in response to NANC nerve stimulation,

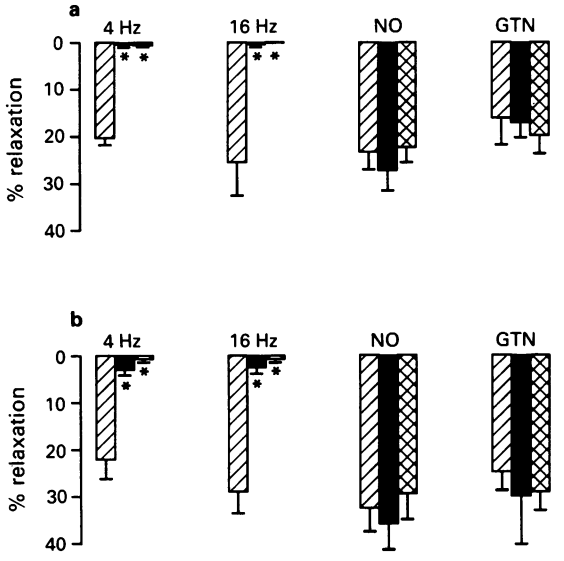


**Figure 2** Effect of tetrodotoxin (2 μM, solid columns) and L-$N^G$-nitro-arginine (L-NOARG, 0.1 mM, cross hatched columns) on the relaxations of the rabbit aorta in response to the release of NO-R induced by electrical stimulation (4–16 Hz, 2 ms, hatched columns) of the canine ICJ and to bolus injections of NO (5 pmol, hatched columns) and nitroglycerin (GTN, 30 pmol, hatched columns) directly onto the rabbit aorta in the presence of (a) 4-aminopyridine (4-AP, 50 μM) and (b) tetraethylammonium (TEA, 1 mM). Results are expressed as percentage decrease of the noradrenaline (0.1 μM)-induced contraction and shown as mean ± s.e.mean for $n = 4–6$ experiments. $*P < 0.05$, significantly different from control, Student's $t$ test for paired observations.

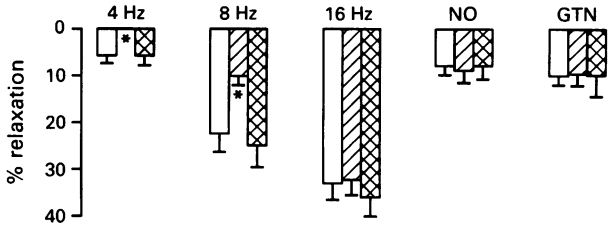


**Figure 3** Effect of UK-14,304 (0.3 μM, hatched columns) and UK-14,304 plus yohimbine (both 0.3 μM, cross hatched columns) on the relaxations of the rabbit aorta in response to the release of NO-R induced by electrical stimulation (4–16 Hz, 2 ms, open columns) of the canine ICJ and to bolus injections of NO (5 pmol, open columns) and nitroglycerin (GTN, 10 pmol, open columns) directly onto the rabbit aorta. Results are expressed as percentage decrease of the noradrenaline (0.1 μM)-induced contraction and shown as mean ± s.e.mean for $n = 4–6$ experiments. $*P < 0.05$, significantly different from control, Student's $t$ test for paired observations.

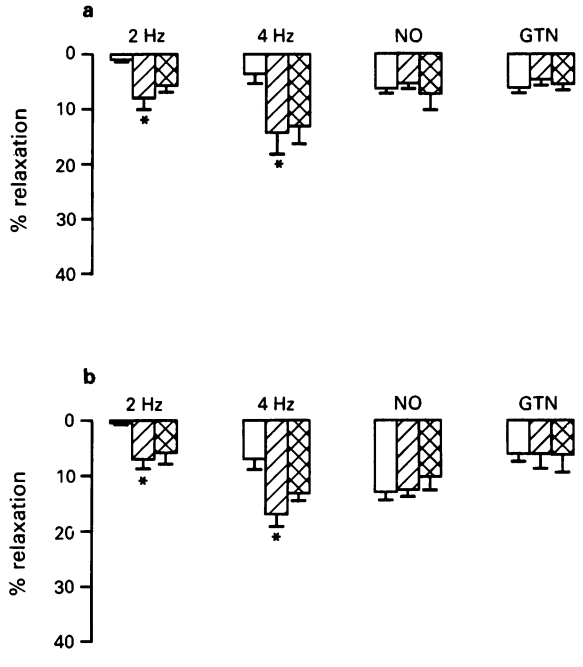


**Figure 4** Relaxations of the rabbit aorta in response to the release of NO-R induced by electrical stimulation (2–4 Hz, 2 ms) of the canine ICJ and to bolus injections of NO (5 pmol) and nitroglycerin (GTN, 10 pmol) in control solution (open columns) and in the presence of (a) 4-aminopyridine (4-AP, 50 μM, hatched columns) or (b) charybdotoxin (LQV, 0.4 μg ml$^{-1}$, hatched columns) alone or in combination with UK-14,304 (0.3 μM) (cross hatched columns). Results are expressed as percentage decrease of the noradrenaline (0.1 μM)-induced contraction and shown as mean ± s.e.mean for $n = 4-6$ experiments. *$P < 0.05$, significantly different from control, Student's $t$ test for paired observations.

whereas blockade of $K^+$ channels increased the NO-R release measured by bioassay. Furthermore, we provided evidence suggesting that the $\alpha_2$-adrenoceptor-mediated modulation of NO-R release in the canine ICJ is coupled to prejunctional $K^+$ channels.

Prejunctional $\alpha_2$-adrenoceptors modulate NANC neurotransmission in various tissues (Fontaine *et al.*, 1984; Kojima *et al.*, 1988; MacDonald *et al.*, 1990; Lefebvre & Smits, 1992), including the canine ICJ. We recently reported that the $\alpha_2$-adrenoceptor agonists, UK-14,304 and clonidine, inhibited electrically-induced nitrergic NANC relaxations without affecting the postjunctional response to NO (Boeckxstaens *et al.*, 1993). In the present study, we also demonstrated that the release of NO-R measured by bioassay, was inhibited by UK-14,304 confirming that the NO-R release, induced by NANC nerve stimulation in the canine ICJ, is indeed subject to $\alpha_2$-adrenoceptor modulation. This inhibitory effect of UK-14,304 was specific since it was completely reversed by the $\alpha_2$-adrenoceptor antagonist, yohimbine. Furthermore, the reactivity of the detector tissue to authentic NO or GTN was not affected by UK-14,304, indicating that the inhibitory effect of UK-14,304 resulted from a decrease in the NO-R release. On the other hand, infusion of yohimbine alone had no effect on the NO-R release. This can be explained by the fact that all experiments were performed in the presence of guanethidine, blocking adrenergic neurotransmission. As such, we cannot determine whether the $\alpha_2$-adrenoceptors on the nitrergic nerves of the canine ICJ are activated by noradrenaline released from adjacent sympathetic nerves. Similarly, in the rat gastric fundus, it was reported that the $\alpha_2$-adrenoceptor antagonist, idazoxan, did not affect NANC nerve-mediated responses, even in the absence of guanethidine (MacDonald *et al.*, 1990). These authors suggested that $\alpha_2$-adrenoceptors, present on NANC nerves of the rat gastric fundus, are inaccessible to noradrenaline released from sympathetic neurones in the myenteric plexus. However, it may be that these receptors can be activated by circulating catecholamines, possibly explaining the motility disturbances observed in stressful situations.

It is believed that receptor-mediated modulation of neurotransmitter release results from the up-and down modulation of the activity of presynaptic ion channels, such as $K^+$ channels (for reviews see Illes, 1986; Belardetti & Siegelbaum, 1988; Westfall & Martin, 1991); $\alpha_2$-adrenoceptor activation is reported to increase $K^+$ conductances (Morita & North, 1981; Bauer, 1985; Zimanyi *et al.*, 1988; Zoltay & Cooper, 1990), thereby decreasing the duration of the action potential and thus reducing the amount of neurotransmitter release (Augustine, 1990). We previously reported that $K^+$ channel blockers enhanced the nitrergic relaxations induced by NANC nerve stimulation, without affecting the postjunctional responses, suggesting the presence of prejunctional $K^+$ channels modulating NO-R release (De Man *et al.*, 1993). By measuring the NO-R release in response to NANC nerve stimulation, we confirmed this hypothesis in the present study; the non-selective $K^+$ channel blockers, 4-AP and TEA and the blocker of large conductance $Ca^{2+}$-activated $K^+$ channels, charybdotoxin (*Leiurus quinquestriatus venom*, Miller *et al.*, 1985), significantly enhanced the release of NO-R. As described previously (De Man *et al.*, 1993), this effect was most pronounced on the release evoked by low frequency stimulation. The release of the transferable factor was almost completely blocked by inhibition of the L-arginine:NO pathway and by blockade of the neuronal conductance, indicating that the increase in vasorelaxant activity was due to the increase of NO-R release and not of another inhibitory neurotransmitter. In addition, the enhancement of the relaxations of the rabbit aorta, induced by electrical stimulation of the ICJ resulted from an increase of NO-R release and not from an altered reactivity of the rabbit aortic detector ring, as the responses of the aortic ring to exogenous NO or GTN were not affected by the $K^+$ channel blockers.

In order to investigate whether these neuronal $K^+$ channels are involved in the $\alpha_2$-adrenoceptor-mediated inhibition of the NO-R release, we also studied the effect of the $\alpha_2$-adrenoceptor agonist, UK-14,304 during $K^+$ channel blockade. In the presence of $K^+$ channel blockers, UK-14,304 failed to inhibit the NO-R release, even that induced by low frequency stimulation. This might be the result of a non-selective effect, as high concentrations of 4-AP (>1 mM) and TEA (>10 mM) were reported to interfere with the binding of ligands to $\alpha_2$-adrenoceptors (Drukarch *et al.*, 1989). However, in the present study, lower concentrations were used and furthermore, charybdotoxin, devoid of such non-selective effects, prevented the inhibitory effect of UK-14,304, excluding this possibility. Therefore, these results suggest that the prejunctional $\alpha_2$-adrenoceptor-mediated inhibition of NO-R release results from an increase in $K^+$ conductance. However, whether this $K^+$ conductance directly regulates neurotransmitter release by affecting the duration of the action potential (Augustine, 1990) or indirectly e.g. by reducing the efficacy of intracellular $Ca^{2+}$ binding (Schoffelmeer & Mulder, 1983) remains to be investigated.

In conclusion, using a bioassay, we demonstrated that the release of NO-R, induced by electrical stimulation of NANC nerves of the canine ICJ, is inhibited by activation of prejunctional $\alpha_2$-adrenoceptors but potentiated by blockade of prejunctional $K^+$ channels. These results confirm that both $\alpha_2$-adrenoceptors and $K^+$ channels prejunctionally modulate NO-R release from nitrergic NANC nerves in the canine ICJ (Boeckxstaens *et al.*, 1993; De Man *et al.*, 1993). Furthermore, blockade of prejunctional $K^+$ channels prevented the $\alpha_2$-adrenoceptor-mediated inhibition of NO-R release suggesting that this inhibition may result from the activation of neuronal $K^+$ channels.

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