



Inhibition of endothelium-dependent hyperpolarization by endothelial prostanoids in guinea-pig coronary artery

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1 In smooth muscle of the circumflex coronary artery of guinea-pig, acetylcholine (ACh, 10^{-6} M) produced an endothelium-dependent hyperpolarization consisting of two components. An initial component that occurs in the presence of ACh and a slow component that developed after ACh had been withdrawn. Each component of the hyperpolarization was accompanied by an increase in membrane conductance.

2 Indomethacin (5×10^{-6} M) or diclofenac (10^{-6} M), both inhibitors of cyclooxygenase, abolished only the slow hyperpolarization. The initial hyperpolarization was not inhibited by diclofenac nor by nitroarginine, an inhibitor of nitric oxide synthase.

3 Both components of the ACh-induced hyperpolarization were abolished in the presence of atropine (10^{-6} M) or high-K solution ($[K^+]_0 = 29.4$ mM).

4 The interval between ACh-stimulation required to generate an initial hyperpolarization of reproducible amplitude was 20 min or greater, but it was reduced to less than 5 min after inhibiting cyclooxygenase activity. Conditioning stimulation of the artery with substance P (10^{-7} M) also caused a long duration (about 20 min) inhibition of the ACh-response.

5 The amplitude of the hyperpolarization generated by Y-26763, a K^+ -channel opener, was reproducible within 10 min after withdrawal of ACh.

6 Exogenously applied prostacyclin (PGI_2) hyperpolarized the membrane and reduced membrane resistance in concentrations over 2.8×10^{-9} M.

7 At concentrations below threshold for hyperpolarization and when no alteration of membrane resistance occurred, PGI_2 inhibited the initial component of the ACh-induced hyperpolarization.

8 It is concluded that endothelial prostanoids, possibly PGI_2 , have an inhibitory action on the release of endothelium-derived hyperpolarizing factor.

Keywords: PGI_2 ; EDHF; autoregulation; hyperpolarization; coronary artery; endothelium

Abbreviations: ACh, acetylcholine; ATP, adenosine triphosphate; EDH, endothelium-dependent hyperpolarization; EDHF, endothelium-derived hyperpolarizing factor; EDRF, endothelium-derived relaxing factor; NO, nitric oxide; PGI_2 , prostaglandin I_2 ; s.d., standard deviation; SP, substance P

Introduction

Endothelial factors such as endothelium-derived relaxing factor (EDRF), endothelium-derived hyperpolarizing factor (EDHF) or prostacyclin (PGI_2) are involved in many agonist-induced vasodilatations, and contribution of these factors in the dilatation differ between agonists and vascular beds (Furchgott, 1984; Vanhoutte *et al.*, 1986; Suzuki & Chen, 1990; Garland *et al.*, 1995). EDRF may be nitric oxide (NO) or a related NO-containing substance and this factor dilates vascular smooth muscle mainly by an increase in production of cyclic GMP through activation of guanylate cyclase (Ignarro *et al.*, 1987; Moncada *et al.*, 1991). This factor has been reported to activate Ca^{2+} -sensitive K^+ -channels of smooth muscle cells in the rabbit aorta (Bolotina *et al.*, 1994) or glibenclamide sensitive K^+ -channels in the rabbit mesenteric artery (Murphy & Brayden, 1995). The chemical nature of EDHF remains unclear; in some arteries, this factor may be a metabolite of arachidonic acid produced through epoxigenase pathways with activation of enzyme cytochrome P450 mono-oxygenase (Hecker *et al.*, 1994; Campbell *et al.*, 1996; Popp *et al.*, 1996; Chen & Cheung, 1996), but in others this is not the

case (Corriu *et al.*, 1996; Vanheel & Van de Voorde, 1997; Chataigneau *et al.*, 1998). EDHF hyperpolarizes the membrane by activating charybdotoxin-sensitive K^+ -channels (Hashitani & Suzuki, 1997) and induces vasodilatation by inhibiting the influx of Ca^{2+} through voltage-sensitive Ca^{2+} -channels (Nelson *et al.*, 1990) or the production of second messenger inositol trisphosphate (Itoh *et al.*, 1992). PGI_2 increases production of cyclic AMP to induce vasodilatation (Gryglewski *et al.*, 1991) and evokes membrane hyperpolarization sensitive to glibenclamide (Parkington *et al.*, 1995).

In the main descending coronary artery of guinea-pig, ACh hyperpolarizes the membrane in an endothelium-dependent manner with three components, i.e., EDRF-, EDHF- and PGI_2 -induced components, and the former and the latter components are inhibited by nitroarginine and indomethacin respectively (Parkington *et al.*, 1993). It remains unclear how these endothelial products interact to each other. In circumflex coronary artery of the guinea-pig, we found that PGI_2 has inhibitory effects on the release of EDHF, and at higher concentrations hyperpolarizes the smooth muscle membrane. Some of the data was reported briefly in the International Society for Autonomic Neuroscience at Cairns, Australia (Yajima *et al.*, 1997).

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Methods

Guinea-pigs of either sex, weighing 200–250 g, were killed by decapitation after asphyxiation with CO₂ gas, and the heart removed. Proximal segments (about 5 mm) of the circumflex coronary artery to the left atrium (outer diameter, 0.1–0.3 mm) were isolated from the heart which was immersed in the oxygenated Krebs solution. The artery was cleaned by removing the surrounding cardiac and connective tissues and mounted in an experimental chamber. The chamber was 8 mm wide, 8 mm deep and 3 cm long, with a rubber plate at the bottom. The artery was pinned down on the rubber plate whilst it was superfused for over 1 h before starting experiments with Krebs solution warmed to 35°C, at a constant flow rate of about 3 ml min⁻¹, so as to maintain the volume of the solution in the chamber at about 0.5 ml. In some vessels, the endothelial cells were damaged by the internal perfusion with distilled water for 15–20 min.

Artery was stimulated with ACh, usually for 1 min, by including ACh in the superfusing solution. Inhibitors of NO-synthase or cyclooxygenase were added to the superfusate 10 min before stimulation with ACh, during which time the membrane potential of the smooth muscle cells was recorded.

Conventional intracellular microelectrode techniques were used to measure the membrane potentials of smooth muscle cells in the coronary arteries. Briefly, a glass capillary microelectrode filled with 3 M KCl (tip resistance; 50–70 MΩ) was inserted into smooth muscle cells from the adventitial side of the vessel. Criteria for successful penetration of the electrode into a smooth muscle cell were a sharp deflection of potential to about -50 mV and the maintenance of a stable membrane potential of between -45 to -55 mV for over 3 min.

The end of an isolated vessel was drawn into a glass capillary with tip diameter of 100–200 μm and was stimulated by rectangular current pulses of 2–3 s duration and up to 0.64 mA intensity through the capillary. Electrotonic potentials thus produced by extracellular stimulation were recorded in close proximity to the edge of the capillary (usually less than 0.1 mm).

The ionic composition of the Krebs solution was as follows (with mM): Na⁺ 137.4, K⁺ 5.9, Ca²⁺ 2.5, Mg²⁺ 1.2, HCO₃⁻ 15.5, H₂PO₄⁻ 1.2, Cl⁻ 134 and glucose 11.5. Solution containing 29.4 mM [K⁺]₀ (high-K solution) was prepared by replacing NaCl with KCl. The solution was oxygenated (95% O₂, 5% CO₂) and had a pH of 7.2–7.3.

Drugs used were acetylcholine chloride (ACh), atropine sulphate, indomethacin and diclofenac sodium salt (all from Sigma, U.S.A.), substance P and N^ω-nitro-L-arginine (nitroarginine, Peptide Institute, Osaka, Japan), Y-26763 (Yoshitomi Pharmac., Osaka, Japan) and prostacyclin (PGI₂, Ono Pharmaceutical Ind., Ltd., Osaka, Japan). These drugs were dissolved in distilled water (ACh, atropine, diclofenac, substance P, nitroarginine), 95.5% ethanol (PGI₂), dimethyl sulphoxide (Y-26763) or 5 × 10⁻³ M Na₂CO₃ solution (indomethacin), and diluted further with Krebs solution to obtain desired concentrations. The final concentration of the solvent did not exceed 1:1000 in the Krebs solution. Addition of drugs or their vehicle to the Krebs solution did not alter the pH of the solution.

The measured values were expressed as the mean ± s.d.mean. Statistical significance was tested using Student's *t*-test, and probabilities of less than 5% (*P* < 0.05) were considered significant.

Results

Endothelium-dependent hyperpolarization produced by ACh

In smooth muscle cells of the circumflex coronary artery of the guinea-pig, the resting membrane potential was stable, ranging between -45 and -55 mV (-52.2 ± 3.5 mV, *n* = 52 arteries). Stimulation of the artery with acetylcholine (ACh, 10⁻⁶ M) for 1 min produced a hyperpolarization with two components. The hyperpolarization appeared during application of ACh (an initial hyperpolarization) and reached a peak amplitude ranging between 15 and 25 mV (19.5 ± 5.5 mV, *n* = 35 arteries) within 15–20 s. The amplitude of the initial hyperpolarization remained constant or often declined gradually by 10–30% during application of ACh. The initial hyperpolarization was followed by a slow hyperpolarization which appeared during the recovery process of the initial hyperpolarization and reached a peak amplitude of 1–8 mV (4.3 ± 1.2 mV, *n* = 35 arteries), 2–4 min after withdrawal of ACh. The duration of the slow hyperpolarization measured after withdrawal of ACh ranged between 5 and 8 min (Figure 1A). Both components of the ACh-induced hyperpolarization were absent after inhibiting muscarinic receptors with 10⁻⁶ M atropine (*n* = 5 arteries, data not shown).

The threshold concentrations of ACh required for generation of the initial and slow components of the hyperpolarization were 10⁻⁸ M and 10⁻⁷ M, respectively. The amplitude of each component of the hyperpolarization increased in a concentration-dependent manner to reach a maximum value of about 20 and 4 mV for the initial and slow hyperpolarizations, respectively, at 10⁻⁶ M (Nishiyama *et al.*, 1998). These properties of the hyperpolarization were essentially the same as those reported by Parkinson *et al.* (1993) in the left descending main coronary artery.

In intact arteries, application of diclofenac (10⁻⁶ M) for over 5 min abolished the slow component of the ACh-induced hyperpolarization, with no significant alteration of the membrane potential (-51.2 ± 2.8 mV, *n* = 15 arteries, *P* > 0.1) or the amplitude of the initial hyperpolarization (20.2 ± 3.8 mV, *n* = 10 arteries; *P* > 0.1) (Figure 1A and B). Indomethacin (5 × 10⁻⁶ M) had effects similar to diclofenac; that is, having no significant effect on the membrane potential (-51.8 ± 2.5 mV, *n* = 8 arteries, *P* > 0.1) or the amplitude of the initial hyperpolarization (19.6 ± 4.8 mV, *n* = 8 arteries, *P* > 0.1) and inhibiting only the slow component in the intact coronary artery (*n* = 8 arteries, see Figure 3). Nitroarginine (10⁻⁵ M) added in the presence of diclofenac did not further alter the membrane potential (-51.8 ± 2.1 mV, *n* = 12 arteries, *P* > 0.1) or the initial component of the hyperpolarization (20.1 ± 4.5 mV, *n* = 6 arteries; *P* > 0.1) (data not shown).

The lumen of the arteries were perfused with distilled water for 20–30 min to damage endothelial cells (four arteries). This procedure caused membrane depolarization by 10–15 mV (-40.5 ± 2.0 mV, *n* = 12 cells, *P* < 0.05), and the change in membrane potential produced by 10⁻⁶ M ACh was negligible (1–3 mV hyperpolarization, *n* = 3 arteries; 2.5 mV depolarization, *n* = 1 artery), suggesting that the endothelial cells were successfully damaged. The results indicate that the ACh-induced hyperpolarization was generated in an endothelium-dependent manner.

In two circumflex coronary arteries with endothelium intact, high-K solution ([K⁺]₀ = 29.4 mM) depolarized the membrane by about 15 mV (from -50.2 ± 1.5 mV, *n* = 5, to -34.4 ± 2.4 mV, *n* = 5), reduced the amplitude of initial component of the ACh-induced hyperpolarization to

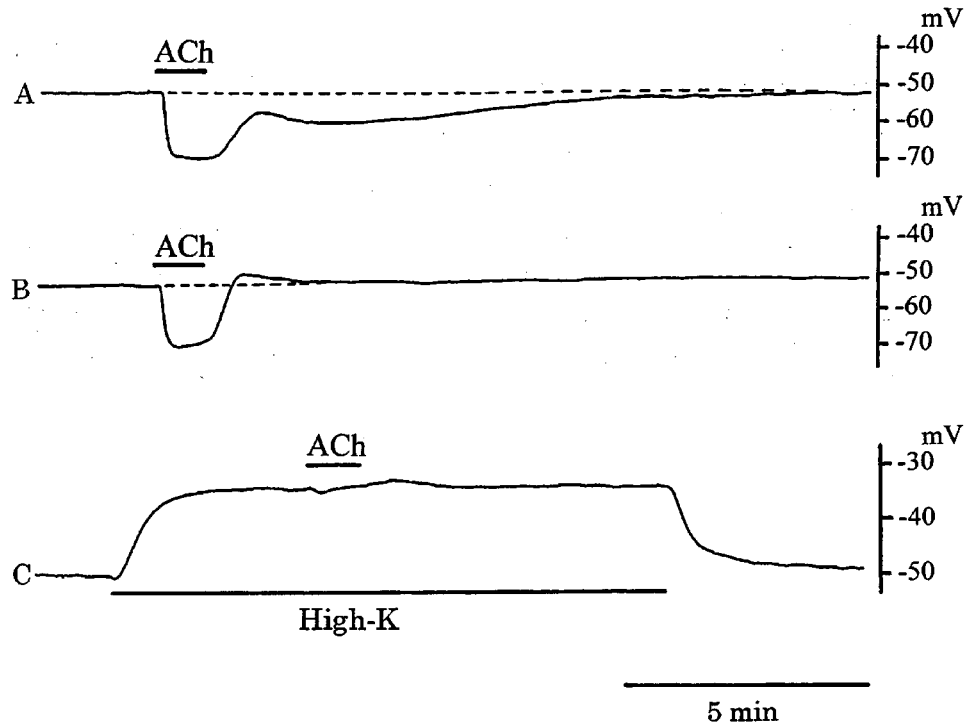


Figure 1 Inhibition by diclofenac of the slow component of the acetylcholine (ACh)-induced hyperpolarization. The circumflex coronary artery of the guinea-pig was stimulated with ACh (10^{-6} M) for 1 min before (A, control) and after application of 10^{-6} M diclofenac (B), or during depolarization with high-K solution ($[K^+]_0 = 29.4$ mM) (C). The dotted lines in A and B represent the level of the resting membrane potential. A, B and C were recorded from different tissues.

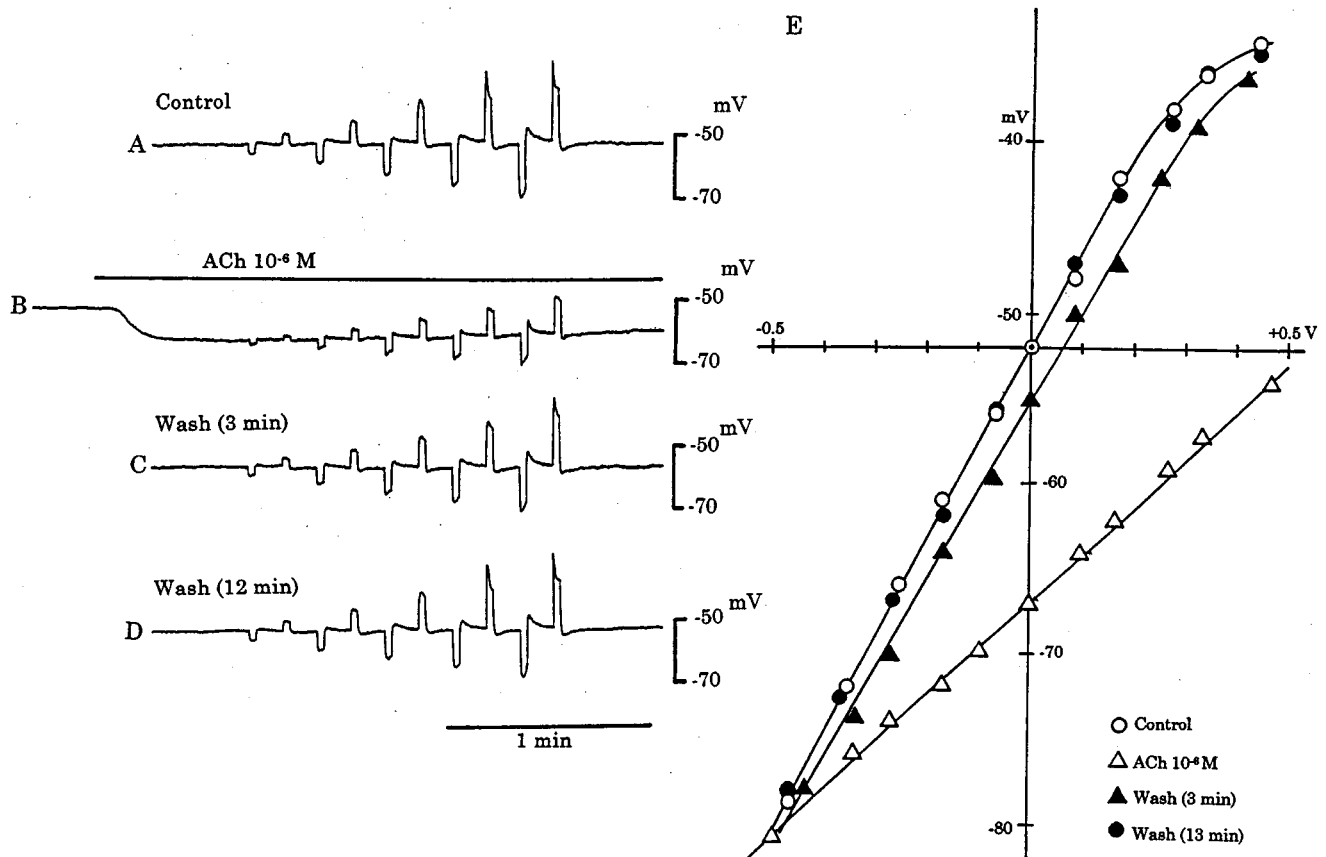


Figure 2 Reduction of electrotonic potentials during the acetylcholine (ACh)-induced hyperpolarization. A coronary artery was stimulated with five sets of rectangular depolarizing and hyperpolarizing current pulses of increasing intensities (2 s duration) at 10 s interval. Electrotonic potentials produced by these stimuli were recorded from the smooth muscle located 0.1 mm from the edge of the capillary before (A), during application of 10^{-6} M ACh (B), at 3 min (C) and 12 min (D) after removal of ACh. Intensity of stimuli for A–D: 0.1, 0.2, 0.31, 0.41 and 0.58 mA. (E) Current-voltage relationships recorded before (Control, ○), during application of 10^{-6} M ACh (△) and after washing ACh for 3 min (▲) and 13 min (●) from the smooth muscle. Amplitudes of electrotonic potentials were plotted as a function of the intensity of stimuli (positive value, outward current; negative value, inward current). All points were recorded from the same cell. A–D are continuous recordings. A–D and E were recorded from different tissues.

1.1 ± 0.1 mV ($n=5$) and abolished the slow component (Figure 1C).

Ionic conductance of the membrane during the ACh-induced hyperpolarization

Extracellular electrical stimulation of the coronary artery with rectangular current pulses (2 s duration, 0.06–0.64 mA intensity) evoked electrotonic potentials in smooth muscle cells located in close proximity (0.1–0.3 mm) to the edge of the suction electrode. In three arteries, the amplitude of the electrotonic potentials produced by alternate application of

inward and outward current pulses at constant intensity (0.1–0.3 mA) and frequency (0.1 Hz) was found to be reduced during the ACh-induced hyperpolarization, indicating that the ACh-induced hyperpolarization was produced by an increase in ionic conductance of the membrane.

The relative changes in membrane resistance during the ACh-induced hyperpolarization were calculated by applying the cable equation (Hodgkin & Rushton, 1946). Provided internal resistances including gap junctional resistance remain the same before and during the ACh-induced hyperpolarization and distance between stimulating and recording electrodes (less than 0.1 mm) is much smaller than the length constant of

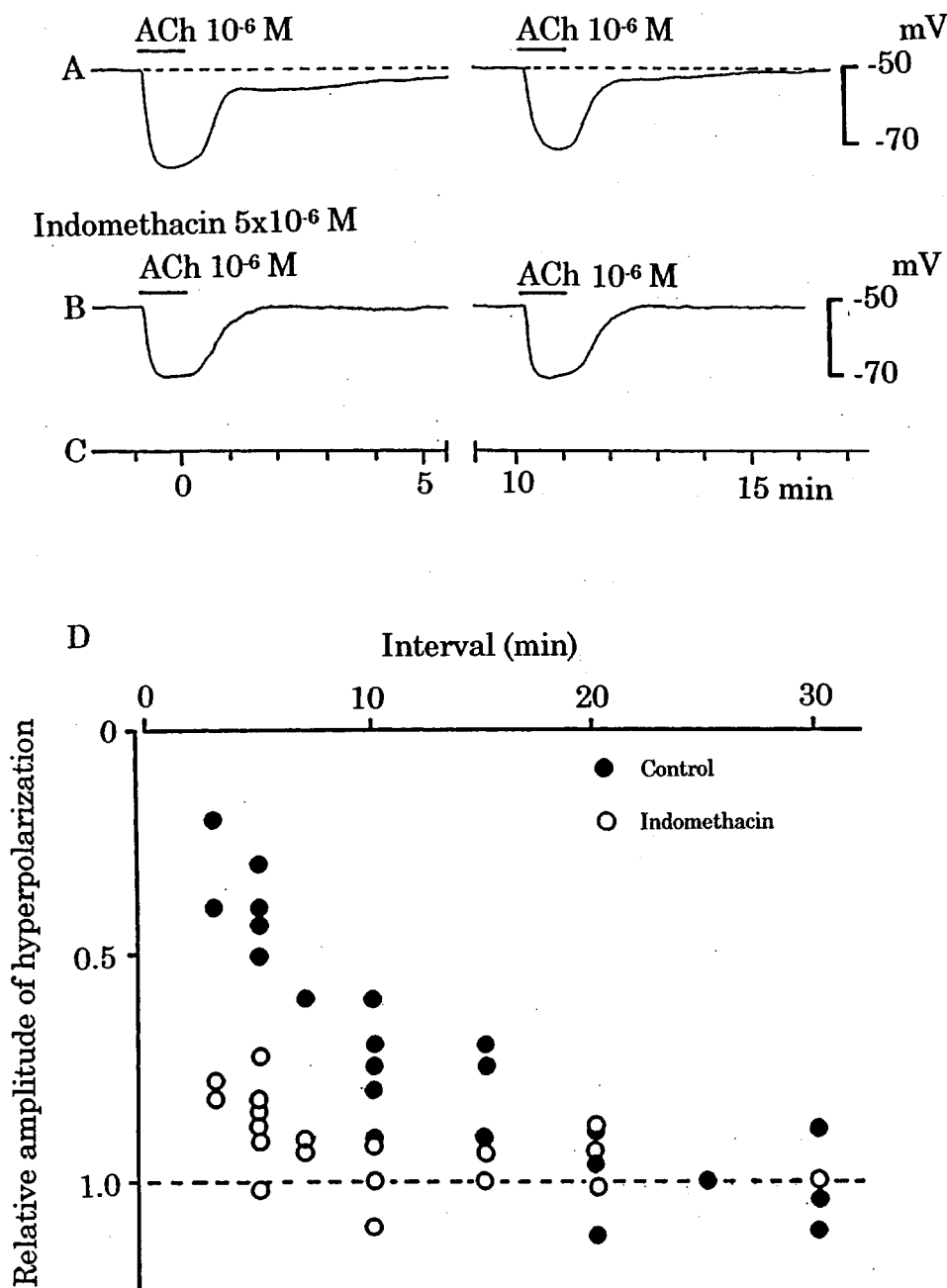


Figure 3 Diminished hyperpolarization produced by repeated application of acetylcholine (ACh) and its recovery by indomethacin. ACh (10^{-6} M) was applied for 1 min at 10 min interval, in the absence (A) and presence of 5×10^{-6} M indomethacin (B). (C) time scale in min with 0 starting at the time of withdrawal of the first ACh application. Dotted line in A, the resting potential level of -50 mV. Each pair of responses shown in A and B was recorded from single cells in different tissues. The lower graph (D) shows the relationship between the amplitude of hyperpolarization produced by the second ACh application (relative to the first ACh-response) and the interval between two ACh-stimuli, measured in the absence (●) and presence (○) of 5×10^{-6} M indomethacin. Data obtained from five tissues were plotted.

the tissue (assuming about 1.0 mm), then the ratio of the amplitude of electrotonic potential $(V'/V)^2 \div \gamma_m'/\gamma_m$ (Suzuki & Kuriyama, 1975), where V and V' or r_m and r_m' are the amplitude of the electrotonic potentials produced by a given intensity of current or the membrane resistances at rest and during the ACh-induced hyperpolarization, respectively. The changes in membrane resistance thus calculated were 0.29 ± 0.11 ($n=4$) for the initial component and 0.71 ± 0.20 ($n=4$) for the slow component of the ACh-induced hyperpolarization.

The ionic mechanisms involved were further investigated from the current-voltage relationships produced by current pulses of different intensities recorded in the absence and presence of ACh. Before application of ACh, the amplitude of electrotonic potentials produced by inward current pulses increased linearly with an increase in stimulus intensity. Amplitude of electrotonic potentials produced by outward current pulses also increased linearly with intensity of stimuli until the membrane potential reached about -40 mV, and the relationship became dissociated from the linear line at stronger intensities (i.e., rectification). Outward current pulses at higher intensities (usually >0.3 mA) evoked a spike potential of amplitude $5-15$ mV at the on-set of the electrotonic potential (Figure 2A). The amplitudes of electrotonic potentials were smaller in the presence than in the absence of ACh (Figure 2A and B). This was also the case when electrotonic potentials were produced 3 min after withdrawal of ACh, i.e., the amplitude of electrotonic potential was reduced during the slow hyperpolarization but less so than during the initial hyperpolarization (Figure 2C). The electrotonic potentials had returned to control amplitudes 12 min after withdrawal of ACh when the membrane potential returned to the control level (Figure 2D).

The peak amplitudes of electrotonic potential obtained in different conditions were plotted on the same scale as a function of the intensity of stimuli (Figure 2E), and the relationship was found to be linear in the absence or presence of ACh, but the slope was reduced during the ACh-induced fast and slow hyperpolarizations. The plot obtained in the presence of ACh crossed that obtained before application of ACh at -81.8 ± 15.6 mV ($n=4$ arteries), while the plot obtained at 3 min after withdrawal of ACh (i.e., during the slow hyperpolarization) crossed at -85.0 ± 8.8 mV ($n=6$ arteries). These two values were not significantly different ($P>0.1$), indicating that both hyperpolarizations had reversed potentials similar to the equilibrium potential for K^+ in arterial smooth muscles (approximately -83 mV, Casteels *et al.*, 1977). Thus, the initial and slow components of the ACh-induced hyperpolarization are produced mainly by an increase in conductance of the membrane to K^+ . The slope of the current-voltage relationship measured 12 min after withdrawal of ACh from the superfusate had returned to the control level, indicating the complete recovery of the resting ionic conductance of the membrane.

Reproducibility of the hyperpolarization produced by repeat stimulation with ACh

In the absence of any inhibitory drugs, a repeat stimulation of the artery with ACh at intervals shorter than 20 min caused a reduction in amplitude of the second response. In the cell shown in Figure 3A, the duration of slow hyperpolarization produced by the first application of ACh was about 6.5 min. The amplitude of the initial and slow hyperpolarizations produced by a second application of ACh 10 min later was $75 \pm 9\%$ and $70 \pm 16\%$ ($n=3$) of the first, respectively.

Experiments were carried out to determine the time required to produce ACh-induced hyperpolarization of reproducible amplitude. It was found that a reproducible amplitude of the initial component of the hyperpolarization was generated when the interval of ACh-stimulation was about 20 min (Figure 3D). The recovery of the slow component of the hyperpolarization was variable, requiring 10–35 min, and therefore the studies on the slow component were not further carried out. Similar experiments were made during application of 5×10^{-6} M indomethacin to inhibit the generation of slow hyperpolarization. It was found that the amplitude of hyperpolarization produced by the second application of ACh was now no longer significantly smaller than the first (Figure 3B; $98 \pm 8\%$ of the initial hyperpolarization, $n=3$) when it was applied at intervals of 5 min or longer (Figure 3D).

These results suggest that the slow hyperpolarization is produced by endothelial prostanoids synthesized through activation of cyclooxygenase, and that the prostanoids have inhibitory actions on the generation of the initial component of the ACh-induced hyperpolarization. Attempts were made to observe whether the prostanoids produced by agonists other than ACh were also effective in inhibiting the generation of the ACh-induced hyperpolarization. In circumflex coronary artery of the guinea-pig, substance P (SP, 10^{-7} M) also hyperpolarized the membrane with two components; in 15 cells from five arteries, the initial hyperpolarization reached the peak amplitude of 20.5 ± 3.5 mV from the resting potential after which it gradually decayed in amplitude, while the second slow hyperpolarization reached the peak amplitude of 5.3 ± 1.2 mV at 3–4 min after withdrawal of SP, and it required 5–8 min (6.8 ± 0.5 min) for complete recovery (Figure 4B). Application of indomethacin (5×10^{-6} M) or diclofenac (10^{-6} M) inhibited only the slow hyperpolarization (data not shown), indicating that this component had properties similar to the slow hyperpolarization produced by ACh.

ACh (10^{-6} M) was applied two times, at least 20 min before and various times after application of 10^{-7} M SP, and the amplitudes of hyperpolarization produced by these two ACh applications were compared. When the second ACh was applied 6 min after withdrawal of SP, the amplitude of the initial and slow components of the hyperpolarization was about 60 and 70% of the first, respectively (Figure 4A and B). The group data (Figure 4C) indicated that a reproducible amplitude of the initial component of the hyperpolarization could be elicited when ACh was applied longer than about 20 min after withdrawal of SP. The effects of conditioning stimulation with SP on the slow component of the ACh-induced hyperpolarization were not investigated, due to unsuccessful measurement of this potential consistently. Thus, these results indicate that the endothelial prostanoids have inhibitory actions on the release of EDHF, irrespective of the agonists used to release the prostanoids.

Hyperpolarization produced by Y-26763

Experiments were carried out to observe the effects of conditioning stimulation of the coronary artery with ACh on the hyperpolarization responses produced by Y-26763, a K^+ -channel opener (Itoh *et al.*, 1994), the objective being to determine possible effects of endothelial prostanoids on the hyperpolarizing responses of arterial smooth muscle. In six arteries in the absence of any stimulation, application of 10^{-6} M Y-26763 for 2 min hyperpolarized the membrane for about 20 min, with a peak amplitude of 12.5 ± 1.5 mV, and a similar hyperpolarization with a peak amplitude of 11.5 ± 2.5 mV ($P>0.1$) was also elicited when this concentra-

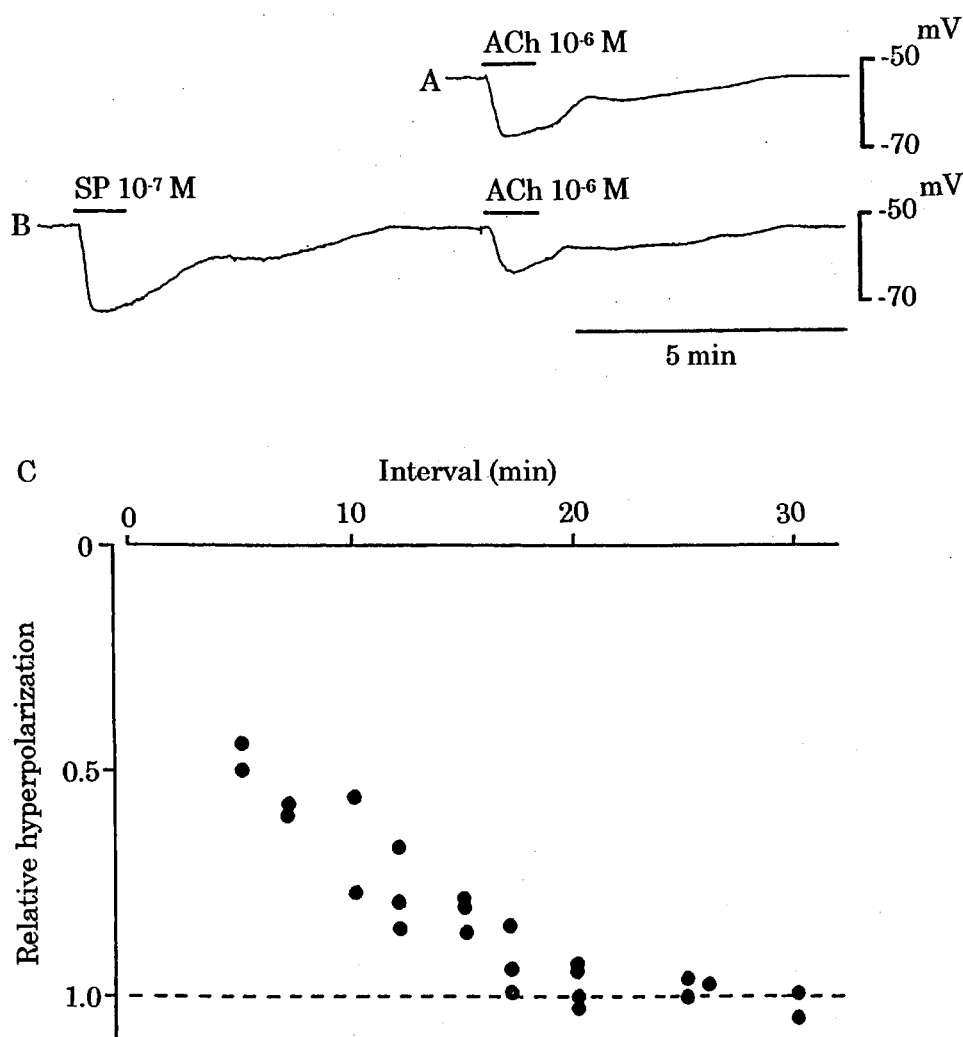


Figure 4 Inhibition of the acetylcholine (ACh)-induced hyperpolarization by conditioning stimulation with substance P. ACh (10^{-6} M) was applied for a duration of 1 min, 25 min before (A) and 6 min after 1 min stimulation of tissue with 10^{-7} M substance P (SP) (B). A and B were recorded from the same cell. (C). The relationship between amplitude of the initial hyperpolarization produced by the second ACh application (relative to the first) and the interval between withdrawal of 10^{-7} M substance P and the second application of ACh. Data obtained from three tissues were plotted.

tion of Y-26763 was applied 10 min after stimulation with ACh. The results indicate that the reduction of the ACh-induced hyperpolarization for periods 10 min or greater is not due to a failure of the smooth muscle membrane of the coronary artery to produce hyperpolarization.

Effects of PGI₂ on smooth muscle of the coronary artery

In smooth muscle of the guinea-pig coronary artery, application of PGI₂ ($>2.8 \times 10^{-9}$ M) hyperpolarized the membrane. A solution containing an ethanol concentration equivalent to that used to make up a solution of 2.8×10^{-6} M PGI₂ did not produce any detectable change in the membrane potential ($n=3$ arteries, data not shown). The PGI₂-induced hyperpolarization remained unaltered in the presence of 5×10^{-6} M indomethacin, 10^{-6} M diclofenac or 10^{-5} M nitroarginine ($n=3$ arteries, data not shown), indicating that the generation of the response did not involve activation of cyclooxygenase or NO synthase and reflects a direct action on smooth muscle. Figure 5A shows the relationship between concentration of PGI₂ and amplitude of hyperpolarization in smooth muscle of the guinea-pig coronary artery. PGI₂ (2.8×10^{-9} – 2.8×10^{-6} M) hyperpolarized the membrane in a

concentration-dependent manner, and the maximum value was obtained at 8.4×10^{-7} M. The amplitude of electrotonic potentials produced by alternate stimulation of the artery with inward and outward current pulses of constant intensity was reduced during the PGI₂-induced hyperpolarization (Figure 5B), indicating that the potential was produced by an increase in ionic conductance of the membrane. Changes in the membrane resistance during the PGI₂-induced hyperpolarization were calculated, according to the cable theory (Hodgkin & Rushton, 1946), and the reduction was 0.61 ± 0.24 ($n=5$) times the control.

Electrotonic potentials were evoked by current steps of increasing intensity (0.1–0.6 mA) in the absence and presence of 8.4×10^{-8} M PGI₂, and the relationship was plotted on the same scale. The relationship was linear for both control and test cases, with the slope less steep in the presence than in the absence of PGI₂ (Figure 5C). The current-voltage relationships crossed at -85.0 ± 8.5 mV ($n=3$ arteries), suggesting that the PGI₂-induced hyperpolarization was produced mainly by an increase in conductance of the membrane to K⁺.

Electrical responses produced by PGI₂ were compared with those of the ACh-induced hyperpolarization in smooth muscle

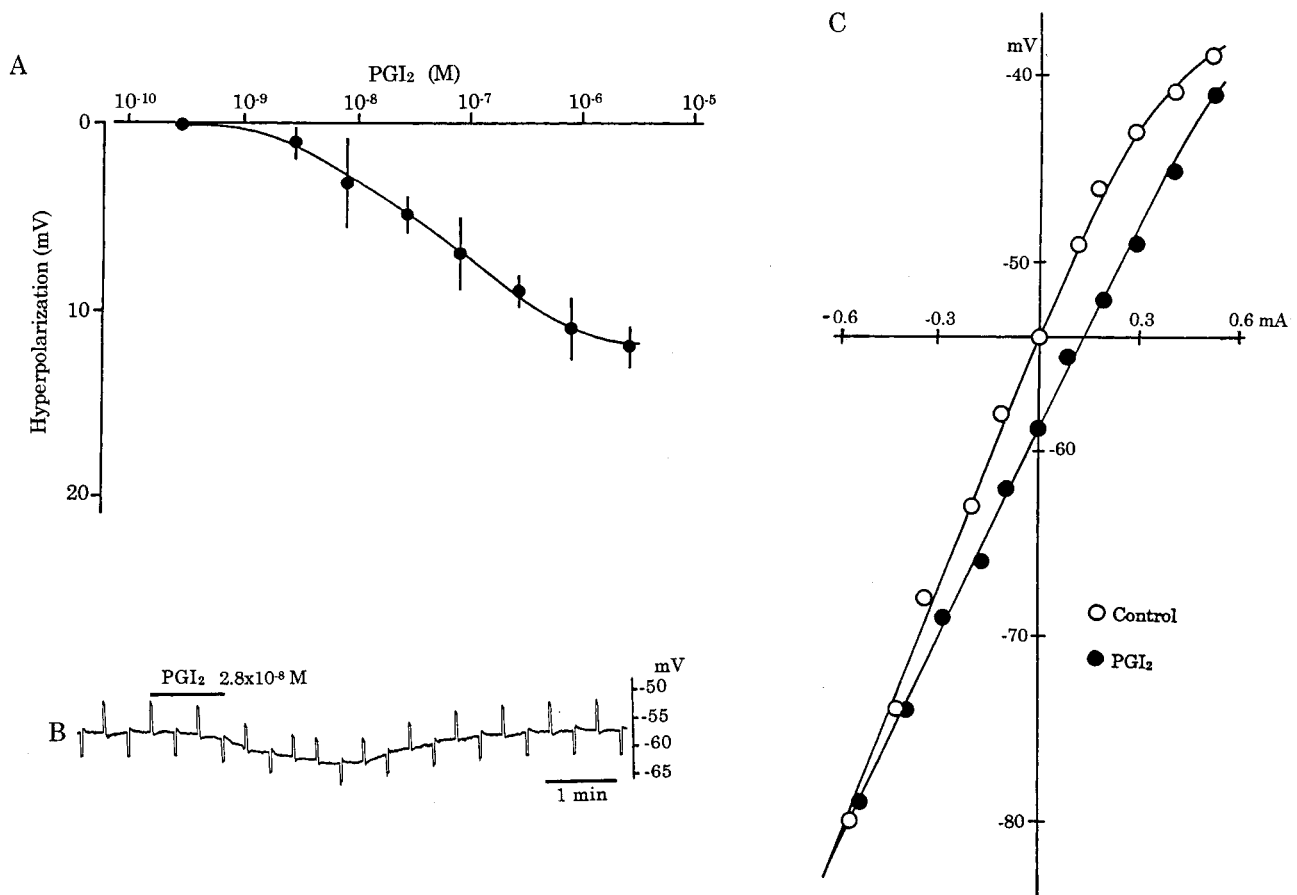


Figure 5 PGI₂-induced hyperpolarization accompanied by a reduction in amplitude of electrotonic potentials. (A) The relationship between concentration of PGI₂ and amplitude of hyperpolarization recorded in the arterial smooth muscle is shown by the means $\pm$ s.d. mean ($n=4-12$). Peak amplitude of the hyperpolarization produced by application of PGI₂ for 3 min was measured. Data obtained from seven tissues are summarized. Significant hyperpolarization was observed at $>2.8 \times 10^{-8}$ M PGI₂. (B) A circumflex coronary artery was stimulated with 2.8×10^{-8} M PGI₂ for 1 min (shown by a bar above the record) while inward and outward current stimuli of a constant intensity (0.26 mA) were applied alternatively to produce electrotonic potentials. (C) Current-voltage relationships recorded before (Control, $\circ$) and during application of 8.4×10^{-8} M PGI₂ ($\bullet$). Amplitudes of electrotonic potentials were plotted as a function of the intensity of stimuli (positive value, outward current; negative value, inward current). All points were recorded from the same cell.

of the guinea-pig. In the hyperpolarizations produced by application of 10^{-6} M ACh for 1 min ($n=4$ cells), the initial component had a latency of onset of 18.1 ± 3.3 s and peak amplitude of 25.5 ± 5.0 mV while the slow hyperpolarization had a peak amplitude of 5.1 ± 2.2 mV and duration of 6.0 ± 0.9 min after withdrawal of ACh (Figure 6A). In the presence of 10^{-6} M diclofenac ($n=3$ cells), ACh produced only an initial hyperpolarization with a latency of 17.8 ± 5.1 s and amplitude of 24.8 ± 3.8 mV (Figure 6B). Application of 8.4×10^{-8} M PGI₂ for 1 min also hyperpolarized the membrane by 6.5 ± 3.1 mV ($n=5$ cells), the value being similar to that produced by ACh, but with a slow time course taking 25–40 s (30.3 ± 8.5 s) to reach the peak amplitude and lasting 4–8 min (5.5 ± 0.7 min) (Figure 6C). The duration of the PGI₂-induced hyperpolarization was not significantly different from the ACh-induced slow hyperpolarization.

ACh (10^{-6} M) and PGI₂ (8.4×10^{-8} M) when applied simultaneously after blockade of cyclooxygenase by diclofenac induced a response which was similar to that produced by ACh in the absence of diclofenac (Figure 6A and B), although the amplitude of the initial hyperpolarization was reduced by about 20% (20.2 ± 2.8 mV, $n=3$ cells). Thus, the slow hyperpolarization produced by ACh could be mimicked by exogenously applied PGI₂.

Possible inhibitory actions of exogenously applied PGI₂ on the ACh-induced hyperpolarization were examined. As shown in Figure 7, application of 2.8×10^{-10} M PGI₂, which itself was without effect on the membrane potential, reduced the amplitude of ACh-induced hyperpolarization to about 60%. In five experiments, the amplitude of the ACh-induced hyperpolarization was significantly reduced to $89.1 \pm 8.8\%$ by 2.8×10^{-11} M PGI₂ and to $77.6 \pm 8.7\%$ by 2.8×10^{-10} M PGI₂. In three experiments, 2.8×10^{-9} M PGI₂ hyperpolarized the membrane by 1.8 ± 0.5 mV and reduced the ACh-induced hyperpolarization to $50.2 \pm 6.1\%$. The inhibitory actions of PGI₂ on the ACh-induced hyperpolarization were also observed in the presence of diclofenac; 2.8×10^{-11} M PGI₂ reduced the initial component of the ACh-induced hyperpolarization to $88.1 \pm 6.9\%$ ($n=3$ cells, $P<0.05$, Figure 7C and D). Thus, PGI₂ in concentrations below threshold for hyperpolarization of smooth muscle membrane inhibits the ACh-induced hyperpolarization.

Discussion

The present experiments have shown that in the circumflex coronary artery of guinea-pig, ACh produces a hyperpolariza-

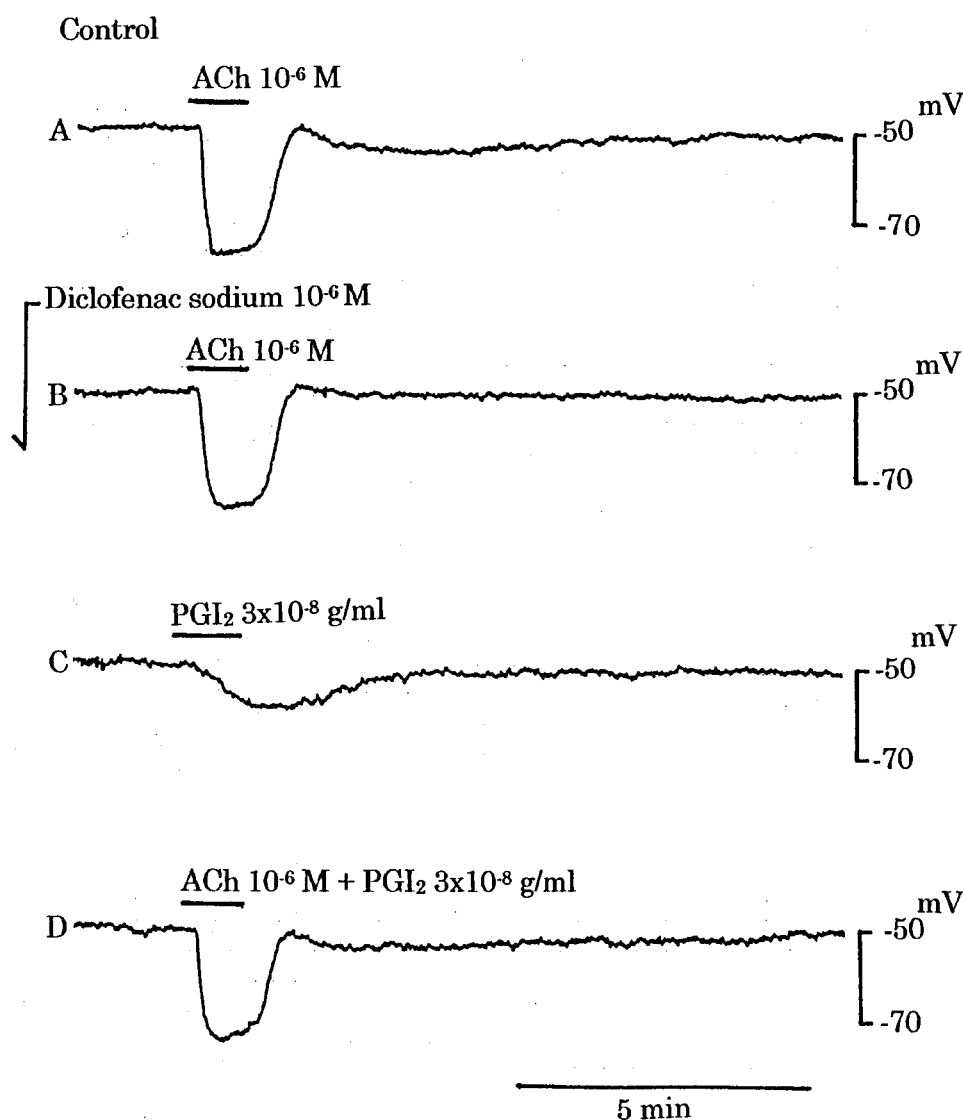


Figure 6 Simulation of the acetylcholine (ACh)-induced slow hyperpolarization by exogenously applied PGI_2 . In the guinea-pig coronary artery, membrane responses produced by ACh and PGI_2 were recorded in the absence (A) and presence of 10^{-6} M diclofenac (B–D). ACh 10^{-6} M (A, B and D) or 8.4×10^{-8} M PGI_2 (C) was applied for 1 min at intervals 20–25 min. A–D were recorded from the same cell.

tion with two components, in an endothelium-dependent manner. The ACh-induced response was abolished after application of atropine, suggesting that the response was produced as a result of stimulation of muscarinic receptors by ACh. Parkinson *et al.* (1993) reported that in the left descending main coronary artery of guinea-pig the ACh-induced response is separated into three components, as the initial hyperpolarization produced by EDHF and the slow hyperpolarization consists of EDRF- and prostanoids-induced components. We found that the slow hyperpolarization was produced by endothelial prostanoids, as this component disappeared completely after inhibiting the activity of cyclooxygenase with indomethacin or diclofenac but not by nitroarginine. The initial component was not sensitive to nitroarginine, indomethacin or diclofenac. Thus, only two components, a nitroarginine and indomethacin-resistant component (possibly produced by EDHF) and indomethacin-sensitive component (produced by prostanoids) were confirmed in all vessels used in the present experiments.

The main finding of the present experiments is the inhibitory actions of endothelial prostanoids on the initial

component of the ACh-induced hyperpolarization. While the duration of the slow hyperpolarization is 5–7 min, the recovery from the inhibition of the ACh-induced hyperpolarization requires over 20 min. Thus, the membrane potential and membrane resistance revert to the resting level before cessation of the inhibitory action on the EDHF-induced hyperpolarization, indicating that the delayed recovery of the ACh-induced response is not due to the changes in biophysical properties of smooth muscle membrane. We speculate that the endogenously released prostanoids inhibit the release of EDHF by autocrine or paracrine actions. The significant shortening of the recovery time for the ACh-response by exposure to a cyclooxygenase inhibitor strongly supports this idea. Furthermore, the inhibitory actions of endogenous prostanoids on the ACh-response could be mimicked by subthreshold concentrations of exogenously applied PGI_2 (equal to $<2.8 \times 10^{-9}$ M). An important function of endothelial PGI_2 is the prevention of blood coagulation by inhibition of the adhesion of platelets to the vessel wall (Gryglewski *et al.*, 1991). We would like to propose that the inhibitory actions on the release of EDHF should also be counted into the role of

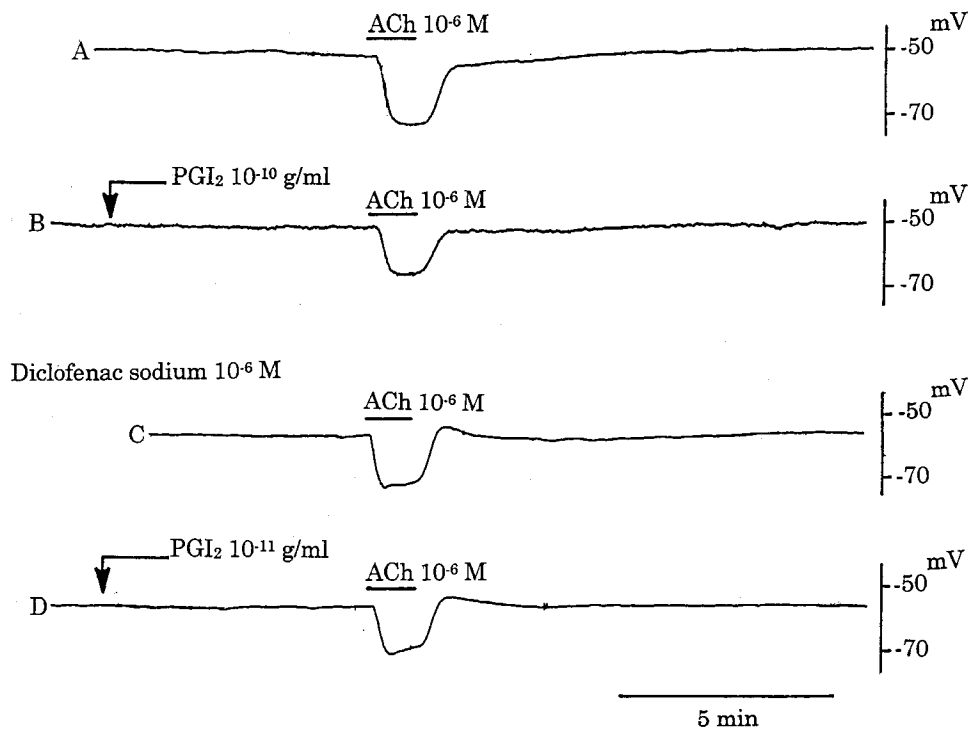


Figure 7 Inhibition of the acetylcholine (ACh)-induced hyperpolarization by subthreshold concentrations of PGI₂. ACh (10^{-6} M) was applied for 1 min at intervals 20–25 min, in the absence (A or C) and presence of 2.8×10^{-10} M PGI₂ (B) or 2.8×10^{-11} M PGI₂ (D). Responses were recorded in the absence (A and B) and presence of 10^{-6} M diclofenac (C and D). A–B and C–D were recorded from single cells in the same tissue.

endothelial PGI₂. This prostanoid is a potent vasodilator in some vessels (Parkington *et al.*, 1995; Zhang *et al.*, 1996), and therefore the inhibitory actions of PGI₂ on the release of EDHF would weaken the endothelium-dependent vasodilatation. The physiological meaning of the inhibitory actions of endothelial PGI₂ on the release of EDHF remains unclear.

Endothelial cells are large source of PGI₂ during stimulation with agonists including ACh (Gryglewski *et al.*, 1991), and in some arteries this prostanoid hyperpolarizes smooth muscle membrane by activating glibenclamide-sensitive K⁺-channels (Siegel *et al.*, 1992; Parkington *et al.*, 1995; Yajima *et al.*, 1997). The present experiments confirmed that the hyperpolarization is indeed accompanied by an increase in K⁺ conductance. In cardiovascular tissues, glibenclamide is reported to inhibit the ATP-sensitive K⁺-channels (Nelson & Quayle, 1995). However, glibenclamide may have additional actions to inhibit prostanoid receptors (Zhang *et al.*, 1991), indicating that contribution of the ATP-sensitive K⁺ channels in the PGI₂-induced hyperpolarization cannot be determined from the effects of glibenclamide alone. The initial hyperpolarization was, on the other hand, inhibited by charybdotoxin (Yajima *et al.*, 1997; Nishiyama *et al.*, 1998), indicating that these two components of the hyperpolarization were generated by activation of different types of K⁺-channels.

The present experiments showed that the time course of the hyperpolarization produced by exogenously applied PGI₂ is similar to that produced by endothelial prostanoids. This property of PGI₂ allowed us to mimic the ACh-induced slow hyperpolarization by exogenous application of PGI₂. These observations suggest that the slow onset and time course of the hyperpolarization produced by endothelial prostanoids are not due to the delayed onset and sustained activation of prostanoid-synthesis systems after withdrawal of ACh, but

probably due to the properties of the prostanoid response. Although the present experiments could not determine the chemical nature of the endothelial prostanoids, the observations strongly suggest that the slow hyperpolarization is produced by PGI₂. This is also supported by the evidence that both the slow hyperpolarization and PGI₂-induced hyperpolarization are inhibited by glibenclamide (Parkington *et al.*, 1995; Yajima *et al.*, 1997). On this assumption, the concentration-response relationship of the PGI₂-induced hyperpolarization allows estimation that the amount of PGI₂ released from endothelial cells in response to 10^{-6} M ACh is in the range of 2.8×10^{-9} – 2.8×10^{-8} M.

The endothelium-dependent hyperpolarization (EDH) is produced by a humoral substance, EDHF, in the coronary artery of guinea-pig (Chen *et al.*, 1991). In the porcine coronary artery, EDHF may be eicosatrienoic acids metabolized from arachidonic acid (Hecker *et al.*, 1994; Popp *et al.*, 1996). However, electrical coupling between endothelial and smooth muscle cells through gap junctions has also been demonstrated in the rat aorta (Marchenko & Sage, 1996), porcine coronary artery (von der Weid & Beny, 1993) and submucosal arterioles of the guinea-pig (Yamamoto *et al.*, 1998). These findings suggest that EDH is produced by an electrotonic spread of potentials from the endothelial cells. However, the rectification of signal transmission noted in the gap junctions between endothelial and smooth muscle cells of the porcine coronary artery, possibly because of electrical mismatch with the large smooth muscle syncytium and the smaller endothelial syncytium (Beny & Pacicca, 1994), suggest that the properties of EDH differ between large and small vessels.

In conclusion, endothelial prostanoids (possibly PGI₂) released by agonists hyperpolarize the smooth muscle membrane by activating K⁺-channels and also inhibit the release of EDHF. Endothelial prostanoids may have inhibitory

actions on the myoendothelial gap junctions, if EDH is produced by the electrotonic spread of potentials from the endothelial cells. The inhibitory actions of EDH appears below threshold concentration to hyperpolarize smooth muscle membrane.

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Effects of Ca^{2+} concentration and Ca^{2+} channel blockers on noradrenaline release and purinergic neuroeffector transmission in rat tail artery

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1 The effects of Ca^{2+} concentration and Ca^{2+} channel blockers on noradrenaline (NA) and adenosine 5'-triphosphate (ATP) release from postganglionic sympathetic nerves have been investigated in rat tail arteries *in vitro*. Intracellularly recorded excitatory junction potentials (e.j.ps) were used as a measure of ATP release and continuous amperometry was used to measure NA release.

2 Varying the extracellular Ca^{2+} concentration similarly affected the amplitudes of e.j.ps and NA-induced oxidation currents evoked by trains of ten stimuli at 1 Hz.

3 The N-type Ca^{2+} blocker, ω -conotoxin GVIA (ω -CTX GVIA, 0.1 μM) reduced the amplitudes of both e.j.ps (evoked by trains of ten stimuli at 1 Hz) and NA-induced oxidation currents (evoked by trains of ten stimuli at 1 Hz and 50 stimuli at 10 Hz) by about 90%.

4 The ω -CTX GVIA resistant e.j.ps and NA-induced oxidation currents evoked by trains of 50 stimuli at 10 Hz were abolished by the non-selective Ca^{2+} channel blocker, Cd^{2+} (0.1 mM), and were reduced by ω -conotoxin MVIIC (0.5 μM) and ω -agatoxin IVA (40 nM).

5 Nifedipine (10 μM) had no inhibitory effect on ω -CTX GVIA resistant e.j.ps and NA-induced oxidation currents.

6 Thus both varying Ca^{2+} concentration and applying Ca^{2+} channel blockers results in similar effects on NA and ATP release from postganglionic sympathetic nerves. These findings are consistent with the hypothesis that NA and ATP are co-released together from the sympathetic nerve terminals.

Keywords: Postganglionic sympathetic nerve; neurotransmitter release; Ca^{2+} channels; Ca^{2+} channel blockers; rat tail artery; noradrenaline; adenosine 5'-triphosphate; electrophysiology; amperometry

Abbreviations: Excitatory junction potential, e.j.p.

Introduction

Depolarization-evoked entry of Ca^{2+} into nerve terminals plays a key role in triggering neurotransmitter release. In postganglionic sympathetic nerve terminals, this Ca^{2+} enters mainly through N-type Ca^{2+} channels (Maggi *et al.*, 1988; Brock *et al.*, 1989; De Luca *et al.*, 1990; Smith & Cunnane, 1996; 1997; Wright & Angus, 1996), although neurotransmitter release can also be evoked by Ca^{2+} entering through other Ca^{2+} channel types. In particular, it has been demonstrated in a number of sympathetically innervated tissues that following the addition of a maximally effective concentration of the N-type Ca^{2+} channel blocker, ω -conotoxin GVIA (ω -CTX GVIA), postjunctional responses to sympathetic nerve stimulation remain (Smith & Cunnane, 1996; 1997; Wright & Angus, 1996; Waterman, 1997). This ω -conotoxin GVIA resistant response is best revealed when the frequency of nerve stimulation is raised. For example, in the guinea-pig vas deferens, ω -CTX GVIA abolished the excitatory junction potentials (e.j.ps) evoked by trains of stimuli at 1 Hz but trains of higher frequency stimuli (≥ 3 Hz) evoked e.j.ps of small amplitude (Smith & Cunnane, 1996). These ω -CTX GVIA resistant e.j.ps were blocked by the non-selective Ca^{2+} channel blocker, Cd^{2+} , indicating that they were due to Ca^{2+} -dependent release of neurotransmitter.

The ω -CTX GVIA resistant e.j.ps in the guinea-pig vas deferens were unaffected by ω -agatoxin IVA (ω -AgTx IVA)

and ω -conotoxin MVIIC (ω -CTX MVIIC) which block P- and Q-type Ca^{2+} channels (Randall & Tsien, 1995) or by nifedipine which blocks L-type Ca^{2+} channels. These findings indicate that ω -CTX GVIA resistant neurotransmitter release in guinea-pig vas deferens is due to the opening of Ca^{2+} channels not blocked by known selective Ca^{2+} channel antagonists. In contrast, in rat and mouse vas deferens (Wright & Angus, 1996; Waterman, 1997) and rat anococcygeus (Smith & Cunnane, 1997) the ω -CTX GVIA resistant responses are dependent on Ca^{2+} entering through P-/Q-type Ca^{2+} channels.

ω -CTX GVIA has a similar blocking action on postjunctional responses mediated by the release of the sympathetic co-transmitters noradrenaline (NA) and adenosine 5'-triphosphate (ATP) in the mouse, rat and guinea-pig vas deferens (Maggi *et al.*, 1988; Brock *et al.*, 1989; De Luca *et al.*, 1990; Waterman, 1997) and the rat anococcygeus (Smith & Cunnane, 1997). These findings suggest that the release of NA and ATP are similarly affected by blockade of N-type Ca^{2+} channels. However, it has recently been reported that ω -CTX GVIA inhibited the nerve stimulation evoked overflow of endogenous NA from the guinea-pig vas deferens to a much greater extent than that of endogenous ATP (Westfall *et al.*, 1996). Conversely, ω -AgTx IVA reduced the stimulus evoked release of ATP to a much greater extent than that of NA. These findings suggest that the release of NA and ATP depend primarily on the activation of N- and P-/Q-type Ca^{2+} channels respectively.

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The main aim of the present study was to compare the action of Ca^{2+} channel blockers on action potential evoked release of NA and ATP from postganglionic sympathetic nerve innervating the rat tail artery. In addition, the Ca^{2+} concentration dependence of NA and ATP release in this tissue was determined. The release of endogenous NA release was measured directly by continuous amperometry (Gonon *et al.*, 1993; Brock *et al.*, 1997). This technique provides a 'real time' measurement of NA release. In separate experiments conventional intracellular recording techniques were used to monitor electrically evoked e.j.ps, which provide a measure of ATP release (Sneddon & Burnstock, 1984; McLaren *et al.*, 1995).

Methods

Female outbred Wistar rats (150–200 g) were killed by an overdose of pentobarbitone sodium (100 mg kg⁻¹, i.p.). Sections of proximal tail artery approximately 10 mm in length were removed and individual preparations pinned to the Sylgard (Dow-Corning) covered base of a 1 ml (electrophysiology) or 2 ml (amperometry) recording chamber. The chambers were perfused continuously at 3–5 ml min⁻¹ with physiological saline of the following composition (mM): NaCl 133.4, KCl 4.7, CaCl_2 2.4, MgCl_2 1.2, NaH_2PO_4 1.3, NaHCO_3 16.3 and glucose 9.8. The physiological saline was gassed with a mixture of 95% O_2 and 5% CO_2 (to pH 7.4) and maintained at 35–36°C. In all experiments, the physiological saline contained the α_1 -adrenoceptor antagonist, prazosin (0.1 μM), to inhibit neurally evoked contraction due to NA release, idazoxan (1 μM) to inhibit α_2 -adrenoceptor mediated auto-inhibition of transmitter release and desipramine (1 μM) to inhibit neuronal uptake of released NA (Uptake_1). In the experiments where the effects of varying the Ca^{2+} concentration were investigated, only the CaCl_2 content of the physiological saline was varied. The proximal end of the artery was drawn into a suction stimulating electrode and the perivascular nerves were excited by electrical field stimulation (1 ms pulse width, 3–20 V for the electrophysiology experiments and 0.3 ms, 20 V for the amperometry experiments).

Electrochemical recording

The release of endogenous NA was monitored by continuous amperometry using a technique similar to that described in Brock *et al.* (1997). This electrochemical method measures the stimulus-evoked increase in NA concentration at the adventitial surface of the artery as an oxidation current, the amplitude of which is linearly related to the change in NA concentration (Gonon *et al.*, 1993; Brock *et al.*, 1997). The only difference between the present experiments and those described in Brock *et al.* (1997), was that all the carbon fibre electrodes were pretreated by dipping the electrode tips for 15–30 min in a 2.5% (w/v) solution of Nafion (Aldrich Chemical Company, Castle Hill, NSW, Australia). After dipping, the electrodes were allowed to dry for at least 1 h before use. This treatment reduced the decline in electrode sensitivity observed during prolonged periods of recording (see Brock *et al.*, 1997).

Intracellular recording

Intracellular recordings were made using the technique described in Brock *et al.* (1997). To avoid the variable amplitude e.j.ps with an early fast component recorded in

superficial cells (see Cassell *et al.*, 1988), recordings were made preferentially from cells two or more cells deep in the media in which the e.j.ps decayed monoexponentially. To facilitate comparisons between different preparations and to reduce the effects of non-linear summation on e.j.p. amplitude, the stimulus intensity was adjusted at the start of each experiment to ensure that e.j.ps evoked by single stimuli were approximately 10 mV in amplitude.

The effects of varying Ca^{2+} concentration and of Ca^{2+} channel blockers on e.j.p. amplitude were determined in single cell experiments in which both control and test recordings were made during the same impalement.

Ca^{2+} concentration dependence experiments

In experiments where the effects of varying the Ca^{2+} concentration on oxidation currents and e.j.ps were investigated, the tissues were stimulated at 10 min intervals with three trains of ten pulses at 1 Hz, each train being separated by 90 s. Initially the tissues were superfused with physiological saline containing 2.4 mM Ca^{2+} for two periods of stimulation and then, immediately following the second and subsequent periods of stimulation, the Ca^{2+} concentration of the physiological saline was varied to 0.6, 0.9, 1.2, 1.8, 3.6 or 4.8 mM. The sequence of the changes in Ca^{2+} concentration in each experiment was randomized. In the electrochemistry experiments, only three changes in Ca^{2+} concentration were made before returning to 2.4 mM Ca^{2+} for the final period of stimulation. This procedure was followed because during control experiments ($n=6$), in which the Ca^{2+} was maintained throughout at 2.4 mM, the amplitudes of oxidation currents did not change detectably during six cycles of stimulation but, during experiments of longer duration, the amplitudes of the oxidation currents decreased significantly (Wilcoxon signed rank tests). No change in e.j.p. amplitude was detected during ten cycles of stimulation in the presence of 2.4 mM Ca^{2+} ($n=6$). Therefore in most electrophysiology experiments the full range of Ca^{2+} concentrations was tested before returning the Ca^{2+} concentration to 2.4 mM for the last period of stimulation. However, in some experiments, the impalements were lost before all the Ca^{2+} concentrations could be tested. All the data presented are normalized with respect to the initial values obtained under control conditions (2.4 mM Ca^{2+}) at the start of each experiment.

Ca^{2+} channel blocker experiments

In all experiments investigating the effects of the Ca^{2+} channel blockers on oxidation currents and e.j.ps, the effects of adding ω -CTX GVIA were first determined. In this part of the experiments, the tissues were stimulated at 10 min intervals with three trains of ten pulses at 1 Hz or three trains of 50 pulses at 10 Hz (electrochemistry experiments only), each train being separated by 90 s. Immediately following the second period of stimulation, ω -CTX GVIA (0.1 μM) was applied to the tissues and the effects of this agent followed for two or three periods of stimulation. In the second part of each experiment, which investigated the effects of adding nifedipine (10 μM), ω -AgTx IVA (40 nM) or ω -CTX MVIIC (0.5 μM) on the ' ω -CTX GVIA resistant' responses, all tissues were stimulated at 10 min intervals with three trains of 50 pulses at 10 Hz. The Ca^{2+} channel blocker was applied to the tissues immediately following the second period of stimulation and the effects of the agent followed for three periods of stimulation. At the end of all experiments, a further period of stimulation was recorded 20 mins after applying Cd^{2+}

(0.1 mM) to block fully neurotransmitter transmitter release. In control experiments ($n=6$) in which the superfusing solution contained ω -CTX GVIA (0.1 μ M), the amplitudes of the oxidation currents and the depolarizations evoked by trains of 50 pulses at 10 Hz declined significantly during a recording period of 30 mins (Wilcoxon signed rank test, $P<0.05$). For this reason the results obtained in the second part of the experiment were compared statistically with those for time matched ω -CTX GVIA-treated controls.

Data analysis

All data were digitized (sampling frequencies of 0.04–0.2 kHz) and collected with a MacLab recording system and the program Scope (ADInstruments Pty Ltd, Castle Hill, N.S.W., Australia). Subsequent analysis was made with the computer program Igor Pro (Wavemetrics, Lake Oswego, OR, U.S.A.). To assess the effect of changing the Ca^{2+} concentration or of drug treatments, the electrophysiological or electrochemical records for individual tissues under each condition were averaged before measurements were made. The amplitudes of the electrophysiological and electrochemical signals evoked during the trains of stimuli at 1 Hz were determined as previously described in Brock *et al.* (1997). The amplitudes of the depolarizations evoked by 50 stimuli at 10 Hz were determined during last the 10 stimuli in the train. The amplitudes of the oxidation currents evoked by 50 pulses at 10 Hz were measured by averaging the period 100–200 ms following the last stimulus in the train.

Data are presented as means $\pm$ s.e.mean. Statistical comparisons were made using non-parametric tests as indicated in the text. For comparisons between the effects of the various manipulations on oxidation currents and e.j.ps it was assumed that both provide measures of neurotransmitter release. Prior to analysing the Ca^{2+} concentration data, the number of measures for each Ca^{2+} concentration was standardized by assigning each measure a random number and using the nine highest values. Comparison between the selected data sets and the complete data sets using Mann-Whitney U -tests revealed no significant difference. In all tests P values <0.05 were considered to be significant.

Drugs

Prazosin, idazoxan, desipramine and nifedipine were supplied by Sigma Chemical Company (Castle Hill, N.S.W., Australia) and ω -CTX GVIA and ω -CTX MVIIC were supplied by Auspep Pty Ltd (Parkville, Vic, Australia). ω -AgTx IVA was a kind gift from Pfizer (Groton, CT, U.S.A.). Nifedipine was made up as a stock solution in ethanol. The peptides were prepared as stock solutions in water and stored at -20°C (the ω -AgTx IVA solution contained 1 mg ml $^{-1}$ cytochrome C). Drugs were added to the solution superfusing the tissue. During peptide application, a recirculation system of 20 ml volume was used to minimize the amounts of the toxins used.

Results

General observations

Under control conditions (2.4 mM Ca^{2+}) the mean increase in oxidation current evoked by each stimulus during a train of 10 stimuli at 1 Hz was 0.5 ± 0.1 pA ($n=39$) and the amplitude of the oxidation current measured at the end of a train of 50 stimuli at 10 Hz was 7.8 ± 0.9 pA ($n=25$). Also under these

conditions the mean amplitude of e.j.ps evoked during trains of 10 stimuli at 1 Hz was 9.7 ± 0.4 mV and the membrane potential was -63.9 ± 0.3 mV ($n=30$ tissues).

Effects of Ca^{2+} concentration

Both the amplitudes of the oxidations currents (Figure 1a) and the e.j.ps (Figure 1b) evoked during trains of 10 pulses at 1 Hz increased as the concentration of Ca^{2+} was raised from 0.6 to 4.8 mM. Figure 1c shows graphically the effects of Ca^{2+} concentration on the amplitudes of the oxidation currents and e.j.ps, relative to the values measured under control conditions (2.4 mM Ca^{2+}) at the start of the experiment. The relationship between the Ca^{2+} concentration and the amplitudes of oxidation currents and e.j.ps was similar and statistical comparison revealed no significant difference between effects of Ca^{2+} concentration on the two data sets (Mann-Whitney U -test, $P=0.53$).

In the electrophysiological experiments the effect of Ca^{2+} concentration on membrane potential was not determined. However, the time constant of decay of the e.j.ps (Table 1), which provides a measure of the membrane time constant (see Cassell *et al.*, 1988), did not change significantly over the range of Ca^{2+} concentrations used (Kruskal-Wallis test, $P=0.31$). In addition, the smooth muscle membrane potential measured at the start (-63.1 ± 0.7 mV) and end (-62.6 ± 0.7 mV) of each experiment did not differ significantly (Wilcoxon signed rank test, $P=0.16$, $n=11$ tissues).

Effects of ω -CTX GVIA

ω -CTX GVIA (0.1 μ M) markedly reduced the amplitudes of the oxidation currents and e.j.ps evoked during trains of ten pulses at 1 Hz (Figure 2a,c, Wilcoxon signed rank test; oxidation current $P<0.05$, $n=8$; e.j.p $P<0.001$, $n=22$) and the amplitudes of the oxidation currents evoked by 50 pulses at 10 Hz (Figure 2b, $P<0.001$, $n=26$). This inhibitory effect was clearly apparent within 10 min of application and reached a plateau level after 20 min (Figure 2d). At 20 min, the inhibitory effect of ω -CTX GVIA on oxidation currents and on e.j.ps did not differ significantly (Kruskal-Wallis test, $P=0.52$).

In a small number of experiments the concentration of ω -CTX GVIA was raised to 0.3 μ M, 20 min after the initial application of the 0.1 μ M solution. The amplitude of the oxidation currents evoked by 50 pulses at 10 Hz and the mean amplitude of e.j.ps evoked by 10 pulses at 1 Hz, measured 20 min after applying 0.3 μ M ω -CTX GVIA, decreased by $14 \pm 4\%$ ($n=3$) and $6 \pm 8\%$ ($n=5$) respectively compared to the values measured just prior raising the concentration of ω -CTX GVIA. In tissues exposed only to 0.1 μ M ω -CTX GVIA, the amplitude of the oxidation currents and e.j.ps decreased respectively by $16 \pm 8\%$ ($n=6$) and $9 \pm 5\%$ ($n=14$) over the same time interval. Thus in the rat tail artery, as in other sympathetically innervated tissues (see e.g. Smith & Cunnane, 1996; 1997), 0.1 μ M ω -CTX GVIA is a maximally effective concentration for blocking neurotransmitter release.

Effects of nifedipine, ω -AgTx IVA and ω -CTX MVIIC on ω -CTX GVIA resistant responses

In tissues treated with the ω -CTX GVIA (0.1 μ M), stimulation with 50 pulses at 10 Hz produced clearly discernible oxidation currents and membrane depolarizations due to summation of e.j.ps (Figure 3a,b). The amplitude of the oxidation currents measured at the end of the train of stimuli was 1.0 ± 0.2 pA

($n=28$) and the amplitude of membrane depolarization was 7.9 ± 0.8 mV ($n=26$). Application of the inorganic Ca^{2+} channel blocker, Cd^{2+} (0.1 mM), abolished these ω -CTX GVIA resistant responses (Figure 3a(i) and b(i)), confirming that they were due to Ca^{2+} -dependent release of neurotransmitter.

Figure 3 and 4 show the effects of applying the Ca^{2+} channel blockers nifedipine (10 μM , Figure 3a(ii) and b(ii)), ω -AgTx IVA (40 nM, Figure 3a(iii) and b(iii)) or ω -CTX MVIIC (0.5 μM , Figure 3a(iv) and b(iv)) on the oxidation currents and membrane depolarizations. The effects of these agents were compared 30 min following their addition to the superfusing solution (Figure 4). At this time there was a significant difference between the effects of the Ca^{2+} channel blockers on the amplitudes of both the oxidation currents and membrane depolarizations (Kruskal-Wallis test, $P < 0.01$ for both oxidation currents and membrane depolarizations).

In comparison with ω -CTX GVIA treated control tissues, ω -CTX MVIIC and ω -AgTx IVA significantly reduced the amplitude of both the oxidation currents and membrane

depolarizations (see Figure 4). This statistical analysis was made by a nonparametric comparison of the ω -CTX GVIA control group data to that of the other test groups (see Zar, 1984). Nifedipine had no inhibitory effect on either the oxidation currents or the membrane depolarizations. Comparison between the effects of each Ca^{2+} blocker on the oxidation currents and membrane depolarizations revealed no significant

Table 1 Effects of Ca^{2+} concentration on the e.j.p. time constant of decay (τ e.j.p.; $n=9$ for all values)

Ca^{2+} concentration (mM)	Control (2.4 mM Ca^{2+}) τ e.j.p. (ms)	Test τ e.j.p. (ms)
0.6	289 ± 26	265 ± 19
0.9	275 ± 25	303 ± 16
1.2	292 ± 26	308 ± 27
1.8	275 ± 25	282 ± 19
2.4	265 ± 12	289 ± 16
3.6	292 ± 26	293 ± 19
4.8	282 ± 27	273 ± 25

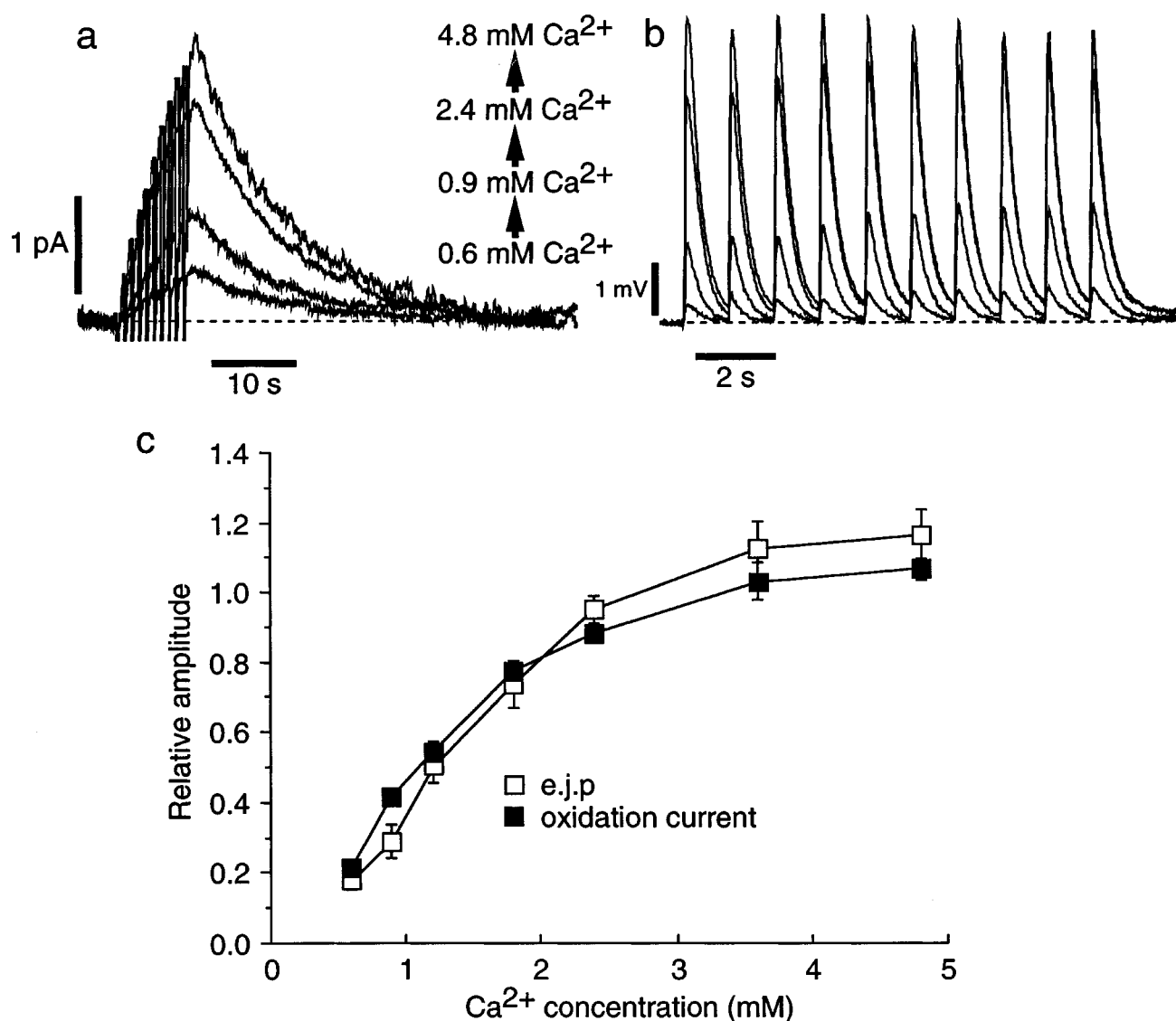


Figure 1 Effects of Ca^{2+} concentration on oxidation currents and e.j.ps evoked by trains of 10 stimuli at 1 Hz. (a and b) Overlaid traces showing oxidation currents (a) and e.j.ps (b) recorded in a range of Ca^{2+} concentrations (0.6–4.8 mM). (c) Graph showing the effects of Ca^{2+} concentration on the amplitudes of oxidation currents ($n=9$) and e.j.ps ($n=9$) evoked during the trains of stimuli. The data presented are normalized with respect to values obtained under control conditions (2.4 mM Ca^{2+}) at the start of each experiment.

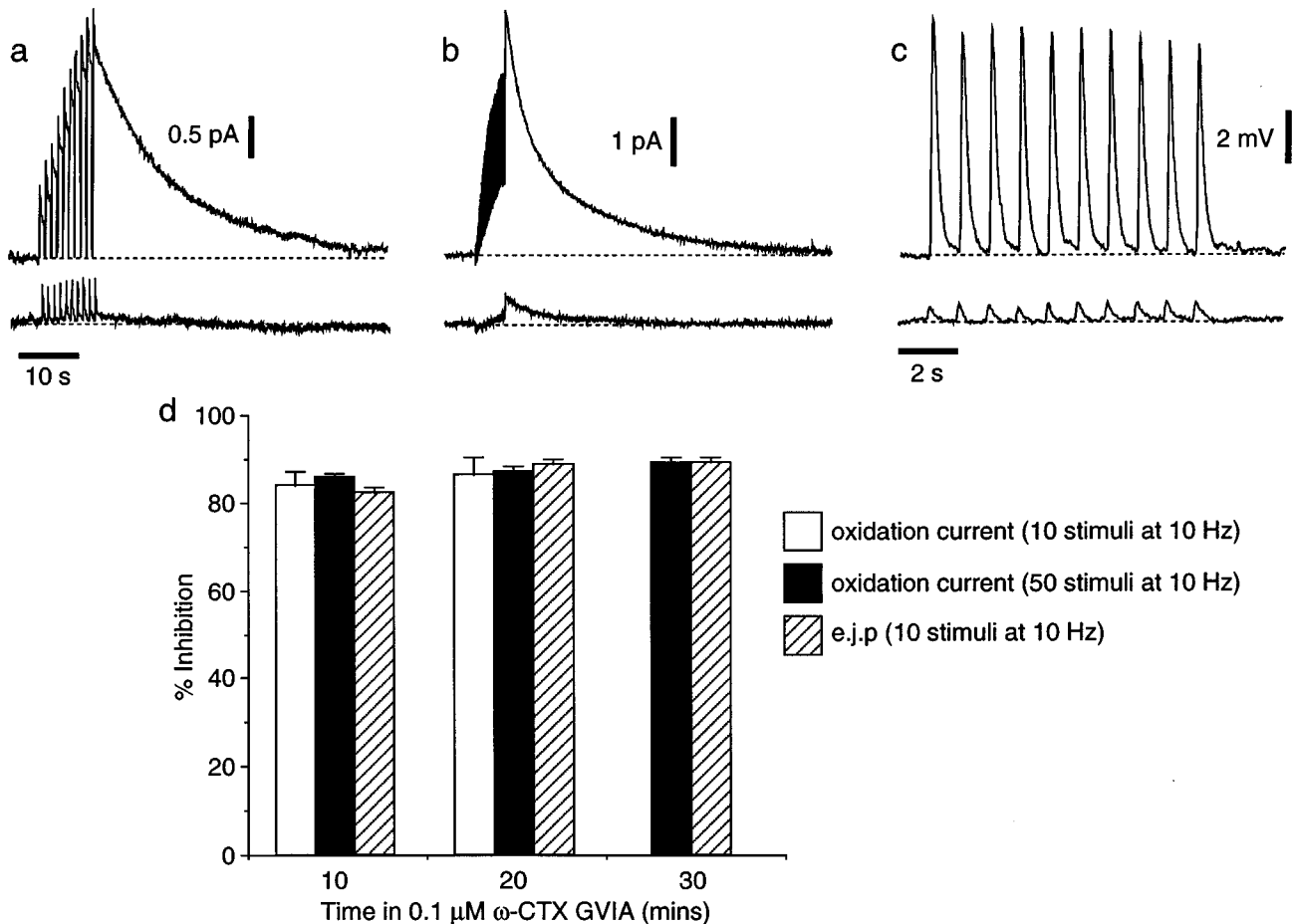


Figure 2 Effects of ω -CTX GVIA (0.1 μ M) on oxidation currents and e.j.ps. (a and b) Oxidation currents evoked by trains of ten stimuli at 1 Hz (a) and 50 stimuli at 10 Hz (b) before (upper traces) and 20 min after application of ω -CTX GVIA (lower traces). (c) E.j.ps evoked by trains of ten stimuli at 1 Hz before (upper trace) and 20 min after application of ω -CTX GVIA (lower trace). (d) Graph showing the mean inhibitory effect of ω -CTX GVIA on the amplitude of oxidations currents evoked by ten stimuli at 1 Hz ($n=8$) and 50 stimuli at 10 Hz ($n=26$) and e.j.ps evoked by ten stimuli at 1 Hz ($n=22$).

difference (Mann-Whitney U -tests; nifedipine $P=0.26$; ω -AgTx IVA $P=0.08$; ω -CTX MVIIC $P=0.42$).

Table 2 shows the effects of the Ca^{2+} channel blockers on both the time constant of decay of the stimulation-evoked membrane depolarizations and the smooth muscle membrane potential measured at the end of the electrophysiological experiments, following the addition of Cd^{2+} (0.1 mM). Comparison of the differences in time constant and membrane potential measured at the start and end of the experiments revealed no significant difference between the effects of the Ca^{2+} blockers (Kruskall-Wallis test $P=0.07$ for the time constant and 0.91 for the membrane potential).

Discussion

The present study demonstrates that both the NA-induced oxidation currents and the e.j.ps evoked by electrical stimulation of the perivascular sympathetic nerves supplying the tail artery have a similar dependence on extracellular Ca^{2+} concentration and sensitivity to the blocking action of a range of Ca^{2+} channel antagonists. As e.j.ps in the rat tail artery are most probably caused by neuronally released adenosine 5'-triphosphate (ATP) acting at P2X-purinoreceptors (Sneddon & Burnstock, 1984; McLaren *et al.*, 1995), the present findings

indicate that the release of both NA and ATP is modulated in parallel.

A potential problem with the electrophysiology experiments investigating the effects of Ca^{2+} concentration on e.j.p amplitude, is that released ATP activates non-selective cation channels with a relatively high Ca^{2+} permeability (see Benham, 1989; Evans *et al.*, 1996). However, it is unlikely that changing the Ca^{2+} concentration significantly affects the size of the ATP-gated unitary currents for the following reasons. (1) In vascular smooth muscle bathed in a physiological solution, Ca^{2+} carries only 5–10% of the inward current through ATP-activated channels (Benham, 1989). (2) The ATP-evoked current in cells homologously expressing the P2X₁-purinoreceptor subunit, which is the predominant subunit present in vascular smooth muscle (Kennedy, 1996), is unaffected by changes in the extracellular Ca^{2+} concentration (0.3–10 mM, Evans *et al.*, 1996). (3) The amplitudes of spontaneous excitatory junction currents recorded in guinea-pig vas deferens, which are believed to result from release of single quanta of ATP from the postganglionic sympathetic nerve terminals, are unaffected by changes in the extracellular Ca^{2+} concentration (0.3–1.8 mM, Cunnane & Ziogas, 1990).

The Ca^{2+} channel blocker ω -CTX GVIA reduced the amplitude of both the oxidation currents and e.j.ps by about 90%, suggesting that neurotransmitter release from sympathetic nerves innervating the tail artery is triggered primarily

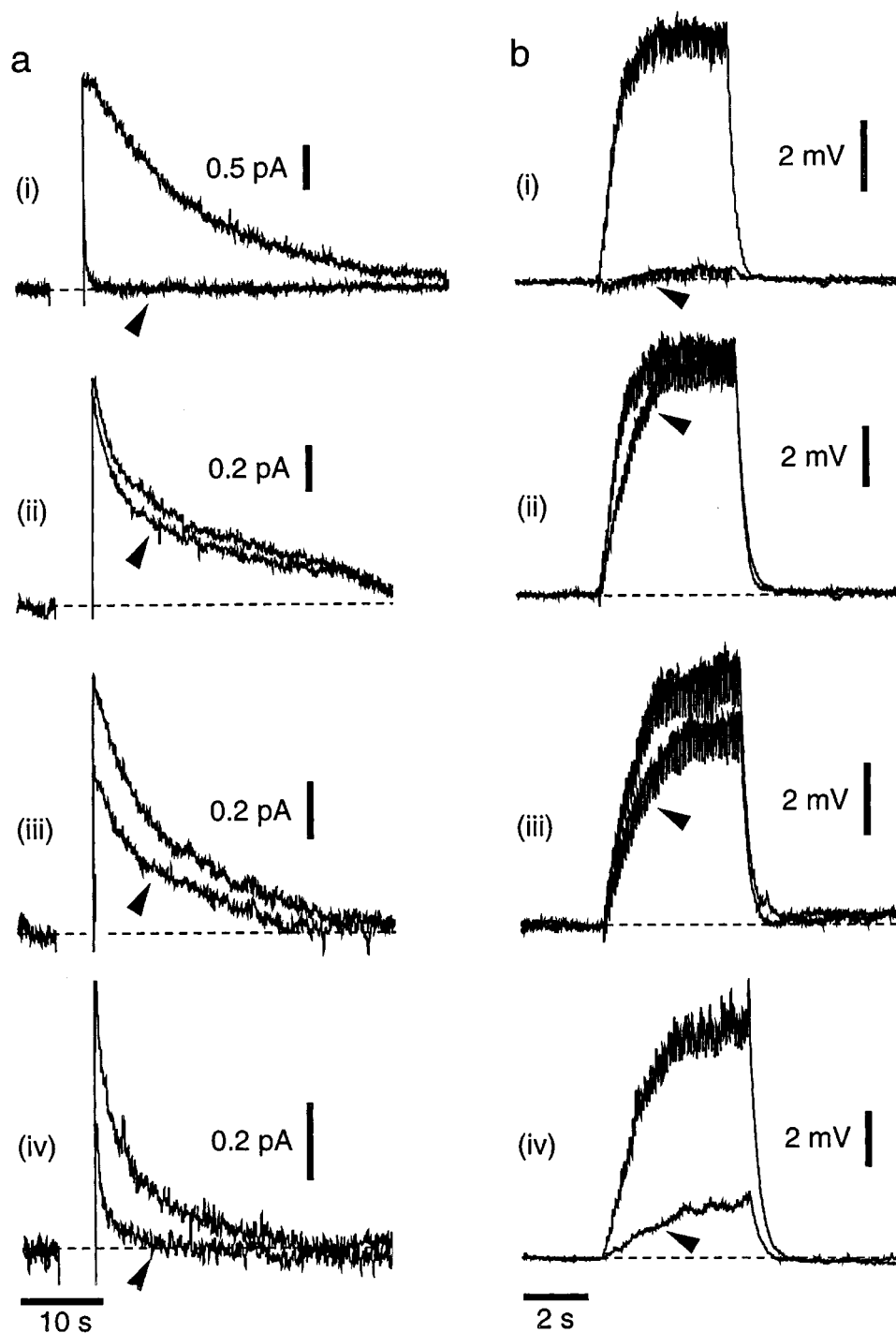


Figure 3 Effects of various Ca²⁺ channel blockers on ω -CTX GVIA resistant oxidation currents (a) and e.j.ps (b) evoked by trains of 50 stimuli at 10 Hz. (a and b) Overlaid traces recorded before and 30 min after (indicated by the arrowhead) application of Cd²⁺ (0.1 mM, a(i) and b(i)), nifedipine (10 μ M, a(ii) and b(ii)), ω -AgTx IVA (40 nM, a(iii) and b(iii)) and ω -CTX MVIIC (0.5 μ M, a(iv) and b(iv)).

by the opening of N-type Ca²⁺ channels. A similar finding has also been reported for rat tail artery labelled with [³H]NA, in which ω -CTX GVIA (0.1 μ M) reduced the stimulus evoked release of tritium by about 90% (Clasbrummel *et al.*, 1989).

In the presence of a maximally effective concentration of ω -CTX GVIA, electrical stimulation of the nerves still evoked oxidation currents and e.j.ps. These resistant responses were blocked by the non-selective Ca²⁺ channel blocker, Cd²⁺, demonstrating that they were due to Ca²⁺-dependent release of neurotransmitter. The resistant responses were also substan-

tially reduced in amplitude by ω -CTX MVIIC (80–90% inhibited), which, under the conditions of the experiment, would be expected to block P- and Q-type Ca²⁺ channels (Randell & Tsien, 1995). A low concentration of ω -AgTx IVA (40 nM), which should fully block P-type Ca²⁺ channels (K_d ~1 nM in rat cerebellar Purkinje neurons, Mintz & Bean, 1993), also reduced the amplitude of the ω -CTX GVIA resistant oxidation currents and membrane depolarizations. The L-type Ca²⁺ channel blocker, nifedipine, had no inhibitory effect on either the oxidation currents or membrane

Table 2 Effects of Ca^{2+} channel blockers on the smooth muscle resting membrane potential and the time constant of decay of the membrane depolarization

Treatment	Control (0.1 μM ω -CTX GVIA)		Test	
	r.m.p. (mV)	τ (ms)	r.m.p. (mV)	τ (ms)
0.1 μM ω -CTX GVIA	-62.8 ± 0.8	276 ± 27	-60.5 ± 1.7	287 ± 40
10 μM Nifedipine	-63.3 ± 0.4	273 ± 17	-60.0 ± 1.9	325 ± 14
40 nM ω -AgTx IVA	-63.3 ± 1.0	262 ± 13	-59.7 ± 1.6	263 ± 17
0.5 μM ω -CTX MVIIC	-64.5 ± 0.6	263 ± 16	-59.7 ± 1.4	341 ± 27

The time constant of decay of the depolarization (τ) was measured before and 30 min after adding the drug. The resting membrane potential (r.m.p.) was measured at the start of the experiment and following the addition of Cd^{2+} (0.1 mM) at the end of the experiment. The ω -CTX GVIA data is for time matched controls. For all values $n=6$.

depolarizations. Comparison of the effects of ω -CTX MVIIC, ω -AgTx IVA and nifedipine on oxidation currents and e.j.ps revealed no significant difference. These findings indicate that ω -CTX GVIA resistant release of NA and ATP is due primarily to opening of P-/Q-type Ca^{2+} channels.

There was no significant difference in the magnitude of the inhibitory effect of ω -CTX GVIA on oxidation currents evoked by trains of ten pulses at 1 Hz and 50 pulses at 10 Hz, or on e.j.ps evoked by ten pulses at 1 Hz. The effects of ω -CTX GVIA on e.j.ps evoked by higher frequencies of stimulation were not investigated because, under control conditions, e.j.ps summate and trigger muscle action potentials. However, the findings with the NA-induced oxidation current suggest that the magnitude of the inhibitory effect of ω -CTX GVIA is not changed if the frequency of stimulation is altered. This finding contrasts with those of previous studies in which the postjunctional response has been used to monitor neurotransmitter release from post-ganglionic sympathetic nerves. For example, in mouse, rat and guinea-pig vas deferens, the ω -CTX GVIA resistant responses of the smooth muscle were only revealed when the frequency of stimulation was increased (Smith & Cunneane, 1996; Wright & Angus, 1996; Waterman, 1997). These findings have led to the conclusion that raising the frequency of stimulation recruits the ω -CTX GVIA resistant component of neurotransmitter release. This does not appear to be the case in rat tail artery.

The effects of ω -CTX GVIA on oxidation currents and e.j.ps in rat tail artery suggests that NA and ATP release are modulated in parallel. Similar conclusions have been made for the mouse, rat and guinea-pig vas deferens where ω -CTX GVIA had a similar inhibitory effect on the noradrenergic and purinergic components of the neurally evoked contraction (Maggi *et al.*, 1988; Brock *et al.*, 1989; De Luca *et al.*, 1990; Waterman, 1997). Also in rat anococcygeus, both the slow (noradrenergic) and fast (purinergic) components of the e.j.p. evoked by sympathetic nerve stimulation were affected in a similar manner by a range of Ca^{2+} channel antagonists (Smith & Cunneane, 1997). It was therefore surprising that Westfall *et al.* (1996) reported that the release of endogenous NA in guinea-pig vas deferens was markedly inhibited by ω -CTX GVIA (0.1 μM) whereas that of endogenous ATP was only slightly inhibited. The release of ATP was substantially reduced by ω -AgTx IVA (0.1 μM). These findings for ATP release in the guinea-pig vas deferens are at odds with those for the purinergic component of the neurally evoked contraction and for e.j.ps which are very substantially reduced by ω -CTX GVIA (Maggi *et al.*, 1988; Brock *et al.*, 1989; Smith & Cunneane, 1996). In addition, in the guinea-pig vas deferens, e.j.ps are insensitive to ω -AgTx IVA (0.1 μM , Smith & Cunneane, 1996). Since the purinergic component of the

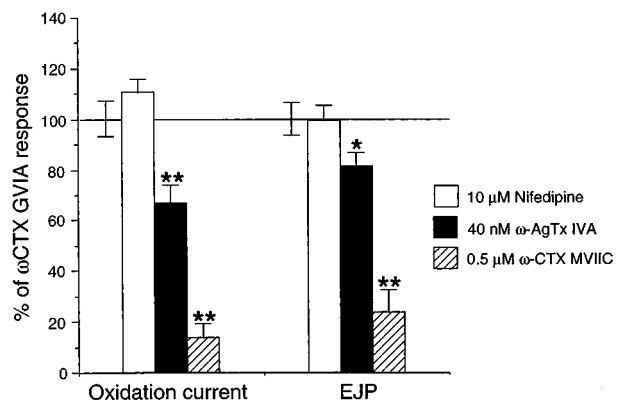


Figure 4 Graph showing the effects of 30 min applications of various Ca^{2+} channel blockers on the amplitude of ω -CTX GVIA resistant oxidation currents and e.j.ps evoked by trains of 50 stimuli at 10 Hz. The % change for tissues treated with 10 μM nifedipine ($n=6$), 40 nM ω -AgTx IVA ($n=6$) or 0.5 μM ω -CTX MVIIC ($n=6$) is plotted relative to that for time matched ω -CTX GVIA (0.1 μM) treated controls ($n=6$). The s.e.mean bars for the control data are plotted on 100% line. * $P<0.05$, ** $P<0.01$ (one-tailed test).

neurally evoked contractions and the e.j.ps are believed to provide a measure of junctionally active ATP in guinea-pig vas deferens (see Stjärne, 1995), the ATP overflow detected in the study of Westfall *et al.* (1996) is unlikely to reflect that released at neuroeffector junctions.

In the present study it has been assumed that e.j.ps provide a measure of ATP release. However, if the ATP overflow detected in the study of Westfall *et al.* (1996) is shown to represent that released at neuroeffector junctions, then it must be concluded that the e.j.ps in the guinea-pig vas deferens are not due to released ATP. Both in muscular arteries and rodent vasa deferentia the e.j.ps evoked by electrical stimulation are resistant to α - and β -adrenoceptor antagonists but are blocked by a range of agents known to block or desensitize smooth muscle P2X-purinoreceptors (see Brock and Cunneane, 1993). Furthermore, the non-cholinergic e.j.ps evoked in guinea-pig bladder detrusor smooth muscle when the parasympathetic nerves are excited are also blocked by agents that prevent activation of purinoreceptors by ATP (Hashitani & Suzuki, 1995; Bramich & Brading, 1996). Taken together these findings provide strong supporting evidence for the view that e.j.ps evoked in muscular arteries and in rodent vasa deferentia are not due to released NA and are mediated through activation of purinoreceptors. As ATP is predominant purine co-localized with NA in synaptic vesicles isolated from sympathetic nerve terminals (see Klein & Lagercrantz, 1981), it is reasonable to assume that the e.j.ps in these tissues are due to activation of purinoreceptors by released ATP.

In conclusion, the results of the present study indicate that the release of NA and ATP from sympathetic nerves supplying the rat tail artery is similarly affected by changes that modify Ca^{2+} entry into the nerve terminals. Previous studies have also shown in tail artery that the amplitudes of oxidation currents and e.j.ps are increased and decreased in a parallel manner activating prejunctional β -adrenoceptors and α_2 -adrenoceptors respectively (Msghina *et al.*, 1992; Brock *et al.*, 1997). Taken together these findings provide strong support for the

hypothesis that NA and ATP are co-released together from the sympathetic nerve terminals.

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Acetylcholine-induced membrane potential changes in endothelial cells of rabbit aortic valve

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1. Using a microelectrode technique, acetylcholine (ACh)-induced membrane potential changes were characterized using various types of inhibitors of K⁺ and Cl[−] channels in rabbit aortic valve endothelial cells (RAVEC).
2. ACh produced transient then sustained membrane hyperpolarizations. Withdrawal of ACh evoked a transient depolarization.
3. High K⁺ blocked and low K⁺ potentiated the two ACh-induced hyperpolarizations. Charybdotoxin (ChTX) attenuated the ACh-induced transient and sustained hyperpolarizations; apamin inhibited only the sustained hyperpolarization. In the combined presence of ChTX and apamin, ACh produced a depolarization.
4. In Ca²⁺-free solution or in the presence of Co²⁺ or Ni²⁺, ACh produced a transient hyperpolarization followed by a depolarization. In BAPTA-AM-treated cells, ACh produced only a depolarization.
5. A low concentration of A23187 attenuated the ACh-induced transient, but not the sustained, hyperpolarization. In the presence of cyclopiazonic acid, the hyperpolarization induced by ACh was maintained after ACh removal; this maintained hyperpolarization was blocked by Co²⁺.
6. Both NPPB and hypertonic solution inhibited the membrane depolarization seen after ACh washout. Bumetanide also attenuated this depolarization.
7. It is concluded that in RAVEC, ACh produces a two-component hyperpolarization followed by a depolarization. It is suggested that ACh-induced Ca²⁺ release from the storage sites causes a transient hyperpolarization due to activation of ChTX-sensitive K⁺ channels and that ACh-activated Ca²⁺ influx causes a sustained hyperpolarization by activating both ChTX- and apamin-sensitive K⁺ channels. Both volume-sensitive Cl[−] channels and the Na⁺-K⁺-Cl[−] cotransporter probably contribute to the ACh-induced depolarization.

Keywords: endothelial cell; aortic valve; acetylcholine; potassium channel; chloride channel

Abbreviations: ACh, acetylcholine; BAPTA-AM, acetoxymethyl ester of bis-(aminophenoxy)ethane-*N, N, N', N'*-tetraacetic acid; ChTX, charybdotoxin; Cl_{vol} channel, volume-sensitive chloride channel; CPA, cyclopiazonic acid; DMSO, dimethyl sulphoxide; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; RAVEC, rabbit aortic valve endothelial cells

Introduction

The vascular endothelium plays an important role in the regulation of vascular tone through the release of vasorelaxing factors such as prostacyclin, endothelium-derived nitric oxide and endothelium-derived hyperpolarizing factor (Chen *et al.*, 1988). Acetylcholine (ACh) is a well-known stimulant that releases these vasorelaxing factors *via* an increase in the cellular concentration of Ca²⁺ ([Ca²⁺]_i) (Lückhoff & Busse, 1990b). In endothelial cells, ACh activates Ca²⁺-activated K⁺ (K_{Ca}) channels through an increase in [Ca²⁺]_i (Himmel *et al.*, 1994), thus causing a membrane hyperpolarization (Brunet & Bény, 1989; Mehrke & Daut, 1990; Chen & Cheung, 1992; Marchenko & Sage, 1993; Carter & Ogden, 1994; Sharma & Davis, 1994; Frieden & Bény, 1995). It has been suggested that this membrane hyperpolarization in endothelial cells in turn increases Ca²⁺ influx due to an increase in the electrical driving force for Ca²⁺ (Lückhoff & Busse, 1990b). Several types of K⁺ channels have been found to be expressed in endothelial cells (Chen & Cheung, 1992; Marchenko & Sage, 1996; Wang *et al.*, 1996; Nilius *et al.*, 1997c). However, exactly which K⁺ channels contribute to the ACh-induced hyperpolarization in

intact endothelial cells under physiological conditions is not entirely clear.

It has been reported that ACh produces not only hyperpolarization but also depolarization in endothelial cells (Marchenko & Sage, 1993). Vascular endothelial cells express both Cl[−] transporters (Klein & O'Neill, 1990; Perry & O'Neill, 1993) and Cl[−] channels (Nilius *et al.*, 1997b), and it is known that Cl[−] plays an important role in the regulation of the membrane potential and cell volume in endothelial cells (Nilius *et al.*, 1997c). However, the roles played by Cl[−] channels and Cl[−] transporters in the ACh-induced membrane potential changes have not yet been clarified.

It has been reported that membrane potential changes evoked in smooth muscle cells can propagate into endothelial cells since these two types of cells are electronically coupled in a unidirectional way (Weid & Bény 1993; Bény & Pacicca, 1994; Marchenko & Sage, 1994b; Bény, 1997). This indicates the difficulty of recording electrical activity that truly arises from endothelial cells when intact vascular tissues are used. An alternative strategy might be to use cultured or isolated endothelial cells, but some alterations in receptors, ion channels and agonist-activated intracellular signal transduction systems have been noted in such cells (Sturek *et al.*, 1991). It has been found that the endothelial cells of canine aortic

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valves can release endothelium-derived relaxing factors (Ku *et al.*, 1990), suggesting that aortic valvular endothelial cells may be functionally similar to vascular endothelial cells. It is also known that the intact rabbit aortic valve is relatively thin and has no smooth muscle cells underlying the surface endothelial monolayer. Thus, the endothelium of the rabbit aortic valve is a preparation that allows us to circumvent the above problems.

The aim of the present study was to elucidate the characteristic features of the membrane potential changes induced by ACh in rabbit aortic valve endothelial cells (RAVEC). The ionic mechanisms underlying the ACh-induced electrical responses were pharmacologically assessed using a range of inhibitors of K^+ channels, Cl^- channels and Cl^- transporters.

Methods

Male Japanese White albino rabbits (supplied by Kitayama Labes Co. Ltd., Japan), weighing 1.9–2.3 kg, were anaesthetized by injection of pentobarbitone sodium (40 mg kg⁻¹, i.v.) and then exsanguinated. The protocols used conformed with guidelines on the conduct of animal experiments issued by Nagoya City University Medical School and by the Japanese government (Law no. 105; Notification no. 6), and were approved by The Committee on the Ethics of Animal Experiments in Nagoya City University Medical School. The heart was rapidly excised and placed in a chamber filled with Krebs solution. The aorta was opened by a longitudinal incision at the attachment to the left ventricle. The aortic valve was dissected out under a binocular microscope and transferred to a chamber of 0.5 ml volume set up on an inverted-microscope (Diaphoto TMD, Nikon, Japan). The chamber was superfused with oxygenated Krebs solution at a rate of 4 ml min⁻¹.

Recording of membrane potential

The membrane potential was measured using conventional glass microelectrodes filled with 1 M KCl. The resistance of the electrode was 120–180 M Ω . To impale an endothelial cell, the electrode was advanced gently towards the surface of the aortic valve using a micromanipulator (model MHW-3; Narishige International, Tokyo, Japan) until the sudden appearance of a negative potential (Weid & Bény, 1992). The experimental protocol was started when the membrane potential had been stable for over 5 min after impalement. Successful recordings were usually obtainable for from 20 min to 5 h. Membrane potentials were recorded using an Axoclamp-2B amplifier (Axon Instruments, Foster, CA, U.S.A.) and were displayed on a cathode-ray oscilloscope (model VC-6020; Hitachi, Tokyo, Japan). The data were stored at an acquisition rate of 20 Hz using an AxoScope 1.1.1/Digidata 1200 data acquisition system (Axon Instruments, Foster, CA, U.S.A.) on an IBM/AT compatible PC.

To observe the concentration-dependent effects of ACh on membrane potential, ACh (0.01–10 μ M) was applied for 3 min at 25 min intervals in an ascending order in the same preparation. Maximum responses were obtained with 3 μ M ACh. The effects of inhibitors of K^+ channels and Cl^- channels were observed on the membrane potential changes induced by 3 μ M ACh. To this end, the ACh-induced response was first recorded, followed by a 25 min intermission. Next, one of a number of inhibitors was applied so that it was added 10 min before the 2nd application of ACh and was present throughout the period for which ACh was

applied. The concentrations of K^+ channel inhibitors were chosen so as to retain their selectivity. To study the effect of ACh in Ca^{2+} -free solution, nominal Ca^{2+} -free solution was first applied for 10 min, then ACh was repetitively applied to the same cell for 3 min at 25 min intervals in Ca^{2+} -free solution. To study the effect of the acetoxymethyl ester of bis-(aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA-AM) on the ACh-induced response, a control response was first recorded, and then BAPTA-AM (30 μ M) was applied for 40 min, followed by a 10 min washout of BAPTA-AM. Finally, ACh was again applied. Ni^{2+} (5 mM) or Co^{2+} (2 mM) was added 10 min before ACh and was present throughout the application of ACh. A23187 (0.1 μ M) was also added 10 min before ACh and was present throughout the application of ACh.

Solutions

The composition of the Krebs solution was as follows (mM): NaCl, 122; KCl, 4.7; $MgCl_2$, 1.2; $CaCl_2$, 2.6; $NaHCO_3$, 15.5; KH_2PO_4 , 1.2; glucose, 11.5. The solutions were bubbled with 95% O_2 and 5% CO_2 and their pH was maintained at 7.3–7.4. High K^+ solution was made by isotonic replacement of NaCl with KCl. Nominal Ca^{2+} -free solution was made by substituting an equimolar concentration of $MgCl_2$ for $CaCl_2$ (no EGTA added). To make hypertonic solutions, 20 mM sucrose or 20 mM D-mannitol was added to the Krebs solution.

Chemicals

Drugs used were acetylcholine hydrochloride (Daiichi Pharmaceutical Co. Ltd., Tokyo, Japan), A23187 and cyclopiazonic acid (CPA) (Calbiochem, La Jolla, CA, U.S.A.), BAPTA-AM (Dojin, Kumamoto, Japan), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) (Research Biochemicals International, Natick, MA, U.S.A.), bumetanide, niflumic acid and glibenclamide (Sigma, St. Louis, MO, U.S.A.), sucrose and D-mannitol, (Wako Pure Chemical, Osaka, Japan), apamin and charybdotoxin (ChTX) (Peptide Institute, Osaka, Japan).

A23187, CPA, BAPTA-AM and glibenclamide were dissolved in dimethyl sulphoxide (DMSO, Dojin, Kumamoto, Japan). The final concentration of DMSO when diluted in Krebs solution was 0.06% at its maximum. This concentration of DMSO had no effect on the ACh-induced electrical responses. NPPB was dissolved in ethanol (stock, 10 mM). Further dilutions were made using Krebs solution. The powdered forms of bumetanide, niflumic acid, sucrose and mannitol were directly dissolved in Krebs solution.

Statistics

All values are expressed as means $\pm$ s.e.mean; *n* denotes the number of valves. Statistical significance was determined using Student's paired and unpaired *t*-tests. Differences were considered to be significant at $P < 0.05$.

Results

ACh-induced membrane potential changes

The resting membrane potential of RAVEC was -49.6 ± 1.2 mV ($n = 29$). ACh (0.01–3 μ M) concentration-dependently produced a transient, followed by a sustained membrane hyperpolarization (Figure 1Aa). In the presence of

3 μM ACh, the sustained hyperpolarization could be maintained for over 10 min, but with a progressive reduction in its amplitude (Figure 1Ab). Following the removal of ACh, a transient membrane depolarization appeared. The depolarization reached its peak within 2 min and the membrane then repolarized to the original level within 15 min. The amplitude of this depolarization was dependent on the concentration of the pre-applied ACh (up to 1 μM) (Figure 1Aa and Ab). These responses to ACh were reproducible when ACh was applied at 25 min intervals.

High K^+ (50 mM) depolarized the membrane from -54.5 ± 1.7 to -23.5 ± 0.2 mV ($n=3$), and ACh (3 μM) did not modify the membrane potential in the presence of high K^+ (-23.5 ± 0.2 mV, $n=3$). Low K^+ (1.2 mM) did not alter the resting membrane potential (-49.3 ± 2.9 to -51.8 ± 2.9 mV, $P>0.05$, $n=4$) but it did enhance the ACh-induced transient (18.7 ± 1.7 to 38.6 ± 2.4 mV, $P<0.01$, $n=4$) and sustained (17.5 ± 3.0 to 37.6 ± 2.8 mV, $P<0.01$, $n=4$) hyperpolarizations.

Effects of K^+ -channel inhibitors on ACh-induced electrical responses (Figure 2)

Apamin (0.1 μM) did not modify the resting membrane potential (-51.5 ± 1.3 to -53.2 ± 1.0 mV, $P>0.05$, $n=6$) but it significantly attenuated the ACh-induced sustained hyperpolarization (15.3 ± 1.2 to 11.1 ± 1.5 mV, $P<0.05$, $n=6$) with no effect on the ACh-induced transient hyperpolarization (17.1 ± 1.3 to 16.4 ± 1.1 mV, $P>0.05$, $n=6$). Apamin significantly enhanced the depolarization seen after washout of ACh (8.8 ± 0.9 to 10.8 ± 1.5 mV, $P<0.05$, $n=6$). ChTX (50 nM) did not modify the resting membrane potential

(-49.7 ± 1.2 to -49.4 ± 0.9 mV, $P>0.05$, $n=5$) but it significantly attenuated the ACh-induced transient (16.5 ± 2.1 to 2.8 ± 2.8 mV, $P<0.01$, $n=5$) and sustained (16.6 ± 2.2 to 8.7 ± 1.1 mV, $P<0.05$, $n=5$) hyperpolarizations (Figure 2b). ChTX significantly enhanced the membrane depolarization

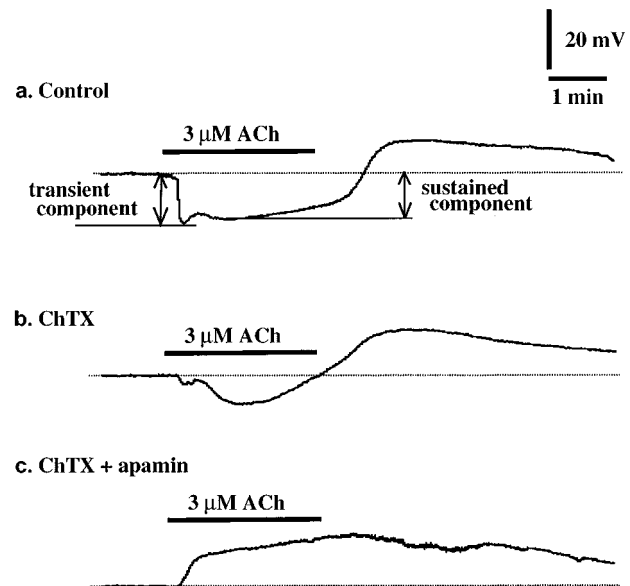


Figure 2 Effects of ChTX and apamin on ACh-induced membrane potential changes. Actual tracings of the effects of ChTX and apamin. (a) Control; (b) in the presence of ChTX (50 nM); (c) in the presence of both ChTX (50 nM) and apamin (0.1 μM). Recordings were all obtained from the same cell.

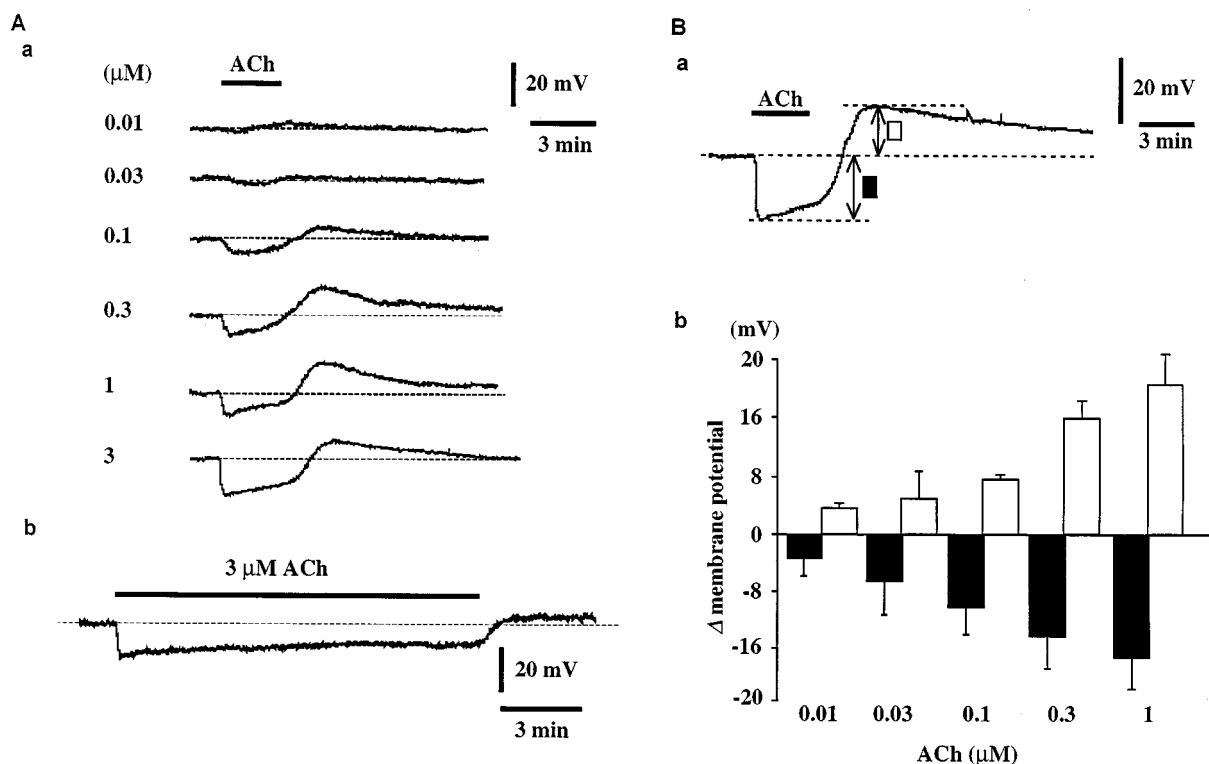


Figure 1 Effects of ACh on membrane potential in RAVEC. (Aa) Concentration-dependent effects of ACh on membrane potential. ACh (0.01–3 μM) was applied for 3 min. Recordings were all from the same preparation. (Ab) Hyperpolarization was maintained throughout a 10-min application of 3 μM ACh. (Ba) and (Bb) Summary of the effects of ACh on membrane potential. Bb shows the maximum hyperpolarization and depolarization elicited by each concentration of ACh (mean of data from three preparations, with s.e. shown by vertical lines). Ba indicates how these maxima were obtained.

seen after washout of ACh (8.4 ± 1.3 to 13.0 ± 1.2 mV, $P < 0.05$, $n = 5$). In the presence of ChTX (50 nM), application of apamin (0.1 μ M) did not modify the resting membrane potential (-51.2 ± 4.3 to -49.4 ± 4.7 mV, $P > 0.05$, $n = 3$). However, under these conditions, ACh produced a sustained depolarization, not a hyperpolarization, with a relatively slow onset (Figure 2c).

Glibenclamide (10 μ M) modified neither the resting membrane potential nor the ACh-induced hyperpolarization ($n = 3$). Ba^{2+} (0.1 mM) significantly depolarized the membrane by 10.4 ± 0.5 mV ($n = 3$, $P < 0.01$) but did not modify the ACh-induced hyperpolarization.

Effects of Ca^{2+} -free solution and BAPTA-AM on ACh-induced responses

At the end of a 10 min application of nominal Ca^{2+} -free solution, the membrane was depolarized by 9.3 ± 3.2 mV ($n = 4$). Repetitive application of ACh in Ca^{2+} -free solution at 25 min intervals resulted in a progressive alteration in the ACh-induced hyperpolarization: it changed from a sustained hyperpolarization (with a depolarization after removal of ACh) to a transient hyperpolarization followed, while the ACh was still present, by a depolarization ($n = 4$) (Figure 3a).

When RAVEC were pre-treated with 30 μ M BAPTA-AM for 40 min, the membrane potential was not significantly changed (-50.4 ± 0.2 to -53.2 ± 1.1 mV, $P > 0.05$, $n = 4$). In such BAPTA-AM-treated cells, ACh produced only a sustained depolarization (3.2 ± 1.8 mV, $n = 4$, Figure 3b).

Effects of Co^{2+} and Ni^{2+} on ACh-induced responses

Ni^{2+} (5 mM) and Co^{2+} (2 mM) depolarized the membrane by 6.4 ± 1.5 mV ($n = 5$) and 5.4 ± 2.5 mV ($n = 3$), respectively. In the presence of Ni^{2+} or Co^{2+} , ACh produced a transient hyperpolarization while the ACh was still present (Figure 4a and b). When Ni^{2+} was applied during the ACh-induced sustained hyperpolarization, the membrane response was converted to a depolarization ($n = 4$) (Figure 4c).

Effects of CPA and A-23187 on ACh-induced responses

CPA (3 μ M) itself produced a small hyperpolarization (3.4 ± 1.4 mV, $P < 0.05$, $n = 3$). In the presence of CPA, ACh produced a hyperpolarization which lasted long after the washout of ACh (Figure 5a). When ACh was again applied after a 30 min interval (in the continued presence of CPA), it produced only a small hyperpolarization. The membrane potential level reached as a result of the second application of ACh (-67.0 ± 3.0 mV, $P > 0.05$, $n = 3$) was similar to that produced by the first. Under these conditions, Co^{2+} converted the membrane response to a depolarization ($n = 3$) (Figure 5b).

A23187 (0.1 μ M) had no significant effect on the resting membrane potential but it did significantly attenuate the ACh-induced transient hyperpolarization (17.3 ± 1.7 to 6.5 ± 2.0 mV, $P < 0.01$) with no change in the amplitude of the sustained component (though its duration was increased) ($n = 4$, Figure 6).

Effects of inhibitors of Cl^- channels and Cl^- transporters on ACh-induced responses

Neither NPPB (20 nM) nor bumetanide (10 μ M) modified the resting membrane potential or the ACh-induced maximum hyperpolarization ($n = 4$). However, these agents did significantly attenuate the depolarization seen after the washout of ACh. The depolarizations were by 7.8 ± 0.9 and 4.4 ± 0.3 mV in the absence and presence of NPPB, respectively ($P < 0.05$, $n = 4$, Figure 7a), and by 6.9 ± 1.0 and 2.1 ± 0.9 mV in the absence and presence of bumetanide, respectively ($P < 0.01$, $n = 5$, Figure 7b). Niflumic acid (0.5 mM) hyperpolarized the membrane by 14.5 ± 2.5 mV ($n = 4$) but it had no effect on the depolarization seen after the removal of ACh. The peak amplitude of the depolarization was 10.2 ± 2.2 and 11.2 ± 0.1 mV in the absence and presence of niflumic acid, respectively ($P > 0.05$, $n = 3$).

Hypertonic solution containing 20 mM D-mannitol did not significantly modify either the resting membrane potential (-53.5 ± 1.1 to -54.3 ± 1.7 mV, $P > 0.05$, $n = 9$) or the ACh-induced maximum hyperpolarization (14.6 ± 1.6 to 13.1 ± 1.2 mV, $P > 0.05$, $n = 7$). This hypertonic solution,

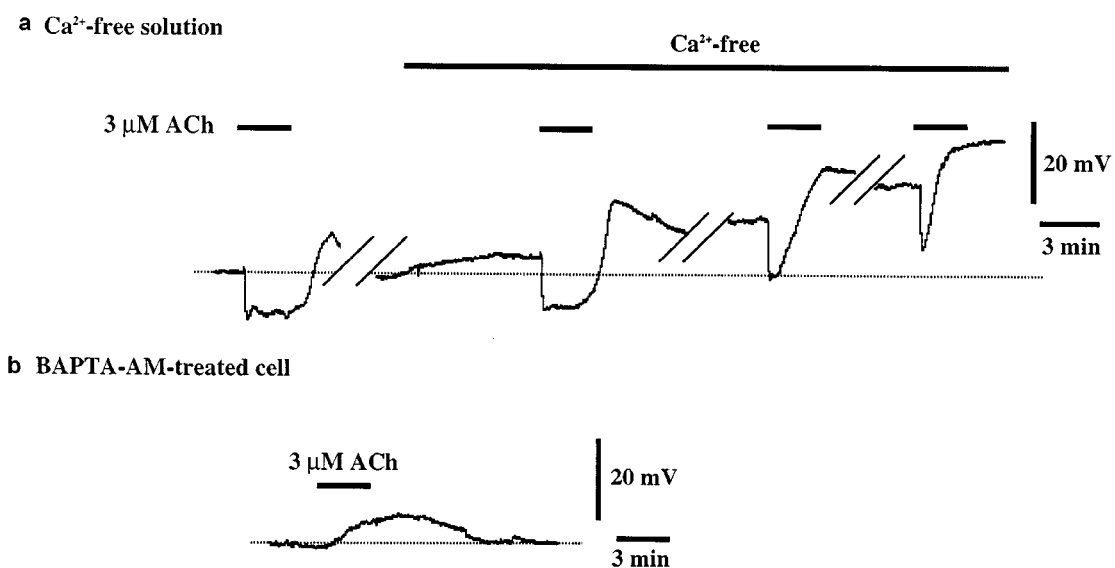


Figure 3 Effects of Ca^{2+} -free solution (a) and BAPTA-AM (b) on ACh-induced membrane potential changes. (a) ACh (3 μ M) was first applied in normal Krebs solution (as control). The preparation was then exposed to Ca^{2+} -free solution for 10 min, and ACh was subsequently applied at 25 min intervals. The traces were obtained from the same cell. Similar observations were made in two other cells. (b) BAPTA-AM (30 μ M) was pretreated for 40 min followed by a 10 min washout, then ACh (3 μ M) was applied. Similar observations were made in two other cells.

however, did significantly attenuate the depolarization seen after the removal of ACh (5.9 ± 1.1 to 1.9 ± 0.6 mV, $P < 0.05$, $n = 8$, Figure 7c). Similar results were obtained when the hypertonic solution used was made by the addition of 20 mM sucrose ($n = 4$, data not shown).

Discussion

ACh produces a transient, followed by a sustained hyperpolarization in the endothelial cells of the porcine coronary artery (Chen & Cheung, 1992) and in those of the rabbit aorta (Wang

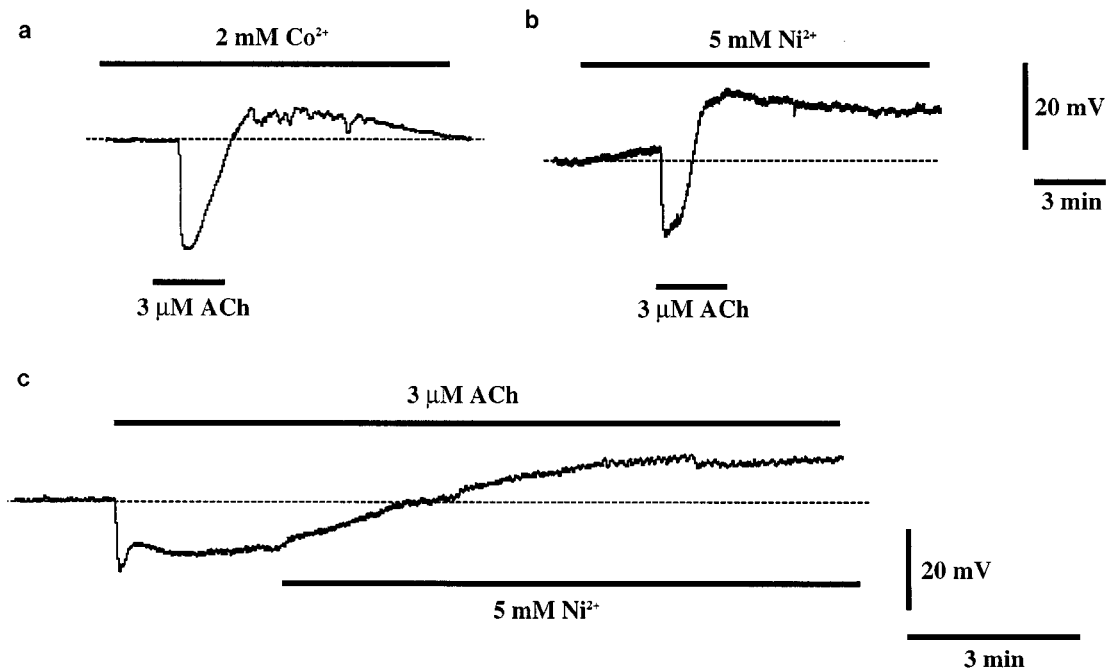


Figure 4 Effects of 2 mM Co²⁺ (a) and 5 mM Ni²⁺ (b and c) on ACh-induced membrane potential changes. ACh (3 μM) was applied for 3 min as indicated by bars (a and b). Co²⁺ and Ni²⁺ were added 10 min before ACh and were present throughout the application of ACh. (c) Ni²⁺ (5 mM) was applied during the sustained component of the hyperpolarization induced by 3 μM ACh.

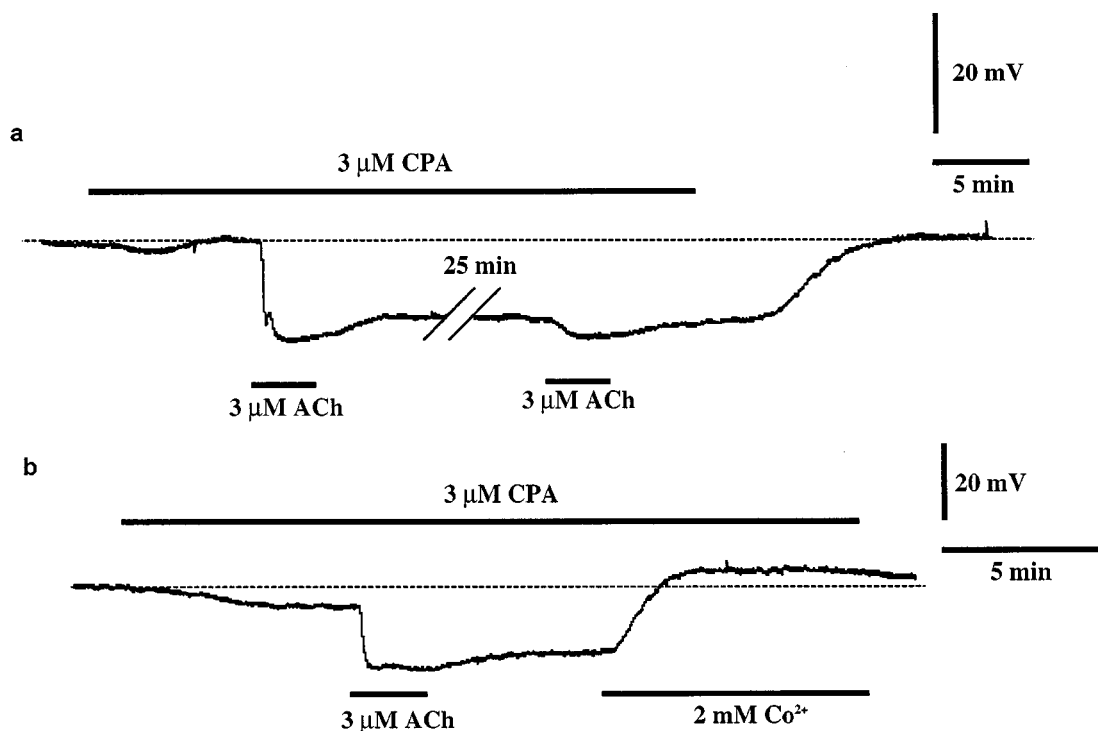


Figure 5 Effect of CPA (3 μM) on ACh-induced membrane potential changes. (a) In the presence of CPA (3 μM), the recovery from the ACh-induced hyperpolarization that occurred after washout of ACh was largely prevented. When ACh was again applied after an interval of 25 min (in the continued presence of CPA), ACh evoked only a small hyperpolarization. The hyperpolarization was maintained for as long as CPA was present. (b) In the presence of CPA, ACh produced a hyperpolarization that was maintained after the removal of ACh. When Co²⁺ (2 mM) was applied during this maintained hyperpolarization, the membrane response was converted to a depolarization.

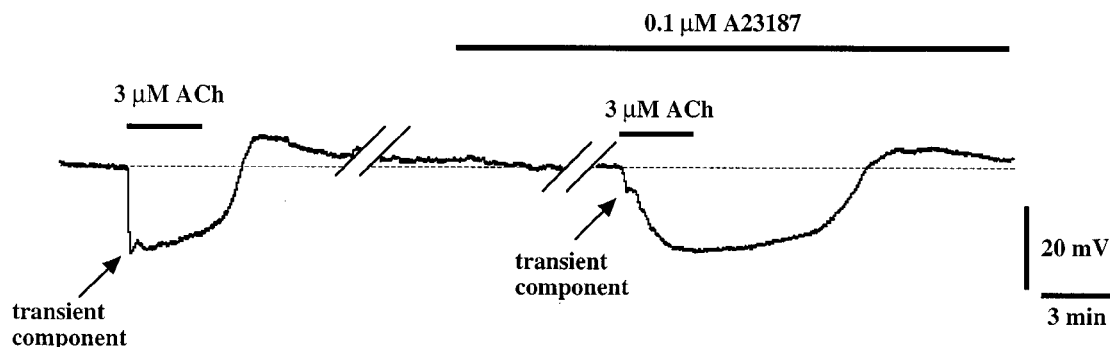


Figure 6 Effect of A23187 ($0.1 \mu\text{M}$) on ACh-induced membrane potential changes. The transient component of the ACh-induced hyperpolarization was attenuated in the presence of A23187.

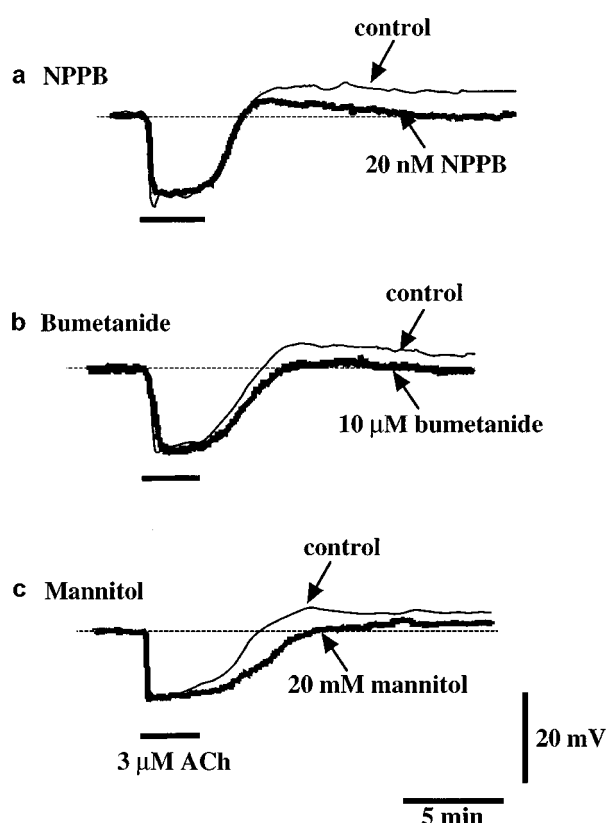


Figure 7 Effects of NPPB, bumetanide and mannitol on ACh-induced membrane potential changes. ACh-induced responses obtained before and after application of inhibitors [NPPB (20 nM , a), bumetanide ($10 \mu\text{M}$, b) and mannitol (20 mM , c)] in the same cell.

et al., 1996). In the endothelial cells of the intact rat aorta, ACh also produces a biphasic response: a transient hyperpolarization followed in that case by a depolarization (Marchenko & Sage, 1993; 1994a). In the present experiments, ACh produced a transient, then a sustained hyperpolarization (which could be maintained for at least 10 min) followed by a transient depolarization that occurred after the removal of the ACh. Thus, the ACh-induced membrane potential changes in endothelial cells may vary by species and by region.

ACh-induced hyperpolarization

In the present experiments, ACh produced a transient, followed by a sustained hyperpolarization. These two ACh-

induced responses were both abolished in high K^+ (50 mM) solution and enhanced in low K^+ (1.2 mM) solution. Furthermore, the ACh-induced hyperpolarization was abolished in BAPTA-AM-treated cells. These results indicate that in RAVEC, the ACh-induced hyperpolarization is due to an activation of those K^+ channels that are regulated by $[\text{Ca}^{2+}]_i$.

The ACh-induced transient hyperpolarization was preserved in Ca^{2+} -free solution and it was resistant to both Ni^{2+} and Co^{2+} . Furthermore, a low concentration of A23287 ($0.1 \mu\text{M}$), which is thought to act selectively on the Ca^{2+} storage sites (Itoh *et al.*, 1985), greatly attenuated the ACh-induced transient hyperpolarization but not the sustained hyperpolarization. These results suggest that the transient hyperpolarization is due to a release of Ca^{2+} from the storage sites. In contrast, the ACh-induced sustained hyperpolarization was abolished in Ca^{2+} -free solution and by Co^{2+} or Ni^{2+} . Furthermore, in the presence of CPA (an inhibitor of Ca^{2+} -ATPase in the Ca^{2+} storage sites: Li & van Breemen, 1996; Sasajima *et al.*, 1997), the ACh-induced hyperpolarization was maintained long after the washout of this agonist. This result is consistent with a previous finding in the endothelial cells of the guinea-pig coronary artery (Cheung & Chen, 1992). The sustained ACh-induced hyperpolarization seen in the presence of CPA was blocked by Co^{2+} . These results suggest that ACh activates Co^{2+} -sensitive Ca^{2+} influx, thus causing a sustained membrane hyperpolarization.

Several types of K^+ channels have been identified in endothelial cells (Lückhoff & Busse, 1990a; Sakai, 1990; Chen & Cheung, 1992; Sharma & Davis, 1994; Song & Davis, 1994; Nilius *et al.*, 1997c). It has been found that ChTX and tetraethylammonium, inhibitors of K_{Ca} channels, both inhibit the ACh-induced hyperpolarization seen in the endothelial cells of the guinea-pig coronary artery (Chen & Cheung, 1992) and in those of the rabbit aorta (Wang *et al.*, 1996). Recently, it has been reported that an apamin-sensitive K^+ channel is functionally expressed in the endothelial cells of the rat aorta (Marchenko & Sage, 1996) and in those of the human umbilical vein (Muraki *et al.*, 1997). In the present experiments, the transient component of the ACh-induced hyperpolarization was markedly attenuated by ChTX (but not by apamin). In contrast, the sustained component of the ACh-induced hyperpolarization was inhibited by ChTX and by apamin. Furthermore, a combined application of ChTX and apamin completely blocked both components of the ACh-induced hyperpolarization. Neither glibenclamide (an inhibitor of ATP-sensitive K^+ channels) nor Ba^{2+} (an inhibitor of inwardly rectifying K^+ channels) affected the ACh-induced hyperpolarization. These results suggest that the ACh-induced transient hyperpolarization is probably due to an activation of

ChTX-sensitive K_{Ca} channels, while the sustained hyperpolarization is due to an activation of both ChTX-sensitive and apamin-sensitive K_{Ca} channels.

In the present experiments, CPA ($3 \mu\text{M}$) and A23187 ($0.1 \mu\text{M}$) by themselves produced only minor hyperpolarizations. In preliminary experiments, however, we found that $0.3 \mu\text{M}$ A23187 produced a profound hyperpolarization (~ 12 – 15 mV), suggesting that the small hyperpolarization induced by either agent in the experiments reported here might have been due to them being in a concentration barely sufficient to produce hyperpolarization. In line with this hypothesis, it was reported a few years ago that in pig coronary artery endothelial cells, A23187 produces a hyperpolarization at $0.5 \mu\text{M}$ (Weid & Bény, 1992).

ACh-induced depolarization

In the present experiments, a transient membrane depolarization was seen after the removal of ACh. Although the membrane hyperpolarization could be sustained as long as ACh was present (at least up to 10 min), application of Ni^{2+} or Co^{2+} converted this response to a depolarization. Furthermore, in Ca^{2+} -free solution, the transient hyperpolarization was followed by a depolarization while the ACh was still present. Moreover, in the combined presence of ChTX and apamin, ACh evoked only a sustained depolarization of slow onset. The sustained component of the hyperpolarization diminished in amplitude as a function of time despite continuous stimulation by ACh, so these results, taken together, suggest that the depolarization phase was being generated while ACh was still present and that it was being masked by the hyperpolarization. This idea is consistent with previous findings by Marchenko & Sage (1993, 1994a).

In the present experiments, an ACh-induced depolarization was observed in BAPTA-AM-treated cells. Furthermore, niflumic acid (an inhibitor of Ca^{2+} -activated Cl^- channels, Nilius *et al.*, 1997a) failed to inhibit the ACh-induced depolarization, suggesting that $[\text{Ca}^{2+}]_i$ does not play an important role in the production of the ACh-induced depolarization. It has been suggested that endothelial cell volume is increased by vasoactive agonists (bradykinin,

angiotensin II, vasopressin) through an activation of Na^+ - K^+ - Cl^- co-transport (O'Donnell, 1993). Furthermore, it has been found that the volume-sensitive chloride current is cross-correlated with changes in cell volume in endothelial cells of the bovine pulmonary artery (Nilius *et al.*, 1996; 1997b; Voets *et al.*, 1998). In the present experiments, the ACh-induced depolarization was markedly attenuated by bumetanide (an inhibitor of the Na^+ - K^+ - Cl^- transporter) (Cabantchik & Greger, 1992), by NPPB (a non-selective Cl^- channel inhibitor) (Cabantchik & Greger, 1992) and by the presence of hypertonic conditions. It is thought that Na^+ - K^+ - Cl^- co-transport could be electrically neutral (Haas, 1994). Thus, we speculate that the final pathway for the ACh-induced depolarization may involve an activation of volume-sensitive Cl^- (Cl_{Vol}) channels. Taken together, these results could be taken to suggest that ACh increases cell volume through an activation of the Na^+ - K^+ - Cl^- co-transporter, thus leading to Cl_{Vol} -channel activation. However, it remains to be clarified how Cl_{Vol} channels might be activated during stimulation with ACh.

In the present study, neither NPPB nor hypertonic solution changed the resting membrane potential. In accordance with the findings of Nilius *et al.* (1996) in the endothelial cells of the bovine pulmonary artery, this result suggests that Cl_{Vol} channels may not be activated under resting conditions.

In conclusion, in RAVEC, ACh produces a transient, followed by a sustained membrane hyperpolarization due to a co-activation of ChTX-sensitive and apamin-sensitive K^+ channels. It is also suggested that volume-sensitive Cl^- channels most likely contribute to the ACh-induced membrane depolarization (a phase of the response that is largely concealed by the ACh-induced hyperpolarization under physiological conditions).

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Long term orexigenic effect of a novel melanocortin 4 receptor selective antagonist

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1 We designed and synthesized several novel cyclic MSH analogues and tested their affinities for cells expressing the MC1, MC3, MC4 and MC5 receptors.

2 One of the substances HS028 (cyclic [AcCys¹¹, dichloro-D-phenylalanine¹⁴, Cys¹⁸, Asp-NH₂²²]- β -MSH_{11–22}) showed high affinity (K_i of 0.95 nM) and high (80 fold) MC4 receptor selectivity over the MC3 receptor. HS028 thus shows both higher affinity and higher selectivity for the MC4 receptor compared to the earlier first described MC4 receptor selective substance HS014.

3 HS028 antagonised a α -MSH induced increase in cyclic AMP production in transfected cells expressing the MC3 and MC4 receptors, whereas it seemed to be a partial agonist for the MC1 and MC5 receptors.

4 Chronic intracerebroventricularly (i.c.v.) administration of HS028 by osmotic minipumps significantly increased both food intake and body weight in a dose dependent manner without tachyphylaxis for a period of 7 days.

5 This is the first report demonstrating that an MC4 receptor antagonist can increase food intake and body weight during chronic administration providing further evidence that the MC4 receptor is an important mediator of long term weight homeostasis.

Keywords: Melanocortin (MC) receptor subtypes; MSH (melanocyte stimulating hormone); ligand binding; cyclic AMP; HS028; chronic i.c.v. administration; food intake

Abbreviations: ACSF, artificial cerebrospinal fluid; ACTH, adrenocorticotropin; cAMP, adenosine 3':5'-cyclic monophosphate; SV40, CV-1 Origin; COS-1, DIEA, N,N-diisopropylethylamine; DMF, N,N-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, O-[7-azabenzotriazol-1-yl]1,1,3,3-tetramethyluronium hexafluorophosphate; pentafluorophenyl; HOAt, OPfp-hydroxy-7-azabenzotriazole; HPLC, high performance liquid chromatography; MC, melanocortin; MeCN, acetonitrile; MSH, melanocyte stimulating hormone; NDP-MSH, [Nle⁴, D-Phe⁷] α -MSH; IBMX, isobutylmethylxanthine; Tris, tris-(hydroxymethyl)aminomethane

Introduction

The melanocortin (MC) receptors include five subtypes and belong to the super family of G-protein coupled receptors (Chhajlani *et al.*, 1993; Chhajlani & Wikberg, 1992; Gantz *et al.*, 1993a,b; Mountjoy *et al.*, 1992). The MC1 receptor is well characterized and has an important role in skin and fur pigmentation in a variety of vertebrates (Cone *et al.*, 1996). It also plays a role in immune responses (Lipton & Catania, 1997). The MC2 receptor (i.e. the ACTH (adrenocorticotropin) receptor) participates in the regulation of steroid production in the adrenal gland. The MC3 receptor is found in the brain and in placenta and gut tissues (Gantz *et al.*, 1993a; Roselli-Refuss *et al.*, 1993) but has still a much less defined physiological role. The MC4 receptor has only been found in the CNS, where it is expressed in several different sites, including in the cortex, thalamus, hypothalamus, brain stem and spinal cord (Gantz *et al.*, 1993b; Mountjoy *et al.*, 1994). Studies in a strain of mouse in which the MC4 receptor has been knocked out have related the MC4 receptor to control of weight homeostasis (Huszar *et al.*, 1997) while work on MC5

receptor knockouts has suggested a role of this receptor in exocrine gland function (Chen *et al.*, 1997).

The 131 amino acids long agouti protein is an antagonist at the MC receptors, including the MC3 and MC4 receptors. Ectopic over-expression of the agouti protein gives rise to an obesity syndrome. Disruption of the MC4 receptor in knockout mice caused obesity that showed similar characteristics as that found for the obesity syndrome in agouti mice (Huszar *et al.*, 1997). Single injections of the non-selective MC3/MC4 receptor agonist MTII and the non-selective antagonist SHU9119 (Fan *et al.*, 1997) and the selective MC4 receptor antagonist HS014 (Kask *et al.*, 1998b) influenced food intake in experimental animals providing further evidence to the hypothesis that melanocortinergic neurones exert a tonic inhibition of feeding behaviour.

The natural melanocortin peptides (α -MSH, β -MSH, γ -MSH and ACTH) do not clearly discriminate between the different MC receptor subtypes, with the exception that α -MSH is selective for the MC1 receptor and ACTH is selective for the MC2 receptor (Schiöth *et al.*, 1995; 1996a,b). Development of MC receptor antagonist and selective MC receptor active compounds for, in particular, the MC3, MC4 and MC5 receptors is still an important task as only few such substances have been forwarded.

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In this study we synthesized several new cyclic MSH analogues and discovered a novel substance, HS028, which is a selective MC4 receptor antagonist showing both higher affinity and much higher selectivity for the MC4 receptor above the MC3 receptor than the earlier discovered HS014. We also report the effects of HS028 on food intake and body weight during 7 days of i.c.v. infusion in rats.

Methods

Chemicals

[Nle⁴, D-Phe⁷]- α -MSH (NDP-MSH) was radio iodinated by the chloramine T method and purified by HPLC (high performance liquid chromatography). NDP-MSH and all amino acid derivatives were purchased from Neosystem, France.

Peptide synthesis

The novel peptides tested in this study were synthesized in our laboratory using the solid phase approach with subsequent purification by HPLC. The correct molecular weights of the peptides were confirmed by mass spectrometry. The peptide sequences were assembled by using Pioneer, PerSeptive Biosystems peptide synthesis system. Fmoc(9-fluorenylmethoxycarbonyl)-amino acid derivatives were used in coupling steps. When OPfp (pentafluorophenyl) esters were used the synthesis cycle was as follows: (a) the Fmoc group was removed by 20% piperidine in DMF (N,N-dimethylformamide) (5 min); (b) to form a new peptide bound side chain-protected Fmoc-amino acid OPfp ester (4 eq.) and HOAt (1-hydroxy-7-azabenzotriazole) (4 eq.) were dissolved in DMF and circulated through the column for 30–60 min; (c) to cap residual amino groups the support was treated with 0.3 M Ac₂O (acetic anhydride) in DMF for 5 min. If free acids were used, then in step (b) side chain-protected Fmoc-amino acid (4 eq.), HATU (O-[7-azabenzotriazol-1-yl]-1,3,3-tetramethyluronium hexafluorophosphate) (4 eq.) and DIEA (N,N-diisopropylethylamine) (4 eq.) were applied. For deprotection a reagent mixture (trifluoroacetic acid) – phenol – anisole – 1,2-ethanedithiol – water, 82:2:2:2:2, 2.5 h) was used. The raw peptides formed were purified by HPLC (10 × 250 mm column, Vydac RP C18, 90A, 201HS1010, eluent – 20–35% MeCN (acetonitrile) in water + 0.1% trifluoroacetic acid, detection at 240 nm).

Expression of receptor clones

The human MC1 and human MC5 receptors (Chhajlani & Wikberg 1992; Chhajlani *et al.*, 1993) were cloned into the expression vector pRc/CMV (InVitrogen). The human MC3 and human MC4 receptors, cloned into the expression vector pCMV/neo, were a gift from Dr Ira Gantz (Gantz *et al.*, 1993a,b). For receptor expression COS-1 (CV-1 Origin, SV40) cells were grown in Dulbecco's modified Eagles medium with 10% foetal calf serum. Eighty per cent confluent cultures were transfected with the DNA (approximately 1 μ g DNA for every 1 × 10⁶ cells) mixed with liposomes in serum free medium (for details see Schiöth *et al.*, 1996b). The number of receptors, using this amount of DNA was about 4,000–20,000 binding sites/cell for all of the receptor types derived from the B_{max} of saturation binding studies which were performed as earlier reported (Schiöth *et al.*, 1995). After transfection, the serum-free medium was replaced by serum-containing medium and the cells were cultivated for about 48 h. Cells were then scraped off, centrifuged, and used for radioligand binding.

Binding studies

The transfected cells were washed with binding buffer (see Schiöth *et al.*, 1995) and distributed into 96-well non-culture-coated plates, which were centrifuged and the binding buffer was removed. The cells were then immediately incubated in the well plates for 2 h at 37°C with 0.05 ml binding buffer in each well containing a constant concentration of [¹²⁵I]-NDP-MSH and appropriate concentrations of the competing unlabelled ligand. After incubation the cells were washed with 0.2 ml of ice-cold binding buffer and detached from the plates with 0.2 ml of 0.1 N NaOH. Radioactivity was counted (Wallac, Wizard automatic gamma counter) and data were analysed with a software package suitable for radioligand binding data analysis (Wan System AB, Umeå, Sweden). Data were analysed by fitting to formulas derived from the law of mass action by the method generally referred to as computer modelling. The K_d values for [¹²⁵I]-NDP-MSH for the MC receptors were taken from Schiöth *et al.*, 1995; 1996b. The binding assays were performed in duplicate wells and repeated three times. Untransfected COS-1 cells did not show any specific binding for [¹²⁵I]NDP-MSH. The non-specific binding was generally low, typically being less than 5% of the total binding at 2 nM concentration of labelled NDP-MSH and 3 μ M cold NDP-MSH, measured by saturation analysis which were performed as earlier reported (Schiöth *et al.*, 1995).

cyclic AMP assay

The transfected cells were harvested and incubated for 30 min at 37°C with 0.05 ml serum free Dulbecco's modified Eagles medium in each tube, containing 0.5 mM IBMX (isobutylmethylxanthine) and appropriate concentrations of α -MSH or HS028. After incubation with the indicated drugs, cyclic AMP (adenosine 3':5'-cyclic monophosphate) was extracted with perchloric acid at final concentration 0.4 M. After centrifugation, the protein free supernatants were neutralised with 5 M KOH/1 M Tris (tris-(hydroxymethyl)aminomethane). 0.05 ml of the neutralised cyclic AMP extract or a cyclic AMP standard (dissolved in distilled water) was added to a 96 well microtiter plate. The content of cyclic AMP was then estimated essentially according to Nordstedt & Fredholm (1990), by adding to each well [³H]-cyclic AMP (0.14 pmol, approximately 11,000 c.p.m., specific activity 54 Ci/mmol⁻¹, Amersham) and bovine adrenal binding protein and incubating at 4°C for 150 min. Standards containing non-labelled cyclic AMP were also assayed concomitantly with the samples. The standard curve ranged from 0.125–8.0 pmol/sample. Samples from each experiment were always assayed with the corresponding standard curve, using the same labelled cyclic AMP and binding protein solutions diluted from stocks directly before use. The incubates were thereafter harvested by filtration on Whatman GF/B filters using a semiautomatic Brandel cell harvester. Each filter was rinsed with 3 ml 50 mM Tris/HCl pH 7.4. The filters were punched out and put into scintillation vials with scintillation fluid and counted. The cyclic AMP assays were performed in duplicate wells and repeated three times. Both inter and intra-assay coefficients of variation were below 25%.

Drug treatment, food consumption and body weight

Thirty-four male Wistar rats weighing 200–300 g were fed a standard, commercial laboratory diet (rat and mouse maintenance diet No. 1, Special Diets Services, Essex, U.K.) and water *ad libitum* under standard conditions using a 12:12-h

light-dark cycle. One-week before the administration of drugs by i.c.v. infusion, the rats were housed individually in plastic cages. On the day the operation treatment started, the rats were assigned randomly to one of the following six treatment groups: (a) 1.0 $\mu\text{L h}^{-1}$ i.c.v. vehicle (buffered artificial cerebrospinal fluid, ACSF, control group; $n=6$); (b) 0.0007 nmol h^{-1} i.c.v. HS028 ($n=3$); (c) 0.0007 nmol h^{-1} i.c.v. HS028 ($n=6$); (d) 0.007 nmol h^{-1} i.c.v. HS028 ($n=6$); (e) 0.07 nmol h^{-1} i.c.v. HS028 ($n=5$); (f) 0.7 nmol h^{-1} i.c.v. HS028 ($n=4$). An additional group received 0.1 nmol h^{-1} i.c.v. HS014 ($n=4$). HS028 and HS014 were dissolved in ACSF. Prior to the infusions, a brain infusion kit connected to an osmotic minipump (Alzet, 2001, Alza corp., Palo Alto, CA, U.S.A.) was implanted into the right lateral cerebral ventricle (1.0 mm posterior and 1.5 mm lateral to the bregma in rats anaesthetized with methohexital sodium (50 mg kg^{-1} i.p. Brietal®, Lilly, Indianapolis, IN, U.S.A.). Dental glass ionophor (ChemFil(R) Superior, Dentsply DeTray GmbH, D-78467 Konstanz, Germany) and a stainless steel screw were used to secure the infusion cannula in position. The osmotic minipumps were placed s.c. in the midscapular region. The osmotic minipumps were activated by immersion in 0.9% saline at room temperature 12 h before the implantation, so that the infusion started immediately upon implantation of the pumps. The room temperature was monitored at $22 \pm 1^\circ\text{C}$ and the humidity $50 \pm 5\%$. Food intake and body weight were recorded in the first 3 h of light phase every other day for 1 week prior to the implantation of the infusion device and for the 1-week infusion period. After the 7 days infusion, the animals were sacrificed and injected with a small amount of a dye through the infusion kit where after the brains were dissected and positive staining of the cerebral ventricles verified.

Statistical analysis

The average 24-h body weight gain and food intake for each animal was measured during the last 4 days before the operation (control period), as well as during the last 5 days of the 7 days infusion period. These values were then subtracted to obtain each animals changes in weight gain and food intake from the control period to the infusion period. In addition, each animals initial weight was defined as its weight

24 h before the first control measurement. The differences in weight gain and food intake from the control period to the infusion period were then analysed with analysis of covariance using the animals initial weight as a covariate (JMP version 3.02, SAS Institute Inc., Cary, NC, U.S.A.) calculating the least squares means for the change in each parameter for each infusion protocol. Additionally, specific preplanned contrasts were applied comparing each dose of HS028 to ACSF as well as 0.7 nmol h^{-1} HS028 to 0.0007 nmol h^{-1} HS028, utilizing Bonferroni corrections of P values keeping the error rate less than 0.05.

Animal ethics

Experimental procedures were carried out in accordance with guidelines of the European Community, local laws and policies.

Results

We designed and synthesized several new cyclic MSH analogues. The structure of the novel substances are aligned with α -MSH, NDP-MSH, MTII, SHU9119 and HS014 in Figure 1. The human DNAs for the MC1, MC3, MC4 and MC5 receptors were transiently and independently expressed in COS-1 cells for competitive receptor binding assays using [^{125}I]-NDP-MSH as radioligand. The expression levels of the different receptor subtypes were similar (data not shown). The K_i values for the different peptides resulting from calculations of the competition curves of binding with [^{125}I]-NDP-MSH are summarized in Table 1. For comparison, we also included in Table 1 the K_i values of α -MSH, NDP-MSH, MTII, SHU9119 and HS014, which values we recently reported (Schiöth *et al.*, 1997b; 1998a) using the same method as in the present study.

In our recent study (Schiöth *et al.*, 1998a), we generated substances with a disulphide bridge between Cys residues in positions 4 and 11 and found them to be clearly selective for the MC4 receptor. In this study, we made several modifications on this basic ring structure (represented in HS964) as well as the previously most potent and MC4 receptor selective substance HS014 which has the same ring structure as HS964,

Position nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Peptide															
α -MSH	Ser	Tyr	Ser	Met	Glu	His	Phe	Arg	Trp	Gly	Lys	Pro	Val		
NDP-MSH	Ser	Tyr	Ser	Nle	Glu	His	D-Phe	Arg	Trp	Gly	Lys	Pro	Val		
MTII				Nle	<u>Asp</u>	His	D-Phe	Arg	Trp	<u>Lys</u>					
SHU9119				Nle	<u>Asp</u>	His	D-Nal	Arg	Trp	<u>Lys</u>					
HS964				<u>Cys</u>	Glu	His	D-Nal	Arg	Trp	Gly	<u>Cys</u>				
HS014				<u>Cys</u>	Glu	His	D-Nal	Arg	Trp	Gly	<u>Cys</u>	Pro	Pro	Lys	Asp
HS015				<u>Cys</u>	Glu	Pro	D-Nal	Arg	Trp	Gly	<u>Cys</u>				
HS017				<u>Cys</u>	Glu	Glu	D-Nal	Arg	Trp	Gly	<u>Cys</u>				
HS023				<u>Cys</u>	Glu	Gly	D-Nal	Arg	Trp	Gly	<u>Cys</u>				
HS016				<u>Cys</u>	Arg	His	D-Nal	Arg	Trp	Gly	<u>Cys</u>	Asp	Arg	Phe	
HS018			<u>Cys</u>	Glu	His	Gly	D-Nal	Arg	Trp	<u>Cys</u>					
HS019	Pro	Tyr	Arg	<u>Cys</u>	Glu	His	D-Nal	Arg	Trp	Gly	<u>Cys</u>	Pro	Pro	Lys	Asp
HS028				<u>Cys</u>	Glu	His	dCl-D-Phe	Arg	Trp	Gly	<u>Cys</u>	Pro	Pro	Lys	Asp
HS029				<u>Cys</u>	Glu	His	pF-D-Phe	Arg	Trp	Gly	<u>Cys</u>	Pro	Pro	Lys	Asp
HS030				<u>Cys</u>	Glu	His	pNO-D-Phe	Arg	Trp	Gly	<u>Cys</u>	Pro	Pro	Lys	Asp

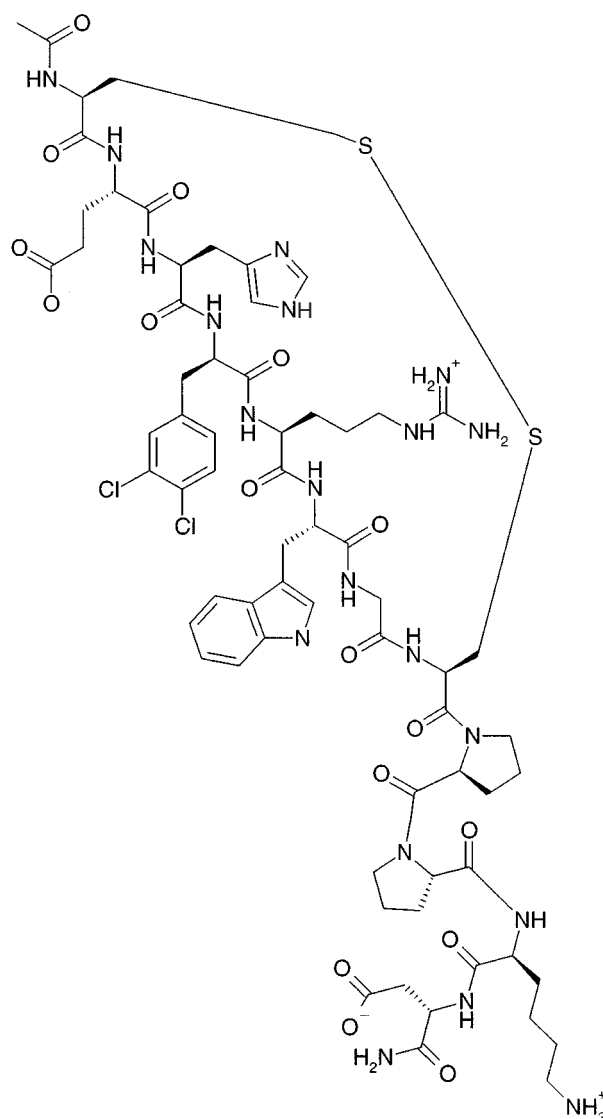
Figure 1 Alignment of α -MSH, NDP-MSH, MTII, SHU9119 and HS014 to the new analogues evaluated in this study. All peptides have an acetyl-group on the N-terminus and an amide group on the C-terminus. The amino acid residues which make up the ring closure in the cyclic compounds are shown underlined in italics.

Table 1 K_i values (means $\pm$ s.e.mean) obtained from competition curves, for MSH analogues on MC1, MC3, MC4 and MC5 receptor transfected COS cells

Ligand	MC1 K_i (nmol/l)	MC3 K_i (nmol/l)	MC4 K_i (nmol/l)	MC5 K_i (nmol/l)
α -MSH*	0.230 $\pm$ 0.089	31.5 $\pm$ 3.9	900 $\pm$ 97	7160 $\pm$ 860
NDP-MSH*	0.109 $\pm$ 0.010	0.469 $\pm$ 0.038	2.93 $\pm$ 0.34	5.50 $\pm$ 0.11
MTII*	0.686 $\pm$ 0.109	34.1 $\pm$ 4.4	6.60 $\pm$ 0.82	46.1 $\pm$ 7.9
SHU9119*	0.714 $\pm$ 0.161	1.20 $\pm$ 0.30	0.360 $\pm$ 0.059	1.12 $\pm$ 0.31
HS964**	1,460 $\pm$ 440	281 $\pm$ 40	23.2 $\pm$ 4.4	164 $\pm$ 27
HS014**	108 $\pm$ 62	54.4 $\pm$ 37.6	3.16 $\pm$ 1.16	694 $\pm$ 237
HS015	170 $\pm$ 35	280 $\pm$ 30	238 $\pm$ 22	178 $\pm$ 39
HS017	31,400 $\pm$ 1800	31,800 $\pm$ 9500	3,640 $\pm$ 710	15,000 $\pm$ 1,600
HS023	1,730 $\pm$ 460	3,000 $\pm$ 1600	138 $\pm$ 56	2,260 $\pm$ 1,240
HS016	27.6 $\pm$ 10.9	5.68 $\pm$ 2.01	4.42 $\pm$ 1.28	46.4 $\pm$ 7.0
HS018	> 300,000	> 300,000	604 $\pm$ 251	> 300,000
HS019	108 $\pm$ 27	46.9 $\pm$ 20	5.8 $\pm$ 1.9	51.6 $\pm$ 4.2
HS028	60.1 $\pm$ 16.2	73.7 $\pm$ 11.0	0.95 $\pm$ 0.12	211 $\pm$ 89
HS029	123 $\pm$ 36	962 $\pm$ 299	21.5 $\pm$ 2.2	2,630 $\pm$ 880
HS030	770 $\pm$ 227	407 $\pm$ 257	19.5 $\pm$ 4.7	728 $\pm$ 83

*Data from Schiöth *et al.*, 1997, **1998a by courtesy of the publisher. The structures of the substances can be seen in Figure 1.

but it is distinguished from HS964 by a C-terminal tail mimicking the C-terminal of β -MSH. We made three substances where we changed amino acids in position 6, thus in HS015, HS017 and HS023 we replaced the basic hydrophilic His⁶ by the non-polar and hydrophobic residue Pro⁶ (HS015), the polar hydrophilic residue Glu⁶ (HS017) and the small non-polar residue Gly⁶ (HS023), respectively. For HS015 (Pro⁶), this change cancelled out the selective character as compared with HS964. For HS017 (Glu⁶) the affinities were substantially reduced for all the receptor subtypes even though the MC4 receptor selective binding properties were maintained. For HS023 (Gly⁶) the affinities were lowered for all the receptor subtypes, except for the MC1 receptor. This peptide had about 20 fold selectivity for the MC4 receptor beyond the MC3 receptor which is similar to that of HS014. One of the peptides (HS007) originating from our earlier study (Schiöth *et al.*, 1998a) has Arg in position 5. This peptide displayed selective character for the MC3 receptor. We here synthesized an analogue of HS007, termed HS016, having a C-terminal tail (Asp¹²-Arg¹³-Phe¹⁴) being identical to the C-terminal sequence of γ -MSH, which is a natural peptide shown to have preference for the MC3 receptor (Schiöth *et al.*, 1996b). However, HS016 is virtually non-selective, showing equal affinities for the MC3 and MC4 receptors, and only slightly lower affinities for the MC1 and MC5 receptors. Recently, we introduced this very same sequence in NDP-MSH, also without leading to enhancement of the affinity or selectivity for the MC3 receptor (Schiöth *et al.*, 1998b). We also made another peptide, also having a 26 membered ring as HS964, where the Gly¹⁰ was removed, and a Gly was inserted in position 6 generating a distance between the His⁶ and the D-Nal⁷-Arg⁸-Trp⁹ motifs in the peptide. This change totally abolished the binding to the MC1, MC3 and MC5 receptors, whereas the binding to the MC4 receptor was only lowered 26 fold compared with HS964. We also made four analogues based on HS014. HS019 has an N-terminal tail (Pro¹-Tyr²-Arg³), which is identical to the amino acids of the corresponding positions in the β -MSH peptide. HS019 has very similar affinity for all the receptors as HS014, except that the affinity for the MC5 receptor was increased 13 fold. Moreover, we made three replacements of the D-Nal⁷ with three different bulky hydrophobic residues with D-conformation showing resemblance with D-Nal. The replacements of D-Nal⁷ by 4-parafuoro-D-phenylalanine (pF-D-Phe⁷) (HS029) and 4-paranitro-D-phenylalanine (pNO₂-D-Phe⁷) (HS030) lowered the affinity for all the receptor subtypes for both the peptides.

**Figure 2** Primary structure of the MC4 receptor selective analogue; HS028.

However, when D-Nal⁷ was replaced by 3,4-dichloro-D-phenylalanine (dCl-D-Phe⁷) (HS028), the affinity was increased for the MC4 receptor, and lowered for the MC3

receptor compared with HS014. HS028 has 78 fold selectivity for the MC4 receptor beyond the MC3 receptor. This is a considerably higher selectivity compared to that of HS014, which shows 17 fold affinity difference between these same receptors. The primary structure of HS028 is shown in Figure 2. Competition binding curves of HS028 for the human MC1, MC3, MC4 and MC5 receptors are shown in Figure 3.

On the basis of these results we selected HS028 for further testing and investigated the cyclic AMP response of α -MSH and HS028 in COS-1 cells expressing the human MC1, MC3, MC4 and MC5 receptors. These results are shown in Figure 4. As can be seen from the figure, α -MSH stimulated the accumulation of cyclic AMP in all the cell types. COS-1 cells which had not been transfected with any of the MC receptors did not respond to α -MSH (data not shown). HS028 did stimulate accumulation of cyclic AMP in the MC1 and MC5 receptor expressing cells but without the cyclic AMP reaching the same levels as for α -MSH. For the MC3 receptor, HS028 in concentrations up to 100 μ M did not affect the cyclic AMP levels. Instead, 0.1 μ M HS028 was found to completely block the cyclic AMP increase induced by α -MSH. For the MC4 receptor, HS028 blocked the α -MSH response while HS028 on itself did not affect cyclic AMP at least until a dose of 50 nM. At this dose and higher, we observed up to 2 fold increase in cyclic AMP for both HS028 itself and HS028 in combination with α -MSH.

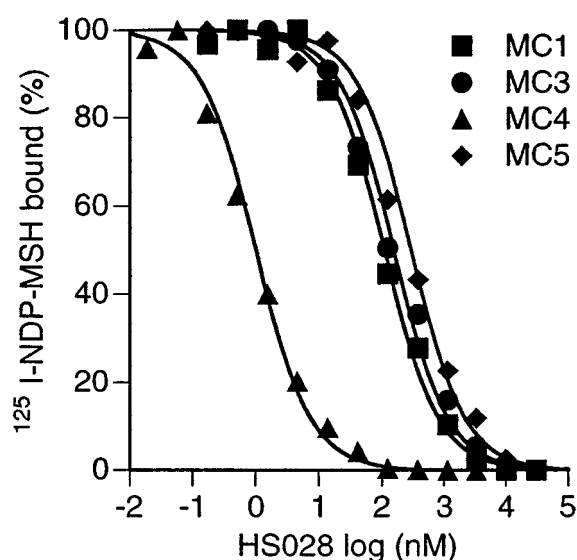


Figure 3 Binding data showing high selectivity of HS028 for the MC4 receptor beyond the other MC receptors. Competition curves of HS028 obtained on COS-1 cells transfected with the MC receptor clones, obtained by using a fixed concentration of [^{125}]NDP-MSH and varying concentrations of the non-labelled competing peptide. Competing peptides used are indicated on abscissa for each panel. Each experiment were performed in duplicates and repeated three times.

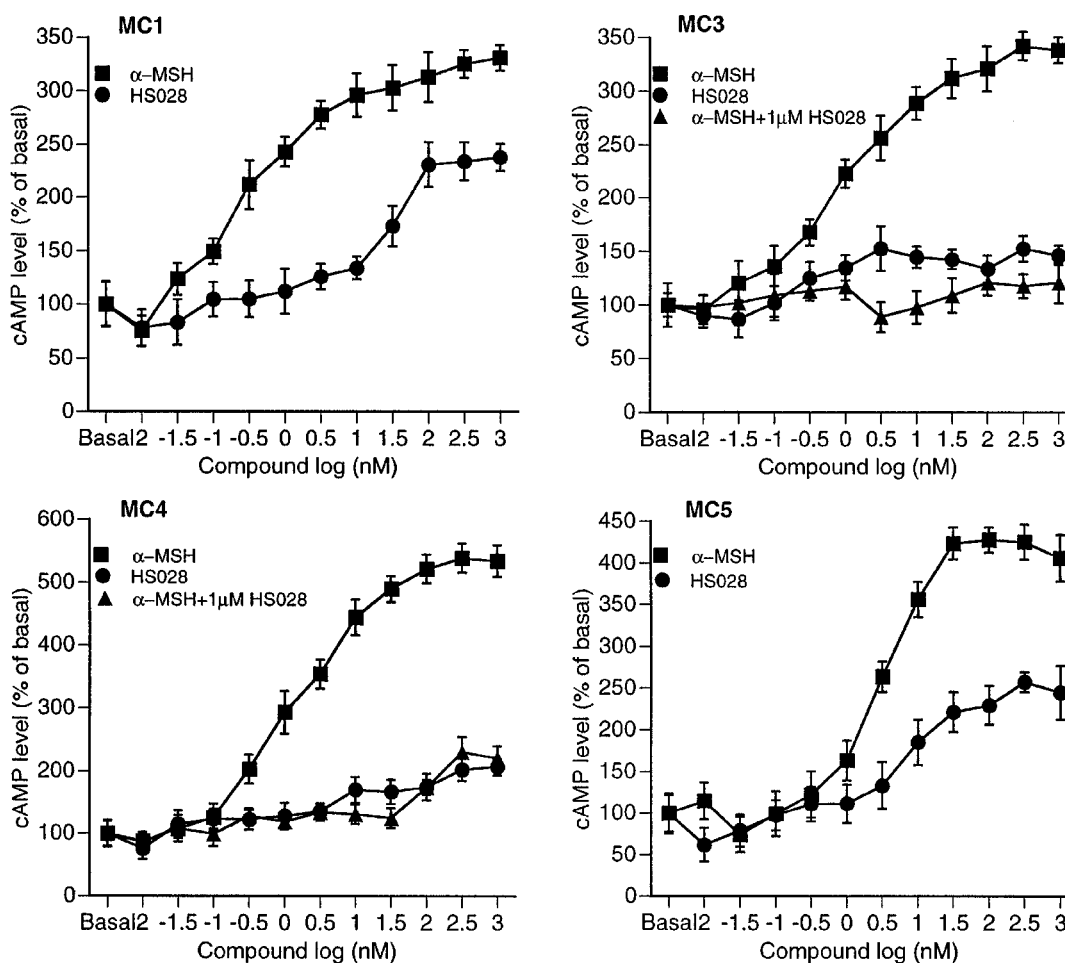


Figure 4 Generation of cyclic AMP in MC receptor transfected COS-1 cells. Each point represents the average $\pm$ s.e. mean ($n=6$).

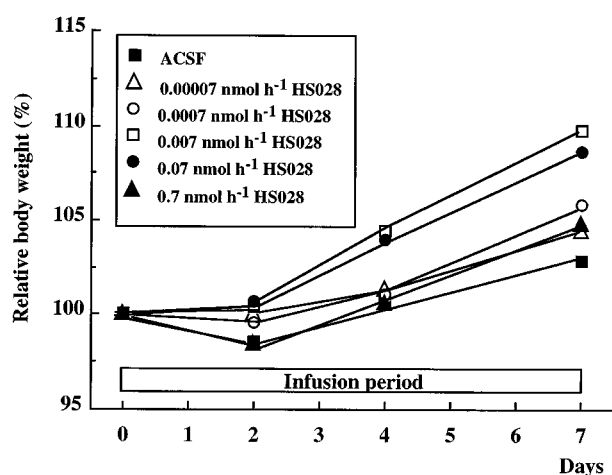


Figure 5 The relative average body weight (%) for each group during 1-week chronic infusion of artificial cerebrospinal fluid (ACSF) or HS028 in doses 0.00007–0.7 nmol h⁻¹. The body weight on the operation day (0) was set as 100%.

We used osmotic minipumps for continuous 7 days infusion of HS028 in free feeding rats. Figure 5 shows the body weight changes, relative to that on the operation day, during the 7 days infusion of vehicle and HS028. In all the experimental groups, including the controls, the weight of the animals decreased during the first 48 h after the start of infusion, but then the animals started to gain weight and continued to do so until the end of the infusion (Figure 5). At the end of the treatment period, the relative body weight increase was highest for the groups infused 0.007 and 0.07 nmol h⁻¹ doses of HS028. Figure 6 shows the 24-h average change in food intake (A) and body weight gain (B) during 7 days of chronic infusion of either vehicle (ACSF), HS028 or HS014 compared with that of the baseline within each group. During 7 days of chronic infusion of either vehicle, 0.00007, 0.0007, 0.007 nmol h⁻¹ HS028 or 0.1 nmol h⁻¹ HS014 the 24-h food intake and body weight gain were reduced relative to that of the baseline for each group (Figure 6). HS028 at a dose of 0.07 nmol h⁻¹ was found to significantly ($P < 0.05$) increase the food consumption compared to the vehicle treated animals (Figure 6A). Both the doses 0.07 and 0.7 nmol h⁻¹ of HS028 increased significantly ($P < 0.05$) the 24-h body weight gain during 1-week chronic infusion compared with that of the vehicle (Figure 6B).

Discussion and conclusion

In a recent study we discovered that cyclic disulphide bridged MSH peptides, with 26 membered ring structure that includes the bulky hydrophobic amino acid D-Nal⁷, are selective for the MC4 receptor. Earlier it has been found that cyclic disulphide MSH analogues are highly stable and effective in skin tanning assays relating to the MC1 receptor (for review see Hruby *et al.*, 1993). Discovery of the lactam analogues MTII and SHU9119 showed that replacement of D-Phe⁷ by D-Nal⁷ (in SHU9119) resulted in antagonism at the MC3 and MC4 receptors (Al Obeidi *et al.*, 1989; Hruby *et al.*, 1995). However, MTII is selective for the MC1 receptor and SHU9119 is virtually non-selective for all the MC receptor subtypes (Hruby *et al.*, 1995; Schiöth *et al.*, 1997b). A bulky hydrophobic amino acid like D-Nal⁷ and pI-Phe⁷ in position 7 gives compounds with antagonism at the MC3 and MC4 receptors and contribute also to increase the relative binding affinity for the MC4 receptor (Hruby *et al.*, 1995; Adan *et al.*, 1994; Schiöth *et al.*, 1997a,b).

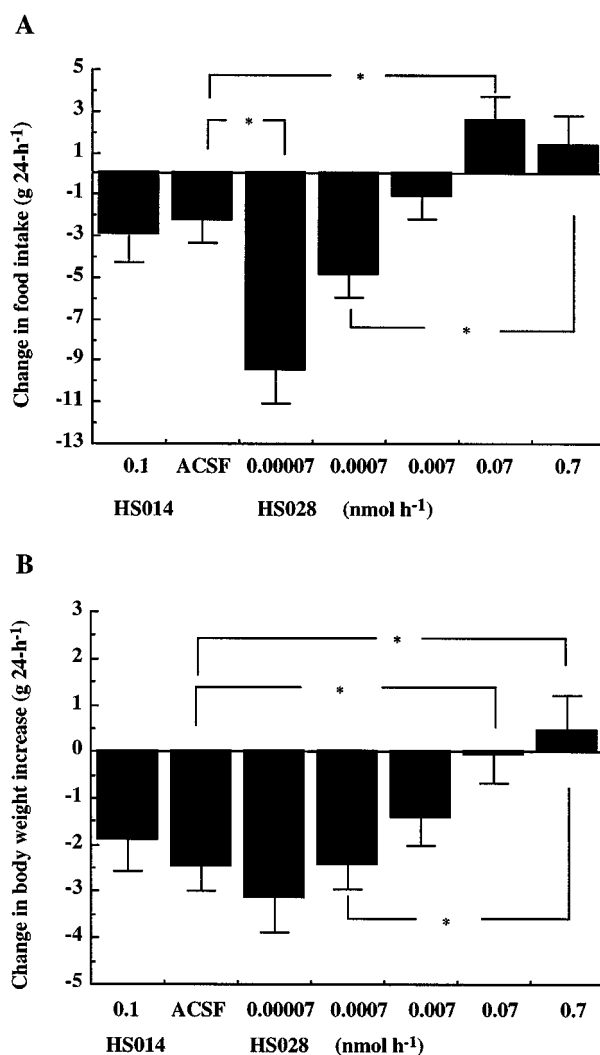


Figure 6 24-h average change in food intake (A) and in body weight increase (B) during 1-week chronic infusion of artificial cerebrospinal fluid (ACSF), HS028 or HS014 relative to baseline period within each dose. Values are least squares means $\pm$ s.e. mean during days 3 to 7 of infusion period. * $P < 0.05$, Bonferroni corrections for multiple comparisons.

This increase in binding affinity for the MC4 receptor by D-Nal⁷ has also recently been shown to exist for linear MSH analogues (Schiöth *et al.*, 1998b). We speculated in our earlier report that His⁶ in the 26 membered cyclic ring structure might not take the same conformation as it may in the linear analogues, and that this might be the reason for the comparatively low affinity of the 26 membered ring peptides for the MC1 receptors. In our present study we elaborated further on position 6. Our present results show that none of our replacements in this position increased the affinity or the selectivity for the MC4 receptor. However, looking at the data for HS018 where the His⁶ was detached for the main pharmacophore (D-Nal⁷-Arg⁸-Trp⁹) within a 26 membered ring structure, the MC4 receptor selective property was maintained (or even increased) even though the all-over affinities were lowered. This may support the hypothesis that His⁶ in these cyclic peptides may not be as important for the MC4 receptor as it may be for the other subtypes.

Addition of a 'β-MSH N-terminal' tail to HS964, giving HS014, both increased the affinity and the selectivity for the MC4 receptor. Addition of a 'γ-MSH N-terminal' tail to our earlier MC3 receptor selective lead HS007, did not result in

increased selective properties. Nor did insertion of ' γ -MSH N-terminal tail' to the linear NDP-MSH peptide result in increase of MC3 receptor selective binding property as reported by our recent study (Schiöth *et al.*, 1998b). It seems thus that fitting of a ' γ -MSH N-terminal' to a specific MC3 receptor binding pocket is not as compatible as the fitting of ' β -MSH N-terminal' tail into a MC4 receptor binding cavity which turned out to be useful when we designed HS014.

As can be deduced from earlier data, position 7 is a key position both for the selectivity and antagonism at the MC4 receptor. Our data show that replacement of D-Nal⁷, with three other un-natural bulky hydrophobic phenylalanine derivatives, had considerable impact on the affinity profiles even though all of the peptides made were selective for the MC4 receptor. HS028 with two chlorine atoms attached to the phenylalanine (dCl-D-Phe⁷) turned out to be highly potent, showing about 3 fold higher affinity than HS014. However, perhaps more importantly, it shows about 80 fold selectivity for the MC4 receptor above the MC3 receptor which is the MC receptor which is most abundantly expressed in the brain besides the MC4 receptor. We selected HS028 for further investigations. Our data on the effect of HS028 on functional coupling to intracellular cyclic AMP production indicate that HS028 is a partial antagonist at the MC1 and MC5 receptors and a potent antagonist at the MC3 and MC4 receptors. This is similar to our findings with HS014. However, at high doses HS028 showed partial agonistic activity at the MC4 receptor. Earlier lactam analogues of MSH, of which several were substituted in position 7 by bulky hydrophobic residues, displayed also both agonist and antagonistic activity at different doses (Hruby *et al.*, 1995).

As HS028 is the most selective MC4 receptor antagonist available and there have not yet been any investigations of the long term i.c.u. administration of MSH peptides, we used HS028 to probe the chronic effects of i.c.u. infusion of a MC4 receptor antagonist. Our data show that the animals in all groups, including the controls, lose their body weight over the first 48 h after the brain operation and osmotic pump installation (Figure 5). This observation can be explained by a long-lasting effect of stress experienced by animals in

association with the brain operation and osmotic pump installation (Levine *et al.*, 1980). From day 2 the animals start gaining weight again. At day 7 of infusion the weight gain was significantly increased beyond the level of the control for the groups receiving the highest doses of HS028 (0.07 and 0.7 nmol h⁻¹) (Figure 6B). Our data thus show that a chronic administration of a selective MC4 receptor antagonist increases food intake with concomitant increase in body weight without inducing any signs of tachyphylaxis. The orexigenic effects of HS028 are conceivably due to its antagonistic action on the MC4 receptor.

There are only few reports about orexigenic effects of MC4 receptor antagonists (Fan *et al.*, 1997; Seeley *et al.*, 1997; Kask *et al.*, 1998a,b). It is interesting in this context that it has been observed that a single injection of the non-selective MC4 antagonist SHU9119 stimulated only nocturnal feeding in mice fed *ad libitum* (Fan *et al.*, 1997). However, the MC4 receptor selective HS014 was highly effective in inducing food intake during daytime in rats (Kask *et al.*, 1998a,b). We did not study the circadian feeding pattern during the long term administration of HS028. Keeping in mind that rats are nocturnal animals and that most food consumption in unstressed as well as in stressed rats occurs during the dark period of the lighting cycle (Marti *et al.*, 1996), the effects of HS028 on feeding behaviour in our study are likely to take place during the dark period of the lighting cycle.

In summary, we have developed a novel high affinity and high selective MC4 receptor antagonist which may become an useful tool to investigate the physiology of the MC4 receptor. Moreover, we have also shown, for the first time, that chronic injection of a MC4 receptor antagonist increases food intake and body weight.

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The cyclo-oxygenase-dependent regulation of rabbit vein contraction: evidence for a prostaglandin E₂-mediated relaxation

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1 Arachidonic acid (0.01–1 μ M) induced relaxation of precontracted rings of rabbit saphenous vein, which was counteracted by contraction at concentrations higher than 1 μ M. Concentrations higher than 1 μ M were required to induce dose-dependent contraction of vena cava and thoracic aorta from the same animals.

2 Pretreatment with a TP receptor antagonist (GR32191B or SQ29548, 3 μ M) potentiated the relaxant effect in the saphenous vein, revealed a vasorelaxant component in the vena cava response and did not affect the response of the aorta.

3 Removal of the endothelium from the venous rings, caused a 10 fold rightward shift in the concentration-relaxation curves to arachidonic acid. Whether or not the endothelium was present, the arachidonic acid-induced relaxations were prevented by indomethacin (10 μ M) pretreatment.

4 In the saphenous vein, PGE₂ was respectively a 50 and 100 fold more potent relaxant prostaglandin than PGI₂ and PGD₂. Pretreatment with the EP₄ receptor antagonist, AH23848B, shifted the concentration-relaxation curves of this tissue to arachidonic acid in a dose-dependent manner.

5 In the presence of 1 μ M arachidonic acid, venous rings produced 8–10 fold more PGE₂ than did aorta whereas 6keto-PGF_{1 α} and TXB₂ productions remained comparable.

6 Intact rings of saphenous vein relaxed in response to A23187. Pretreatment with L-NAME (100 μ M) or indomethacin (10 μ M) reduced this response by 50% whereas concomitant pretreatment totally suppressed it. After endothelium removal, the remaining relaxing response to A23187 was prevented by indomethacin but not affected by L-NAME.

7 We conclude that stimulation of the cyclo-oxygenase pathway by arachidonic acid induced endothelium-dependent, PGE₂/EP₄ mediated relaxation of the rabbit saphenous vein. This process might participate in the A23187-induced relaxation of the saphenous vein and account for a relaxing component in the response of the vena cava to arachidonic acid. It was not observed in thoracic aorta because of the lack of a vasodilatory receptor and/or the poorer ability of this tissue than veins to produce PGE₂.

Keywords: Saphenous vein; vena cava; arachidonic acid; A23187; relaxation

Introduction

In response to acetylcholine, ATP, thrombin or A23187, vascular endothelium produces nitric oxide (NO) and vasoactive metabolites of arachidonic acid as a result of activation of the cyclo-oxygenase pathway (DeMey *et al.*, 1982; DeMey & Vanhoutte, 1982; Singer & Peach, 1983a,b; Miller & Vanhoutte, 1985; Vanhoutte & Miller, 1985; Lüscher *et al.*, 1988; Rubanyi & Vanhoutte, 1988; Yamasaki & Toda, 1991; Yang *et al.*, 1991; Cosentino *et al.*, 1993; Garcia-Villalon *et al.*, 1993). Although cultured arterial and venous endothelial cells synthesize similar amounts of NO and prostaglandins (D'Orléans-Juste *et al.*, 1991; Upchurch *et al.*, 1989), endothelial control of the vasculature appears to be different in veins and arteries isolated from the same species (DeMey *et al.*, 1982; DeMey & Vanhoutte, 1982; Seidel & LaRochelle, 1987; Lüscher *et al.*, 1988; Rubanyi and Vanhoutte, 1988; Yang *et al.*, 1991; Garcia-Villalon *et al.*, 1993; Sellke & Daï, 1993). Using isolated dog vessels, DeMey & Vanhoutte (1982) were the first to report that Ca²⁺-dependent stimulation of the endothelium produced sustained relaxation of precontracted

arteries whereas in the corresponding veins it produced only weak and transient relaxations which were counteracted by contractions when the endothelium was maximally stimulated. Such differences in endothelium-dependent regulation between arteries and veins have subsequently been confirmed in vessels from other species including man (Lüscher *et al.*, 1988; Yang *et al.*, 1991). Presumably, venous and arterial endothelia release different relaxing and constricting mediators and/or the underlying smooth muscles have different reactivities to these mediators. If the first of these two possibilities is true, the weaker endothelium-dependent relaxation in isolated veins than arteries may be due to low NO synthetic activity and not to a weak venous response to NO (Miller & Vanhoutte, 1985; Seidel & LaRochelle, 1987; Vallance *et al.*, 1989).

The mechanism of the differential control of venous and arterial tones by the cyclo-oxygenase pathway of the vascular wall remains unclear. Maximal stimulation of the venous endothelium by acetylcholine, ATP, thrombin or A23187 produces cyclo-oxygenase-dependent contractions which counteract a weak NO-mediated relaxation (Cosentino *et al.*, 1993; Garcia-Villalon *et al.*, 1993; Sellke & Daï, 1993). Such cyclo-oxygenase-dependent contractions result from activation of

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the TP receptors in the smooth muscle (Miller & Vanhoutte, 1985; Lüscher *et al.*, 1988; Yang *et al.*, 1991). *In vitro*, direct stimulation of cyclo-oxygenase activity by exogenous arachidonic acid results in qualitatively and quantitatively different effect in arteries and veins: canine veins exhibit endothelium-dependent, TP receptor-mediated contractions whereas the corresponding arteries exhibit endothelium-dependent relaxations which are blocked by cyclo-oxygenase inhibitors (DeMey & Vanhoutte, 1982; Miller & Vanhoutte, 1985; Vanhoutte & Miller, 1985). Neither the inhibitory prostaglandin or the receptor involved in the vasorelaxant effects of arachidonic acid on canine arteries have been identified.

A highly sensitive, vasodilatory PGE₂ receptor subtype, called EP₄, has been described in isolated veins from pig (Louttit *et al.*, 1992a,b; Coleman *et al.*, 1994) and rabbit (Lydford & McKechnie, 1994a; Milne *et al.*, 1995; Lydford *et al.*, 1996a) but has never been reported in any arterial tissue. In these species, the differential responses of veins and arteries to stimulation of the cyclo-oxygenase pathway have never been explored. We therefore tested whether the venous wall produces enough PGE₂ to stimulate EP₄ receptors and in turn provoke venous relaxation. Thus, we studied the contractile responses of rings of rabbit saphenous vein and vena cava and rings of thoracic aorta from the same animal to exogenous arachidonic acid. The productions of PGE₂, 6keto-PGF_{1 α} and TXB₂ by isolated vascular rings were measured. We conclude that in response to stimulation of the cyclo-oxygenase pathway, isolated rabbit veins produced more PGE₂ than did aorta. In saphenous vein, this provoked an EP₄-mediated response which accounted for arachidonic acid induced relaxation and might contribute to the endothelium-dependent relaxant response to the calcium ionophore, A23187. This mechanism might participate in the arachidonic acid-mediated relaxation in the vena cava, but was not observed in thoracic aorta presumably because of the lack of a vasodilatory receptor and/or the poor ability of this tissue to produce prostaglandins.

Methods

Vessel isolation

Male New Zealand rabbits weighing 3 kg were treated with heparin (200 IU kg⁻¹) and sacrificed by intravenous injection of sodium pentobarbital (50 mg kg⁻¹). External saphenous vein, vena cava and/or thoracic aorta were rapidly dissected and rinsed in bubbled (95% O₂-5% CO₂) Krebs-Henseleit solution (KH-solution) containing (mM): NaCl 118.4, KCl 4.7, MgSO₄ 1.2, CaCl₂ 1.3, KH₂PO₄ 1.2, NaHCO₃ 25 and 11.7 glucose, pH 7.4. Fat was removed and vessels were cut transversely to give eight (saphenous vein), five (thoracic aorta) or three (vena cava) rings approximately 4 mm long. When required, the endothelium was mechanically scraped off by gentle rubbing of the luminal surface with curved forceps. The rings were then mounted on parallel wires, one of which was fixed and the other attached to a force transducer (EMKA, France) allowing tension changes to be recorded isometrically. The rings were bathed in 20 ml KH solution maintained at 37°C and continuously gassed with 95% O₂-5% CO₂.

In vitro measurement of contraction

Rings were stretched at resting tensions of 2 g (thoracic aorta) and 1.6 g (saphenous vein or vena cava). These stretch tensions were optimal according to previous determinations of length-tension relationships. Tissues were equilibrated for 45 min

before challenge with 100 mM KCl KH solution. Once a plateau was attained, endothelial function was checked by addition of acetylcholine (from 0.1–10 μ M) to the bath. Tissues were then washed with 5 mM KCl KH solution to recover their resting tension and tested twice with 100 mM KCl KH-solution and subsequent addition of acetylcholine. The amplitude of contraction after this second 100 mM KCl challenge was the maximal tension which could be developed by the rings: higher KCl concentrations did not further increase the tension. The mean KCl tension was 7.8 ± 0.1 g for saphenous vein ($n=348$), 1.2 ± 0.1 g for vena cava ($n=107$) and 9.6 ± 0.2 g for thoracic aorta ($n=170$). In intact rings, maximal relaxations obtained in response to acetylcholine were $-44.7 \pm 1\%$ ($n=237$) of the maximal contraction of saphenous vein, $-60 \pm 3\%$ ($n=64$) of vena cava and $-32 \pm 1\%$ ($n=93$) of thoracic aorta. No significant acetylcholine-induced relaxation was observed when the endothelium had previously been scraped off ($-5.3 \pm 0.3\%$, $n=110$ for saphenous vein; $-5 \pm 1\%$, $n=30$ for vena cava; $-2.0 \pm 0.4\%$, $n=51$ for thoracic aorta). After these functional tests, rings were maintained at rest for 90 min in 5 mM KCl KH-solution. To detect both contraction and relaxation in response to the various agents tested, rings were treated with KH solution containing 40 mM KCl for saphenous vein, 30 mM KCl for thoracic aorta and 20 mM KCl for vena cava. At these KCl concentrations, the rings were approximately half activated (from 30–70% of the maximal contraction) whether or not the endothelium was present. Once the precontraction plateaued, increasing doses of arachidonic acid, PGE₂, PGI₂ or A23187 were added to the bath. In some experiments, a TP receptor antagonist (GR32191B or SQ29548 at 3 μ M), a cyclo-oxygenase inhibitor (indomethacin 10 μ M), an EP₄ receptor antagonist (AH23848B) or an appropriate vehicle was added 30 min before precontraction and remained until the end of the experiment. Although, the potency of TP receptor antagonists for rabbit tissues is lower than for human or rat tissues (Lumley *et al.*, 1989; Tahraoui *et al.*, 1990; Tymkewycz *et al.*, 1991; Foster *et al.*, 1992), GR32191B and SQ29548 were used at concentrations at least 30 fold higher than their previously reported pA₂ values against rabbit TP-receptors (Lumley *et al.*, 1989; Tahraoui *et al.*, 1990; Foster *et al.*, 1992; Lydford *et al.*, 1996a). Because of the tachyphylaxis of the responses to arachidonic acid (DeMey *et al.*, 1982) and PGE₂ (data not shown), each vascular ring was used for only one dose-response analysis. The contracting and relaxing effects are expressed as the positive and negative mean $\pm$ s.e.mean respectively of the percentage of the precontraction.

Eicosanoid production by vascular rings

Rings of rabbit saphenous vein, vena cava and thoracic aorta dissected as described above were immediately rinsed in Hank's saline solution (HBSS, Gibco-BRL) and cut transversely into 4 mm long segments. The endothelium was mechanically removed from some segments by gentle rubbing of the luminal surface with curved forceps as for functional experiments. Because tissue dissection and preparation might acutely trigger the arachidonic acid cascade, the rings were allowed to recover for 90 min in fresh HBSS, before experiments. After the recovery period, rings were equilibrated for 30 min under constant shaking in 500 μ l physiological buffer containing (in mM): HEPES 20, NaCl 120, KCl 4.7, MgSO₄ 1.2, CaCl₂ 1.25, 10 glucose, KH₂PO₄ 1.2, pH 7.4 with or without 10 μ M indomethacin. At $t=0$, they were transferred into 500 μ l of the same buffer with or without 1 μ M arachidonic acid. Thirty minutes later, they were removed and the medium was frozen at

–80°C. Each ring was superficially dried and weighed. Amounts of PGE₂, 6keto-PGF_{1α} and TXB₂ in the medium were determined within a week using commercially available enzymeimmunoassay kits (Amersham). According to the manufacturer, crossreactivities of the kits with related compounds were less than 1%. Results are expressed as means ± s.e.mean pg of eicosanoid per mg wet weight.

Drugs and statistics

Arachidonic acid, PGE₂, PGI₂ and SQ29548 were purchased from Spibio (Massy, France). All other drugs were from Sigma Chemical (St Quentin Fallavier, France). Solutions were freshly prepared before use. For stock solutions, arachidonic acid was dissolved in ethanol, PGE₂ in water and PGI₂ in Tris buffer (pH 9). A23187 was dissolved in dimethylsulphoxide (DMSO). GR32191B and SQ29548 were prepared at 15 mM in DMSO and ethanol respectively. AH23848B and indomethacin were dissolved at 10 mM in 0.5 M NaHCO₃. N^G-nitro-L-arginine methyl ester (L-NAME) was dissolved at 0.4 M in water. Subsequent dilutions were made in physiological saline solution. Results are expressed as means ± s.e.mean. Differences are considered significant when $P < 0.05$ using the non-parametric transformation of the unpaired *t*-test: standard Mann-Whitney *U*-test.

Results

Responses to arachidonic acid and effects of TP receptor antagonists

Arachidonic acid (0.001–1 μM) provoked potent relaxation of intact rings of the rabbit saphenous vein (–50 ± 4% at 1 μM,

$n = 4$) whereas those of vena cava and thoracic aorta were insensitive to this range of concentrations (Figure 1a). Higher concentrations of arachidonic acid induced contraction of saphenous vein (–18.6 ± 7.8% at 10 μM, $n = 4$), vena cava (+69.6 ± 13.6% at 10 μM, $n = 7$) and thoracic aorta (+29.3 ± 3.6% at 10 μM, $n = 15$). This contractile phenomenon counteracted but did not totally reverse the potent relaxing effect of arachidonic acid on saphenous vein (–18.6 ± 7.8% at 10 μM vs –50 ± 4% at 1 μM; $P < 0.001$). In rings of vena cava the onset of contraction was rapid and plateaued 1–2 min after addition of the following dose. Pretreatment with a TP receptor antagonist (3 μM GR32191B or 3 μM SQ29548, Figure 1b) abolished or reduced the contraction in response to high doses of arachidonic acid in saphenous vein and vena cava respectively but failed to prevent arachidonic acid-induced contraction in aorta. The blockade of TP receptors potentiated the arachidonic acid-induced relaxation observed in saphenous vein but also revealed a dose-dependent inhibitory component in the response of rabbit vena cava (–44.1 ± 4.2% at 1 μM, $n = 24$, $P < 0.001$). Nevertheless, even in the presence of 3 μM GR32191B, a contraction in vena cava still counteracted the relaxing effect at doses higher than 1 μM (–15.4 ± 8.2% at 10 μM).

Effects of cyclo-oxygenase inhibition and removal of the endothelium

We investigated whether the TP receptor-independent effects of arachidonic acid were due to stimulation of the endothelial cyclo-oxygenase pathway. Intact rings and endothelium-free rings were pretreated with a TP receptor antagonist and 10 μM indomethacin (an inhibitor of cyclo-oxygenase activity). Prior blockade of the cyclo-oxygenase pathway provoked a 10 fold rightward shift in the dose-response curve in intact saphenous

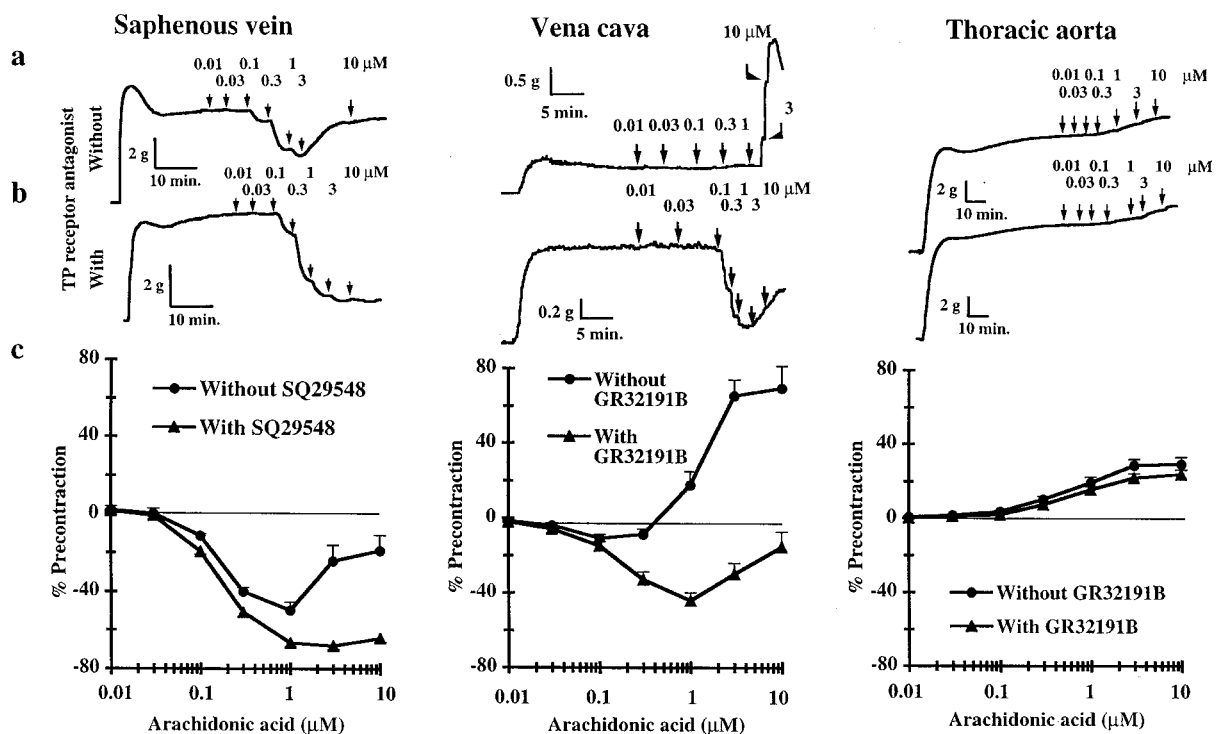


Figure 1 Effects of cumulative doses of arachidonic acid on rings of rabbit saphenous vein, vena cava and thoracic aorta submaximally contracted by external application of KCl. Time courses of representative experiments without (a) and with (b) 3 mM TP receptor antagonist (SQ29548 or GR32191B) pretreatment. (c) Summarized results obtained with untreated rings ($n = 4, 7, 15$) and rings pretreated with the TP receptor antagonist ($n = 40, 21, 25$ for saphenous vein, vena cava and thoracic aorta respectively). Results are expressed as means ± s.e.mean of percentage of KCl precontraction.

vein to arachidonic acid and totally suppressed the relaxing response of intact vena cava (Figure 2). In vena cava, indomethacin pretreatment potentiated the TP receptor-independent contraction (+39.7±7.1% at 10 µM, *n*=21). In thoracic aorta, the cyclo-oxygenase blockade significantly reduced the TP receptor-independent response to arachidonic acid (+8.2±1.1% at 10 µM, *n*=9).

Elimination of the endothelium of the vessels totally suppressed acetylcholine-induced relaxation during preliminary functional tests but did not suppress the relaxation responses in either saphenous vein or vena cava. Endothelium removal caused a 3–10 fold rightward shift in the relaxant dose-response curves for these tissues to arachidonic acid. The remaining vasorelaxant response of endothelium-free saphenous vein and vena cava was inhibited by indomethacin pretreatment. Interestingly, the TP receptor-independent contraction observed in intact vena cava at high dose of arachidonic acid was eliminated by endothelium removal. The contraction observed in intact rings of thoracic aorta was significantly reduced by endothelium removal and indomethacin pretreatment did not further reduce the response.

Response to vasorelaxant prostaglandins and effect of AH23848B on the response of saphenous vein to arachidonic acid

Concentration-response curves of rabbit saphenous vein to exogenous PGE₂, PGI₂ and PGD₂ were obtained with KCl precontracted rings (Figure 3a). As previously described (Lydford *et al.*, 1996a), this tissue appeared 50 and 100 fold more sensitive to PGE₂ than to PGI₂ and PGD₂ respectively ($EC_{50} = 1.6 \pm 0.1$ nM, $E_{max} = -97.5 \pm 0.8\%$, $n_H = 1.00$ for PGE₂; $EC_{50} = 55.8 \pm 2.1$ nM, $E_{max} = -99.1 \pm 0.9\%$, $n_H = 1.20$ for PGI₂; $EC_{50} = 112 \pm 5$ nM, $E_{max} = -92.5 \pm 0.9\%$, $n_H = 0.82$ for PGD₂). This suggested a relaxation primarily mediated by activation of vasodilatory EP₂ and/or EP₄ receptors in the smooth muscle. As in saphenous vein, PGE₂ was more potent than PGI₂ for relaxing vena cava. Nevertheless, the response to PGE₂ was weaker and certainly more complex than that observed in saphenous vein spanning more than 3 log units

($EC_{50} = 29.6 \pm 5.8$ nM, $E_{max} = -63.6 \pm 2.2\%$, *n*=9, $n_H = 0.55$). Unlike venous tissues, rabbit aorta did not relax but exhibited moderate contractions in response to PGE₂ and PGI₂ (+9.9±3.2%, *n*=14, for PGE₂; +12.7±1.2% for PGI₂ at 10 µM).

Because the potent vasodilatory response of rabbit saphenous vein to PGE₂ can be weakly antagonized by AH23848B, an antagonist of the PGE₂ receptor subtype EP₄ ($pA_2 = 4.96$, Lydford *et al.*, 1996a), we tested for the involvement of EP₄-mediated relaxation in the vasodilatory response of the saphenous vein to arachidonic acid (Figure 3b). Saphenous vein was treated with AH23848B in the presence of 3 mM SQ29548 to potentiate the vasodilatory response and to mask the TP receptor-mediated effects of AH23848B. At doses known to prevent PGE₂ interaction with EP₄ receptor ($pA_2 = 4.9 - 5.4$; Coleman *et al.*, 1994; Lydford *et al.*, 1996a), pretreatment with 30 and 100 µM AH23848B produced dose-dependent rightward shifts in the response to exogenous arachidonic acid.

Prostaglandin and thromboxane productions

These results suggested that direct stimulation of the venous wall cyclo-oxygenase by exogenous arachidonic acid causes production of eicosanoids which interact with one or several inhibitory or constricting receptors. In a separate set of experiments, we compared the productions of PGE₂ and of the stable analogues of PGI₂ and TXA₂, 6keto-PGF_{1α} and TXB₂ by intact and endothelium-free rings, in the presence or absence of 1 µM arachidonic acid (Figure 4). Under all experimental conditions, eicosanoid production by venous and arterial rings was totally inhibited by previous pretreatment with 10 µM indomethacin (data not shown). The 'basal' productions of PGE₂, 6keto-PGF_{1α} and TXB₂ by intact rings of rabbit saphenous vein, vena cava and thoracic aorta were similar. In the presence of 1 µM arachidonic acid, intact rings of saphenous vein and vena cava produced 1.5–2 fold more cyclo-oxygenase derivatives than did aorta. This was mainly due to an approximately 10 fold larger production of PGE₂. The amount of 6keto-PGF_{1α} produced was similar and that of TXB₂ 1.5–2 fold higher. Nevertheless, even for venous tissues,

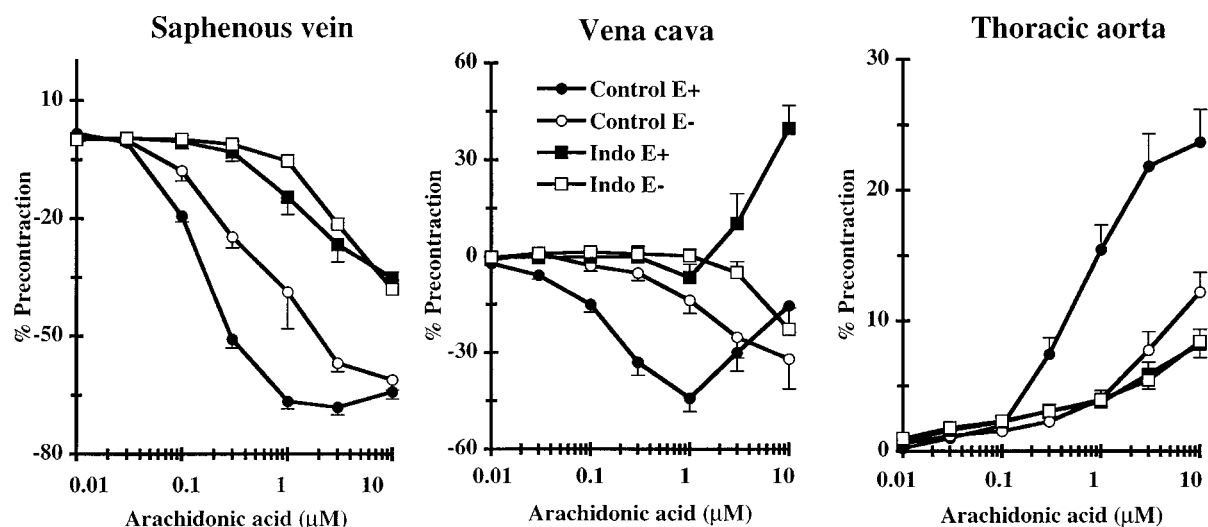


Figure 2 Effects of endothelium removal and/or cyclo-oxygenase inhibition on the effects of arachidonic acid on rabbit saphenous vein, vena cava and thoracic aorta preincubated with 3 mM TP receptor antagonist (SQ29548 or GR32191B). Intact rings (E+) and endothelium-free rings (E-) were incubated without (Control) or with 10 mM indomethacin (Indo). Results are expressed as means ± s.e. mean of 8–40 rings depending on the experimental conditions for saphenous vein, 7–21 rings for vena cava and 9–25 rings for thoracic aorta.

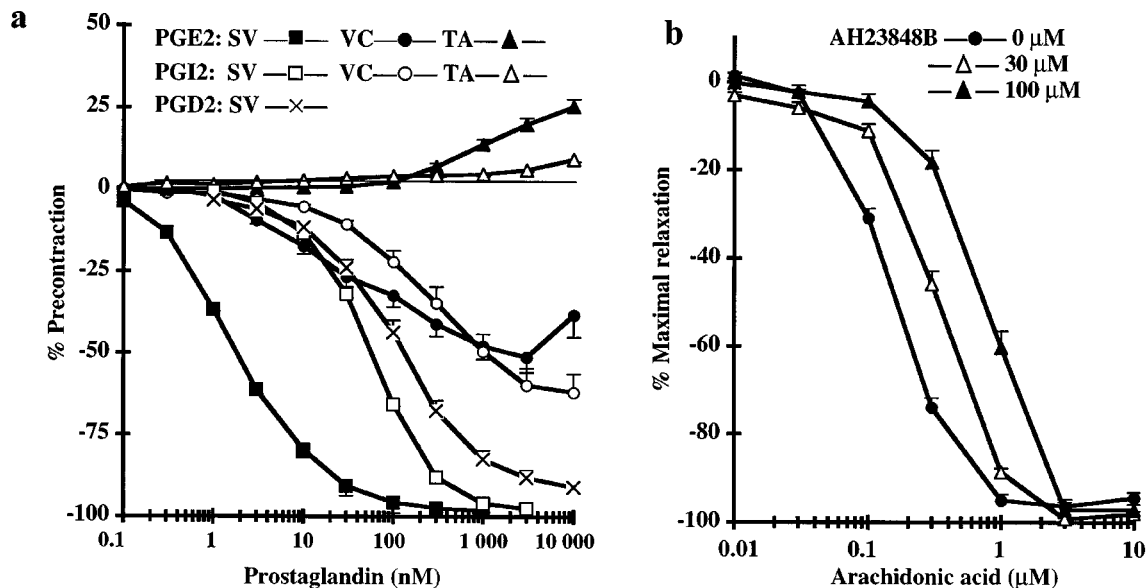


Figure 3 Effects of PGE₂ and EP₄ antagonist receptor, AH23848B, on the arachidonic acid response of saphenous vein. (a) Effects of cumulative doses of PGE₂, PGI₂ and PGD₂ on precontracted saphenous vein (SV, $n=12-15$) and cumulative doses of PGE₂ and PGI₂ on precontracted vena cava (VC, $n=7-9$) and thoracic aorta (TA, $n=8-14$). Experiments were performed on rings without endothelium in the presence of 3 μM TP receptor antagonist. Results are expressed as the means $\pm$ s.e.mean. (b) Effects of pretreatment with 30 μM and 100 μM EP₄ antagonist receptor, AH23848B, or vehicle on the effects of cumulative doses of arachidonic acid on precontracted saphenous vein. Experiments were performed on rings with endothelium in the presence of 3 μM TP receptor antagonist. Results are expressed as means $\pm$ s.e.mean of percentage of maximal relaxation, $n=8-40$ depending on the experimental conditions.

6keto-PGF_{1 α} was the major metabolite produced by intact vascular rings. Removal of the endothelium did not significantly modify unstimulated production of PGE₂, 6keto-PGF_{1 α} or TXB₂ by venous or arterial rings. When stimulated by the fatty acid, the total eicosanoid production by the endothelium-free rings was only 25–30% of that by intact rings of the same origin. Without endothelium, stimulated venous rings still produced 2–3 fold more PGE₂ than rings from aorta although 6keto-PGF_{1 α} remained the most abundant prostaglandin synthesized by both venous and arterial rings.

Effect of indirect stimulation of cyclo-oxygenase by A23187

Because direct stimulation of the cyclo-oxygenase pathway by arachidonic acid relaxed precontracted rings of rabbit saphenous vein even in the absence of TP receptor antagonist, cyclo-oxygenase-dependent relaxation might occur in response to Ca²⁺-dependent release of endogenous fatty acid in this tissue. From 1–100 nM, the Ca²⁺ ionophore A23187 induced a dose-dependent relaxation of intact rings of saphenous vein. At higher doses, this relaxation was counteracted by a contraction. This late phenomenon persisted in the presence of a TP receptor antagonist (data not shown). Pretreatment with either the NO-synthase inhibitor, L-NAME (100 μM), or the cyclo-oxygenase inhibitor, indomethacin (10 μM), reduced the relaxant response to A23187 by 50% (Figure 5). Concomitant pretreatment with L-NAME and indomethacin totally suppressed both the relaxing and contracting responses to A23187. In endothelium-free rings of saphenous vein, A23187 induced desensitized relaxation relative to that observed on intact rings ($\text{EC}_{50}=41.7 \pm 1.5$ nM, $n=32$ vs $\text{EC}_{50}=10.7 \pm 1.5$ nM, $n=43$). This relaxing response was prevented by indomethacin but not L-NAME pretreatment.

Discussion

This study demonstrates that the contraction of both a distal (saphenous vein) and a proximal (vena cava) rabbit vein can be regulated by the cyclo-oxygenase activity of the endothelium. This contrasts with the weak responses observed in aorta from the same animal. As described in venous tissues from dog and human (DeMey & Vanhoutte, 1982; Yang *et al.*, 1991), stimulation of the cyclo-oxygenase activity by arachidonic acid at concentrations higher than 1 μM induced TP receptor-mediated contraction of both rabbit saphenous vein and vena cava. This TP receptor-mediated effect counteracted a potent arachidonic acid-induced vasodilatation in the saphenous vein and totally masked a vasodilatory component in the vena cava response.

The arachidonic acid-induced relaxation of both saphenous vein and vena cava, in the presence of TP receptor antagonist, was inhibited by indomethacin pretreatment and by endothelium removal. This suggests that when the cyclo-oxygenase pathway was stimulated by arachidonic acid, vasodilatory prostaglandins were mostly produced by the venous endothelium. Accordingly, in the presence of 1 μM arachidonic acid, intact but not endothelium-free rings of saphenous vein and vena cava produced large amounts of PGE₂ and PGI₂ (6keto-PGF_{1 α}), both prostaglandins with potent vasodilatory effects in these tissues. In agreement with Lydford's results (1996a), the apparent reactivity of saphenous vein to exogenous PGE₂ appeared 50 fold greater than those to PGI₂ and PGD₂ suggesting that the receptors involved in the relaxant response to arachidonic acid or A23187 could be EP-receptors. Relaxation of the rabbit saphenous vein that in response to exogenous prostaglandins is primarily mediated by EP₄ receptor subtypes although the presence of EP₂ and DP receptors cannot be excluded (Lydford *et al.*, 1996a). In our study, the rightward shifts in the dose-response curve to arachidonic acid provoked by AH23848B were consistent with

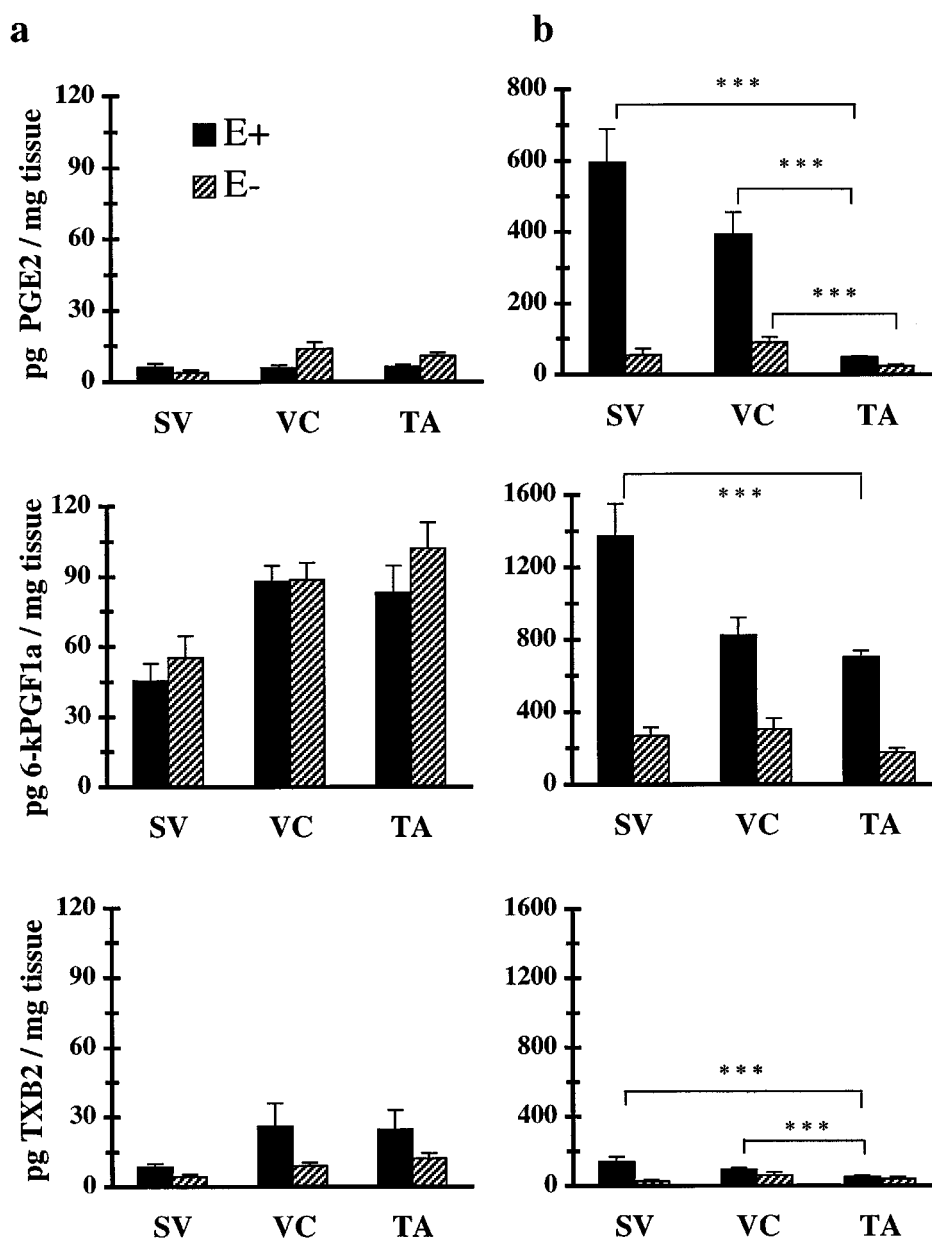


Figure 4 Measurements of PGE₂, 6-ketoPGF_{1α} and TXB₂ production by rings isolated from saphenous vein (SV), vena cava (VC) and thoracic aorta (TA) in basal conditions (a) and stimulated by the addition of 1 μ M arachidonic acid (b). Open columns represent production by intact rings (E+) and hatched columns illustrate production by endothelium-free rings (E-). Results are expressed as means $\pm$ s.e. mean of percentage of 8–35 rings depending on the experimental conditions. ***, $P < 0.001$.

an EP₄-mediated effect of arachidonic acid. Indeed, since the original paper of Coleman *et al.* (1994) describing the pharmacology of an EP₄-mediated response in piglet saphenous vein, AH23848B has been widely used to discriminate EP₄ mediated-relaxant responses from EP₂, IP or DP receptor-mediated relaxation in isolated smooth muscle preparations (Louttit *et al.*, 1992b; Lydford & McKechnie, 1994b; Milne *et al.*, 1995; Lydford *et al.*, 1996a,b). Experiments with rat recombinant prostaglandin receptors confirm the selective antagonism of AH23848B at EP₄ receptors relative to EP₂ receptors (Boie *et al.*, 1997). AH23848B exhibits antagonist properties at almost all human recombinant receptors except the IP receptor. Notably, it similarly antagonizes EP₂-, DP- as well as EP₄-mediated responses in transfected cells. Thus, whereas we could definitely exclude activation of IP receptor, EP₂ and DP receptor activation may participate in the arachidonic acid-induced relaxation observed

in rabbit saphenous vein. Nevertheless, it has been shown that AH23848B fails to antagonize the relaxation induced in rabbit saphenous vein by the EP₂ selective agonists AH13205 and butaprost (Lydford *et al.*, 1996a) ruling out the putative influence of EP₂ receptor activation in the arachidonic acid-induced relaxation that we observed. Furthermore, the relaxant response of rabbit saphenous vein to PGD₂ is effectively antagonized by AH23848B (Lydford *et al.*, 1996b), but PGD₂ is known to activate recombinant human EP₄ receptors at concentrations consistent with the apparent reactivity of the saphenous vein that we report (Wright *et al.*, 1998). Thus, the antagonism of AH23848B on the arachidonic acid concentration response curve was probably due to its interaction with EP₄ receptors in the smooth muscle.

Even in the presence of 10 μ M indomethacin, SQ29548-treated saphenous vein relaxed in response to concentrations of arachidonic acid higher than 1 μ M. This could not be due to

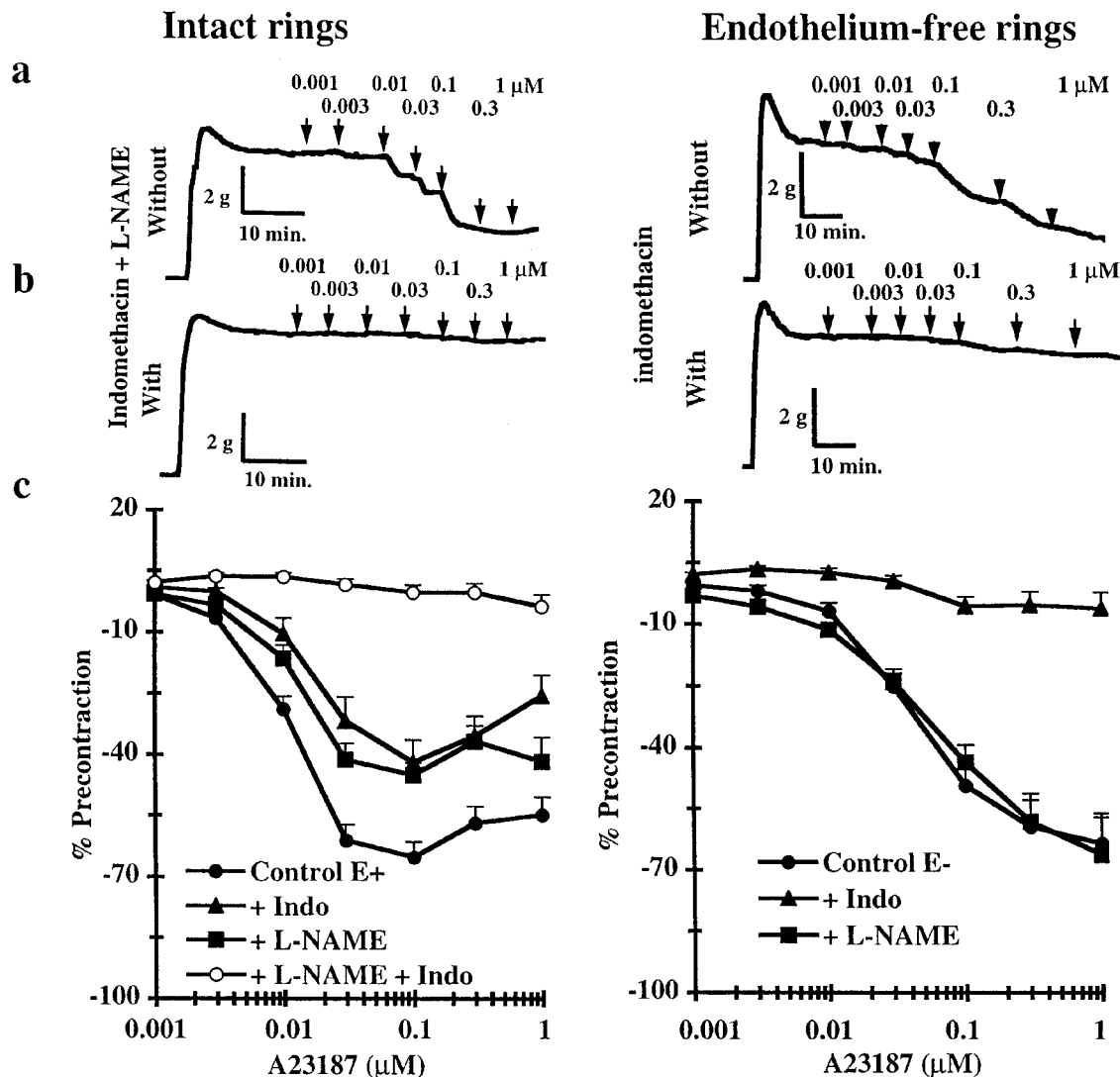


Figure 5 Effects of cumulative doses of A23187 on KCl precontracted intact or endothelium-free rings of saphenous vein rings. Time courses of representative experiments performed without (a) and with concomitant pretreatment with NO synthase inhibitor (L-NAME, 100 μM) and cyclo-oxygenase inhibitor (indomethacin, 10 μM) (b, left) and only with cyclo-oxygenase inhibitor (indomethacin, 10 μM) (b, right). (c) Results obtained with untreated rings ($n=32-43$), and rings pretreated with indomethacin ($n=8-16$) and/or L-NAME ($n=11-25$).

incomplete blockade of cyclo-oxygenase activity since in the presence of 1 μM arachidonic acid, prostaglandin production by rings of saphenous vein was totally inhibited by 10 μM indomethacin. Numerous arachidonic acid derivatives which are not prostaglandins promote relaxation of vascular preparations. Notably, cytochrome P-450 derivatives of arachidonic acid might account for endothelium-derived hyperpolarizing factors (Rubanyi & Vanhoutte, 1987; Komori & Vanhoutte, 1990; Bennett *et al.*, 1992; Hecker *et al.*, 1994; Dong *et al.*, 1997).

The role of cyclo-oxygenase in the relaxation of the rabbit saphenous vein provoked by the calcium ionophore A23187 was explored. A23187 induced a NO-mediated relaxation and a cyclo-oxygenase-dependent vasodilatory process in intact rings. Interestingly, whereas the NO-mediated component of the response was totally suppressed by endothelium removal, cyclo-oxygenase-dependent relaxation was observed in endothelium-free rings. One possible explanation is that both endothelial and smooth muscle cyclo-oxygenase activities were stimulated by A23187 and produced vasodilatory prostaglandin(s), possibly PGE₂. Alternatively, contaminating endothe-

lial cells or endothelium of the vasa vasorum may have produced vasodilatory prostaglandins but failed to produce enough NO to provoke stimulation of the smooth muscle guanylate cyclase. We favour the former hypothesis because the endothelium removal procedure totally suppressed the acetylcholine-induced relaxation observed in intact rings. Furthermore, endothelium removal did not suppress arachidonic acid-induced relaxation in the saphenous vein but provoked a rightward shift of only one order of magnitude in the dose-response curve, suggesting a role for a non-endothelial cyclo-oxygenase activity.

The involvement of PGE₂ in the vasodilatory component of the response of vena cava to arachidonic acid is certainly more debatable than in saphenous vein: (i) In the presence of arachidonic acid, vena cava and saphenous vein rings produced comparable amounts of PGE₂ and PGI₂ but PGE₂ was at least 10 fold less potent in vena cava than in saphenous vein; (ii) In the presence of stimulating concentration of arachidonic acid, rings of vena cava produced twice as much PGI₂ as PGE₂, (iii) Vena cava appeared 10 fold more sensitive to PGE₂ than to native PGI₂ but this difference could result

from the short half-life of PGI₂ in aqueous solution. Although PGI₂ was conserved as a stock solution in ice-cooled basic buffer (pH 9) to ensure stability until the experiments (Johnson, 1976), we could not avoid PGI₂ degradation once it was added to the bath solution. Indeed, the half life in aqueous solution (Dusting *et al.*, 1977) and the time required to reach maximal relaxation in vena cava were comparable (2–4 min). Thus, the true PGI₂ concentration when the plateau was reached could be 2–4 fold lower than that initially added. The dose-response curve of vena cava to PGE₂ spanned a wide range of concentrations (3–4 log units) suggesting activation of more than one vasodilatory receptor in this preparation. Alternatively, a contractile component in the response to PGE₂ via activation of excitatory prostanoid receptors distinct from TP receptor (EP₁, EP₃ or FP) could distort the shape of the curve and account for incomplete relaxation of the vena cava. Further studies are certainly required to characterize the vasodilatory prostanoid receptors in the rabbit vena cava but note that the highly sensitive PGE₂ receptor subtype, EP₄, has been described in several rabbit veins (Milne *et al.*, 1995; Lydford *et al.*, 1996a), but not in any artery. Therefore, we postulate that arachidonic acid-induced relaxation in the rabbit vena cava might be due to production of both PGE₂ and PGI₂ by the endothelium and subsequent activation of vasodilatory EP₂, EP₄ and/or IP receptors on the smooth muscle.

In the absence of TP receptor antagonist, arachidonic acid concentrations higher than 1 μ M induced contraction which either reduced (saphenous vein) or masked (vena cava), cyclo-oxygenase-dependent relaxation in veins (see above). Studies with isolated canine and human veins consistently report such a TP receptor-mediated constriction in response to arachidonic acid or to endothelium stimulation by A23187, acetylcholine and thrombin (DeMey & Vanhoutte, 1982; Miller & Vanhoutte, 1985; Lüscher *et al.*, 1988; Yang *et al.*, 1991). These excitatory responses may result from the endothelium producing eicosanoids other than TXA₂ (mainly the endoperoxide PGH₂) which activate TP receptors (Miller & Vanhoutte, 1985; Van Dam *et al.*, 1986; Pagano *et al.*, 1991; Williams *et al.*, 1994). In our study, although stimulated venous rings produced large amounts of PGE₂ and PGI₂, TXA₂ production accounted for only 10% of the total eicosanoid production. Thus, *in situ*, the local concentration of eicosanoids other than TXA₂ in the vicinity of the smooth muscle cells might be much higher than that of TXA₂, accounting for the TP receptor-mediated contraction observed in saphenous vein and vena cava. Such a TP receptor-mediated contraction in response to arachidonic acid has been previously reported (Pagano *et al.*, 1991) in rabbit aorta. In our experiments, we were unable to prevent cyclo-oxygenase-dependent contraction by a TP-receptor blocking agent. Our results suppose that stimulation of the rabbit aorta cyclo-oxygenase pathway induced synthesis of prostanoids by the endothelium which might activate constricting receptor(s) (Singer & Peach, 1983a) such as FP, EP₁ or EP₃ receptors.

Arachidonic acid concentrations higher than 1 μ M lead to an endothelium-dependent contraction in the vena cava

counteracting the vasodilatory component observed after pretreatment with a TP-receptor antagonist. This contraction was potentiated by indomethacin pretreatment. High concentrations of arachidonic acid in this tissue might result in the production of constricting eicosanoids other than prostaglandins and thromboxane.

One of our main findings is that, in the presence of arachidonic acid, rings of saphenous vein and vena cava produced 8–10 fold more PGE₂ than rings of thoracic aorta whereas the synthesis of PGI₂ by the venous and arterial rings were similar. Because PGI₂ has both vasorelaxant effects in several (but not all, Omini *et al.*, 1977) vascular preparations (Williams *et al.*, 1994) and is an extremely potent inhibitor of platelet aggregation, numerous authors have compared the synthesis of PGI₂ by vascular rings or by cultured endothelial cells from veins and arteries. Published results are conflicting, showing either lower (Chaikhouni *et al.*, 1986; Fann *et al.*, 1989; Nadasy *et al.*, 1992), similar (Carter *et al.*, 1984; Hanley & Bevan, 1985; D'Orleans-Juste *et al.*, 1991) or higher (Upchurch *et al.*, 1989) PGI₂ synthesis in veins than in arteries. Skidgel & Prinz (1978) observed that homogenates of rat arteries generated substantial amounts of PGI₂ but venous homogenates from the same animal did not, producing PGE₂ instead. Our results confirm this observation in the rabbit and provide evidence for a major role for PGE₂ in the cyclo-oxygenase-dependent control of vein contraction. As in arterial rings, the synthesis of prostaglandins by the venous tissues mainly resulted from the cyclo-oxygenase activity of the endothelium. Indeed, endothelium removal reduced the venous production of PGE₂ by 75–90%. Endothelium-free venous rings produced more (2–4 fold) PGE₂ than those from aorta suggesting that the difference between veins and aorta might be due to differences in non-endothelial cyclo-oxygenase and/or PGE₂ isomerase activities. However, we cannot exclude the possibility that the remaining production of prostaglandins after rubbing of the luminal surface of the vessels (to remove the endothelium) resulted from incomplete endothelial elimination. Interestingly, the absence of the arachidonic acid-induced relaxing response in the aorta was due to the absence of vasodilatory eicosanoid receptors rather than to failure of the arterial wall to produce prostaglandins. Indeed, in the presence of 1 μ M arachidonic acid, intact rings of rabbit aorta produced significant amounts of PGI₂ and PGE₂.

Our results highlight the role of PGE₂ in the control of rabbit vein relaxation by the cyclo-oxygenase pathway whereas aorta from the same animal appeared almost insensitive to cyclo-oxygenase stimulation. This venospecific cyclo-oxygenase-dependent relaxation in the rabbit may result from both the expression of a highly sensitive PGE₂ receptor subtype, EP₄, and also the greater capacity of the venous than arterial endothelium to produce PGE₂.

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Cytokine-mediated inflammatory hyperalgesia limited by interleukin-4

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1 The effect of IL-4 on responses to intraplantar (i.pl.) carrageenin, bradykinin, TNF α , IL-1 β , IL-8 and PGE₂ was investigated in a model of mechanical hyperalgesia in rats. Also, the cellular source of the IL-4 was investigated.

2 IL-4, 30 min before the stimulus, inhibited responses to carrageenin, bradykinin, and TNF α , but not responses to IL-1 β , IL-8 and PGE₂.

3 IL-4, 2 h before the injection of IL-1 β , did not affect the response to IL-1 β , whereas IL-4, 12 or 12 + 2 h before the IL-1 β , inhibited the hyperalgesia (–30%, –74%, respectively).

4 In murine peritoneal macrophages, murine IL-4 for 2 h before stimulation with LPS, inhibited (–40%) the production of IL-1 β but not PGE₂. Murine IL-4 (for 16 h before stimulation with LPS) inhibited LPS-stimulated PGE₂ but not IL-1 β .

5 Anti-murine IL-4 antibodies potentiated responses to carrageenin, bradykinin and TNF α , but not IL-1 β and IL-8, as well as responses to bradykinin in athymic rats but not in rats depleted of mast cells with compound 40/80.

6 These data suggest that IL-4 released by mast cells limits inflammatory hyperalgesia. During the early phase of the inflammatory response the mode of action of the IL-4 appears to be inhibition of the production TNF α , IL-1 β and IL-8. In the later phase of the response, in addition to inhibiting the production of pro-inflammatory cytokines, IL-4 also may inhibit the release of PGs.

Keywords: Inflammatory hyperalgesia; interleukin-4; bradykinin; tumour necrosis factor alpha; interleukin-1 β ; interleukin-8; prostaglandin E₂

Abbreviations: BK, bradykinin; Cg, carrageenin; Con-A, concanavalin A; o.p.m., counts per minute; °C, degrees centigrade; h, hour; IFN γ , interferon γ ; IL, interleukin; IU, international unit; IgG, immunoglobulin G; i.pl., intraplantar; LPS, lipopolysaccharide; μ g, microgram; μ l, microlitre; mg, milligram; min, minutes; MAb, monoclonal antibody; ng, nanogram; pg, picogram; PGE₂, prostaglandin E₂; TNF α , tumour necrosis factor α ; Th, T helper; U, unit; ³H, tritiated; w/v, weight per volume.

Introduction

Cytokines constitute a link between cellular injury and recognition of non-self and the development of local and systemic signs and symptoms of inflammation, such as cell migration, oedema, fever and hyperalgesia (Dinarello *et al.*, 1986; Ferreira *et al.*, 1988; Faccioli *et al.*, 1990; Dinarello, 1991). In this context, it was shown, in a model of mechanical hyperalgesia, that carrageenin-evoked hyperalgesia results from the combined effects of the release of cyclo-oxygenase products and sympathomimetic amines (Nakamura & Ferreira, 1987). A cascade of cytokine release preceded the generation of these mediators (Cunha *et al.*, 1992). Carrageenin and LPS caused the release of bradykinin, which stimulated the release of TNF α . TNF α induced the release of IL-1 β and IL-6, which stimulated the production of cyclo-oxygenase products, as well as IL-8, which stimulated production of sympathomimetic mediators (Cunha *et al.*, 1992; Ferreira *et al.*, 1993). In a different model, the 'tail-flick' method, LPS-induced hyperalgesia was also mediated by TNF α and IL-1, and the effect of TNF α was dependent upon IL-1,

although prostaglandins did not appear to be involved in the responses (Watkins *et al.*, 1994, 1995). In addition, TNF α has been suggested to be a mediator of inflammatory pain in man since a monoclonal antibody (MAb) neutralizing human TNF α diminished the pain associated with rheumatoid arthritis (Rankin *et al.*, 1995).

In the last decade, cytokines generally regarded as 'anti-inflammatory' have been described which inhibit the production of cytokines such as IL-1 β , IL-6, IL-8 and TNF α , which are generally regarded as 'pro-inflammatory'. The class of anti-inflammatory cytokines includes IL-4, IL-10, IL-13 and TGF β (Elias *et al.*, 1991; Callard *et al.*, 1996; Hart *et al.*, 1989; Standiford *et al.*, 1990; Fiorentino *et al.*, 1991; Cassatella *et al.*, 1993; Lord & Lamb, 1996). Recently, it was shown that IL-10 (probably originating from local macrophages) inhibited the inflammatory hyperalgesic responses to carrageenin and bradykinin by two mechanisms: inhibition of hyperalgesic cytokine release and blockade of induction of cyclo-oxygenase-2 (COX-2, Poole *et al.*, 1995). Another inhibitory cytokine, IL-4, produced by Th₂ lymphocytes and mast cells, is known to limit production of the cytokines IL-1 β , IL-6, IL-8 and TNF α (Lord & Lamb, 1996; Tunon de Lara *et al.*, 1994). Also, IL-4 can suppress the delayed type hypersensitivity responses in experimental animals and in man, (Rocken *et al.*, 1996),

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possibly because of its capacity to induce Th₂ T cell responses. In addition to inhibition of the production of inflammatory cytokines, IL-4 has been reported to inhibit the induction of COX-2, with a consequent reduction in the production of PGs (Seitz *et al.*, 1994; Endo *et al.*, 1994).

Given the capacity of IL-4 to inhibit the production of pro-inflammatory cytokines, such as TNF α , IL-1 β , and IL-8, we have tested the possibility that IL-4 limits inflammatory hyperalgesia by diminishing the cytokine mediated hyperalgesic responses to carrageenin, bradykinin, and pro-inflammatory cytokines. In addition, the source of the IL-4 released in inflammatory hyperalgesia was investigated.

Methods

Nociceptive test

A constant pressure of 20 mmHg was applied to the hind-paws of rats and discontinued when they presented a typical freezing reaction (reaction time). This reaction was characterized by a reduction of escape movements: animals usually made several attempts to escape from the position imposed by the experimental situation. These were followed by alterations in respiratory frequency with the onset of a typical shivering reaction. The intensity of hyperalgesia was quantified as the variation in reaction time (delta reaction time) obtained by subtracting values measured 3 h after administration of hyperalgesic substances from (control) reaction times measured before injection at time zero (Ferreira *et al.*, 1978). Reaction times were typically 32–34 s (with s.e.means of 0.5–1.0 s) before injection and 2–4 s after stimulation with hyperalgesic agents. Multiple paw treatments did not alter basal reaction times.

Experimental protocol

In vivo measurements Hyperalgesia was measured 3 h after injections (in 100 μ l, i.pl.) of carrageenin (10 or 100 μ g), bradykinin (50 or 500 ng), TNF α (0.25 or 2.5 pg), IL-1 β (0.05 or 0.5 pg), IL-8 (10 or 100 pg), and PGE₂ (10 or 100 ng) into the hind-paws of rats. [The doses of hyperalgesic agents were the smallest that evoked maximum responses and the time interval of 3 h was a time at which responses to the chosen doses of hyperalgesic agents were all at or close to their peak (Ferreira *et al.*, 1988, 1993; Cunha *et al.*, 1991, 1992; Poole *et al.*, 1995). The order of potency of the cytokines was IL-1 β > TNF α > IL-8 for these human sequence cytokines in rats (Cunha *et al.*, 1992). Whether this order of potency reflects the order of potency of the endogenous cytokines of the rat is unknown. The rat sequence cytokines were not available for this study and certain cytokines exhibit species preference or specificity (Lumpkin, 1987; Morstyn & Burgess, 1988; Stefferl *et al.*, 1996) and the native cytokines remain to be tested.] IL-4 (2.5–10 ng in 50 μ l, i.pl.) or saline (50 μ l) was injected in the (same) hind-paws, 30 min, 2, 12, or 12+2 h before hyperalgesic agents. MAbs to murine IL-4 (BVDG or 11B11, 50 μ g in 50 μ l, i.pl.) were injected into hind-paws to be injected with hyperalgesic agents, 30 min before carrageenin, bradykinin, TNF α , IL-1 β , IL-8, and dextran (200 μ g, 100 μ l). BVDG (50 μ g in 50 μ l, i.pl.) was injected into the hind-paws of athymic rats and, 30 min later, bradykinin (50 ng, 100 μ l) was injected into the (same) hind-paws. Hyperalgesia was assessed after a further 3 h. Also, hyperalgesia was measured 3 h after injection of bradykinin into hind-paws of rats depleted of mast cells by chronic administration (in 50 μ l, i.pl.) of

compound 48/80 (day 1: 1 μ g, day 2: 3 μ g, days 3 and 4: 10 μ g). Bradykinin (50 ng, 100 μ l, i.pl.) was injected on day 5.

In vitro measurements Murine peritoneal macrophages, harvested from the peritoneal cavities of mice treated, 3 days earlier, with sterile thioglycollate (2 ml of a 3% w/v solution), were allowed to adhere to 24-well plastic tissue culture plates. The macrophages were seeded at 10⁶ cells ml⁻¹ well⁻¹ in RPMI with 10% foetal calf serum, penicillin (100 U ml⁻¹) and streptomycin (100 mg ml⁻¹), for 1 h at 37°C, in an atmosphere of 5% CO₂ in air. The monolayers that formed were washed three times with phosphate buffered saline (PBS), pH 7.4, and cultured at 37°C with the following: (a) RPMI medium, (b) LPS (3 μ g ml⁻¹), (c) murine IL-4 (1.6 and 16 ng ml⁻¹), (d) murine IL-4 (1.6 and 16 ng ml⁻¹) and, 12 h later, LPS (3 μ g ml⁻¹), (e) murine IL-4 (16 ng ml⁻¹) and, 2 h later, LPS (3 μ g ml⁻¹). After culture for a further 16 h, the concentrations of IL-1 β and PGE₂ in the supernatants were measured as described previously (TNF α : Cunha *et al.*, 1993; PGE₂: Amersham kit, Poole *et al.*, 1995).

Results are presented as means with s.e.means of groups of five animals for the *in vivo* measurements and of triplicate wells for *in vitro* assays. Two independent *in vitro* assays were performed, each using macrophages from different mice. Differences between responses were evaluated by ANOVA, followed by Bonferroni's *t*-test.

Materials

Drugs The following materials were obtained from the sources indicated. Recombinant human IL-1 β , IL-4, IL-8, TNF α , murine IL-1 β and murine IL-4 (NIBSC preparations coded 86/680, 88/656, 89/520, 87/650, 93/668 and 91/656). The specific activities of these materials are IL-1 β : 100,000 international units (IU) 1 μ g⁻¹ ampoule⁻¹, IL-4: 1,000 IU 0.1 μ g⁻¹ ampoule⁻¹, IL-8: 1,000 IU 1 μ g⁻¹ ampoule⁻¹, TNF α : 40,000 IU 1 μ g⁻¹ ampoule⁻¹, murine IL-1 β : 100,000 IU 0.1 μ g⁻¹ ampoule⁻¹, murine IL-4: 10,000 IU 1 μ g⁻¹ ampoule⁻¹. Carrageenin (FMC Corporation, Philadelphia, U.S.A.). PGE₂, bradykinin, dextran, concanavalin A (Con-A) and compound 48/80 (Sigma, St. Louis, U.S.A.). Bacterial endotoxin from *E. coli* 055:B5 (referred to here as lipopolysaccharide, LPS) and thioglycollate (Difco Laboratories Ltd, West Molesey, Surrey, U.K.). PGE₂ assay reagents and [³H]-thymidine (Amersham International plc, U.K.). Monoclonal IgG antibodies (MAbs) to murine IL-4: BVDG and 11B11 (Professor F. Liew, University of Glasgow, U.K.). Control monoclonal antibody was a purified unrelated IgG raised against ovalbumin in our laboratory.

Animals Male Wistar or Nude-Rowett (athymic) rats, weighing 130–180 g, housed in temperature controlled rooms (22–25°C) with water and food *ad libitum* until use.

Results

Effect of IL-4 on hyperalgesic responses to carrageenin, bradykinin, TNF α , IL-1 β , IL-8 and PGE₂

Injection (in 100 μ l, i.pl.) of carrageenin (100 μ g), bradykinin (500 ng), TNF α (2.5 pg), IL-1 β (0.5 pg), IL-8 (100 pg) and PGE₂ (100 ng) into the hind-paws of Wistar rats evoked hyperalgesia, measured 3 h after injections. Treatment with IL-4 (2.5–10 ng, 50 μ l, i.pl.), 30 min before carrageenin, bradykinin, or TNF α inhibited, in a dose-dependent manner,

hyperalgesic responses to these agents. IL-4 (10 ng, i.pl.) inhibited the hyperalgesic responses to carrageenin, bradykinin and TNF α by 81, 84 and 86%, respectively. Hyperalgesic responses to IL-1 β , IL-8 and PGE $_2$ were not affected by IL-4 (Figure 1). Although IL-4 (10 ng, i.pl.) injected 2 h before IL-1 β (0.5 pg, i.pl.) did not affect the response to IL-1 β , when IL-4 was given 12 or 12+2 h before IL-1 β the hyperalgesic response to this cytokine was inhibited by 30 and 74%, respectively (Figure 2A). The hyperalgesic response to IL-8 (100 pg, i.pl.) was not inhibited by IL-4 (10 ng, i.pl.) injected in any of the three schedules (2, 12 and 12+2 h before IL-8, Figure 2B). There was no visible paw inflammation following treatment with IL-4.

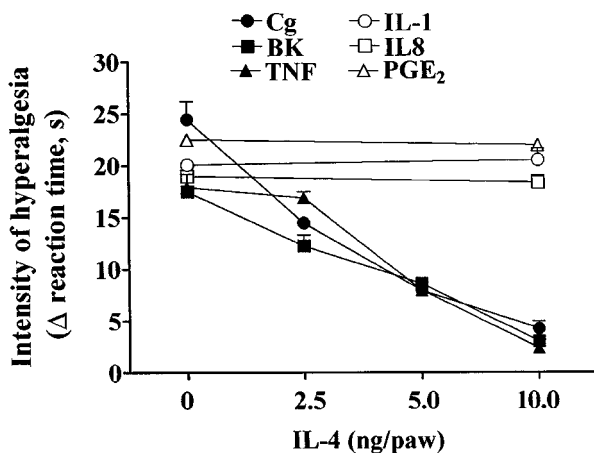


Figure 1 Effect of IL-4 (2.5–10 ng, 100 μ l, i.pl.) on hyperalgesic response to injections (in 100 μ l, i.pl.) of carrageenin (Cg, 100 μ g), bradykinin (BK, 500 ng), TNF α (2.5 pg), IL-1 β (0.5 pg), IL-8 (100 pg), and PGE $_2$ (100 ng). IL-4 was injected into paws to be injected with a hyperalgesic agent, 30 min before the hyperalgesic agent. The intensity of hyperalgesia was measured 3 h after injection of hyperalgesic agents. Means $\pm$ s.e. means in groups of five rats are shown.

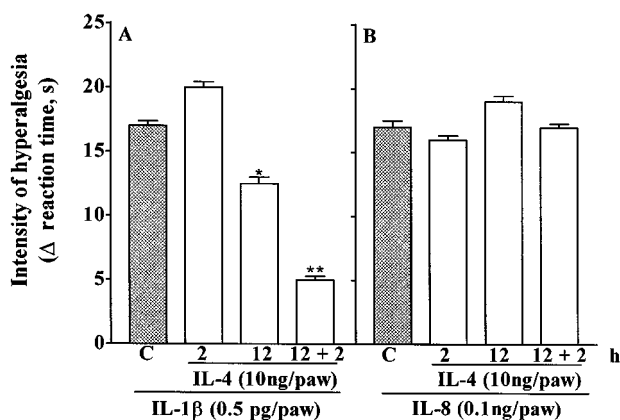


Figure 2 Effect of IL-4 (10 ng, 100 μ l, i.pl.) on hyperalgesic responses to injections (in 100 μ l, i.pl.) of IL-1 β (0.5 pg, panel A) and IL-8 (100 pg, panel B). IL-4 was injected into paws to be injected with a hyperalgesic agent, 2, 12, or 2+12 h before the hyperalgesic agent. The intensity of hyperalgesia was measured 3 h after injection of hyperalgesic agents. Means $\pm$ s.e. means in groups of five rats are shown; * P < 0.001, ** P < 0.0001.

Effect of IL-4 on production of IL-1 β and PGE $_2$ by murine peritoneal macrophages stimulated with LPS

Murine peritoneal macrophages stimulated with LPS (3 μ g ml $^{-1}$ for 16 h) produced IL-1 β and this production was not inhibited by addition to the macrophages of IL-4 (1.6 or 16 ng ml $^{-1}$) for 12 h before stimulation with LPS. Addition to the macrophages of IL-4 (16 ng ml $^{-1}$), for 2 h before stimulation with LPS partially inhibited the production of IL-1 β (–40%, Figure 3A). Stimulation of the macrophages with LPS (3 μ g ml $^{-1}$) also evoked release of PGE $_2$, and this release was inhibited by 40 and 70% by the treatment of the macrophages for 12 h with IL-4 at 1.6 and 16 ng ml $^{-1}$, respectively. Treatment of macrophages with IL-4 (16 ng ml $^{-1}$) for 2 h prior to stimulation with LPS (3 mg ml $^{-1}$) did not inhibit LPS-induced release of PGE $_2$ (Figure 3B).

Potentialiation by monoclonal antibodies to IL-4 of hyperalgesic responses to carrageenin, bradykinin, TNF α , but not IL-1 β and IL-8

Two MAbs to murine IL-4, 11B11 and BVDG (50 μ g, i.pl.), but not a control MAb (50 μ g, i.pl.), injected 30 min before injections (i.pl.) of carrageenin (10 μ g), bradykinin (50 ng) and TNF α (0.25 pg) potentiated responses, measured 3 h after injection of these hyperalgesic agents (responses after 11B11: carrageenin = +74%, bradykinin = +117%, TNF α = +148%; responses after BVDG: carrageenin = +75%, bradykinin = +107%, TNF α = +204%). Hyperalgesic responses to

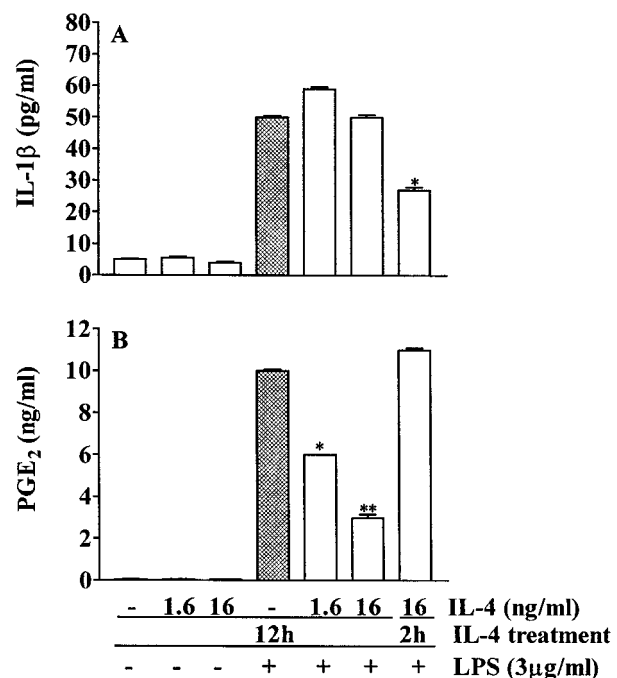


Figure 3 Effect of IL-4 on the production of IL-1 β and PGE by murine macrophages stimulated with LPS (3 μ g ml $^{-1}$). The macrophages (10⁶ cells well $^{-1}$) were cultured with murine IL-4 (1.6 or 16 ng ml $^{-1}$) in medium (+) or medium alone (–). Two h or 12 h later the macrophages were stimulated with LPS (3 μ g ml $^{-1}$) in medium (+) or with medium alone (–) for a further 16 h. At the end of this period the concentrations of IL-1 β (panel A) and PGE $_2$ (panel B) in the macrophage-conditioned media were measured. Means $\pm$ s.e. means of triplicate wells are shown; * P < 0.001, ** P < 0.0001.

IL-1 β (0.05 pg, i.pl.) and IL-8 (10 pg, i.pl.) were not affected by the MABs to murine IL-4 (Figure 4).

Potentialiation by a monoclonal antibody to IL-4 of hyperalgesic response to bradykinin of athymic (nude) rats

As in Wistar rats, in athymic Nude-Rowett rats BVDG (50 μ g, i.pl.), but not a control antibody, potentiated by +101% hyperalgesic responses measured 3 h after injection of bradykinin (50 μ g, i.pl., Figure 5A). The absence of the thymus in the Nude-Rowett rats was confirmed by histopathological analysis and in a splenocyte proliferation assay using Con-A. Proliferative responses (^{3} H-thymidine incorporation, c.p.m.) to Con-A (5 μ g ml $^{-1}$) were for Wistar rats, culture medium alone = 1,320 $\pm$ 140 c.p.m., Con-A = 12,316 $\pm$ 940 c.p.m. and for Nude-Rowett rats, culture medium alone = 1,050 $\pm$ 128 c.p.m., Con-A = 970 $\pm$ 90 c.p.m.

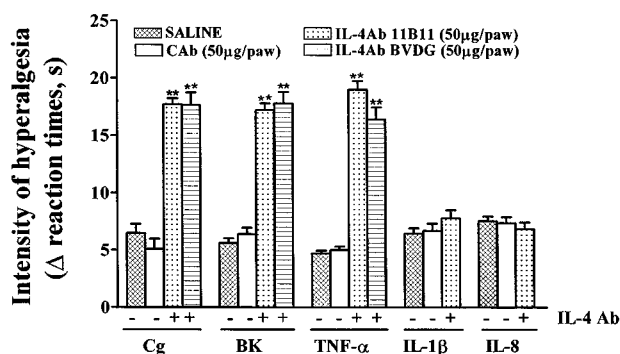


Figure 4 Effect of MABs to IL-4, 11B11 and BVDG (50 μ g in 50 μ l, i.pl.), a control antibody and saline on hyperalgesic responses to injections (in 100 μ l, i.pl.) of carrageenin (Cg, 10 μ g), bradykinin (BK, 50 ng), TNF α (0.25 pg), IL-1 β (0.05 pg), and IL-8 (10 pg). One of the MABs or saline was injected into paws to be injected with a hyperalgesic agent, 30 min before the hyperalgesic agent. The intensity of hyperalgesia was measured 3 h after injection of hyperalgesic agents. Means $\pm$ s.e. means in groups of five rats are shown. ** P < 0.0001.

Failure of a monoclonal antibody to IL-4 to potentiate the hyperalgesic response to bradykinin following mast cell depletion

Potentiation of the hyperalgesic response to bradykinin (50 ng, i.pl.), measured 3 h after its injection, by BVDG (50 μ g, i.pl., given into paws to be injected, 30 min later, with bradykinin) was abolished by the depletion of mast cells by local treatment (i.pl.) with compound 48/80 (Figure 5B). No potentiation of the response to bradykinin by the MAB to IL-4 was observed in animals depleted of mast cells, compared with an increase in response of +104% in wild type (control) animals. There was no visible paw inflammation following treatment with compound 48/80.

Induction by a monoclonal antibody to IL-4 of a hyperalgesic response to dextran

Injection of dextran (200 μ g, i.pl) into one hind-paw of Wistar rats did not evoke hyperalgesia, measured 3 h after injection. BVDG (50 μ g), but not a control MAB, injected into paws to be injected, 30 min later, with dextran, rendered the paws sensitive to dextran, which now evoked a hyperalgesic response (Figure 5C).

Discussion

IL-4 has a number of biological activities, including B cell activation, proliferation of T cells and natural killer cells, upregulation of CD23 expression and stimulation of the expression of the Th $_1$ phenotype by Th $_0$ T helper cells. In addition, IL-4 has co-ordinated anti-inflammatory effects by suppressing the production of IL-1 (and also TNF α , IL-8 and IFN γ) while upregulation production of IL-1ra by LPS-stimulated monocytes (Vannier *et al.*, 1992; Fenton *et al.*, 1992). Other, essentially anti-inflammatory, effects of IL-4 include inhibition of the induction of the nitric oxide and synthase and COX-2 enzymes responsible for the production of nitric oxide and prostaglandins, respectively. In the present study the potential anti-hyperalgesic effect of IL-4 in a model of mechanical hyperalgesia was investigated.

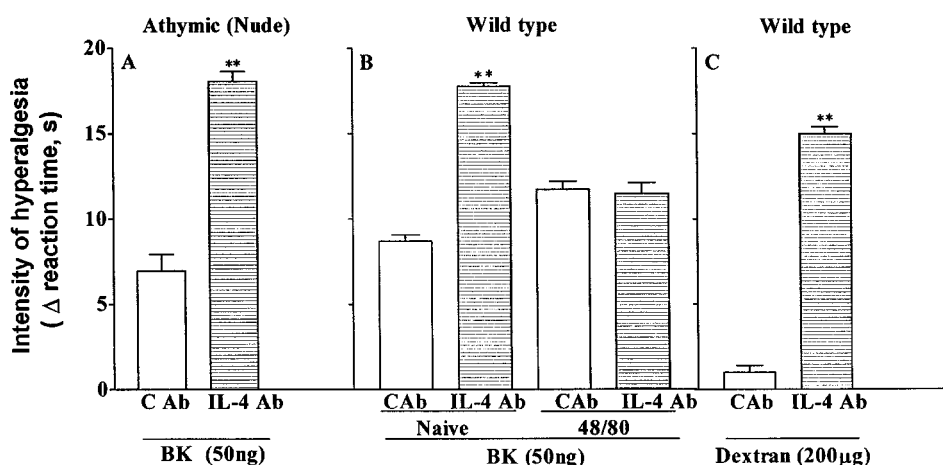


Figure 5 Effect of a MAB to IL-4, BVDG (50 μ g in 50 μ l, i.pl., horizontally hatched columns) and a control MAB (open columns) on hyperalgesic responses to injections (in 100 μ l, i.pl.) of bradykinin (BK, 50 ng, panel A,B) and dextran (200 μ g, panel C) in athymic (nude) rats (panel A), wild type rats (panel C) and wild type rats depleted of mast cells (panel B). The MABs were injected into paws to be injected with a hyperalgesic agent, 30 min before the hyperalgesic agent. The intensity of hyperalgesia was measured 3 h after injection of hyperalgesic agents. Means $\pm$ s.e. means in groups of five rats are shown; ** P < 0.0001.

Local administration of IL-4 inhibited, in a dose-dependent manner, hyperalgesic responses to carrageenin, bradykinin and TNF α . This anti-hyperalgesic effect of IL-4 was likely to have been a consequence of its capacity to inhibit the production of pro-inflammatory cytokines (namely TNF α , IL-1 β and IL-8) since, in carrageenin-evoked hyperalgesia, bradykinin is generated and initiates the release of TNF α , which stimulates the release of IL-1 and IL-8 (Tiffany & Burch, 1989; Ferreira *et al.*, 1993). Consistent with this notion, the release of IL-1 β by activated murine macrophages was partially inhibited by treating the cells with IL-4 for 2 h. The absence of an effect of IL-4 on hyperalgesic responses to IL-8 and PGE $_2$ was expected, since IL-8 stimulates the local production of sympathetic amines (Cunha *et al.*, 1991), which sensitize nociceptors (Ferreira & Lorenzetti, 1981). IL-10, which has a profile of action similar to that of IL-4 in terms of its inhibitory actions on the production of cytokines generally regarded as pro- also inhibited hyperalgesic responses to carrageenin, bradykinin and TNF α , but not those to IL-8 and PGE $_2$ (Poole *et al.*, 1995). In contrast to IL-10, IL-4 injected 0.5 h or 2 h before IL-1 β did not inhibit its hyperalgesic effect. Inhibition was observed only when IL-4 was injected either once, 12 h before, or twice, at 12 + 2 h before the administration of IL-1 β . Since IL-1 β -induced hyperalgesia is mediated by eicosanoid products (Ferreira *et al.*, 1988; Seibert *et al.*, 1994), the effect of IL-4 on PGE $_2$ production by activated macrophages was investigated. Similar to its effect on IL-1 β evoked hyperalgesia, IL-4 inhibited, in a dose-dependent manner, PGE $_2$ production only when the macrophages were pre-treated with IL-4 for 12 h; treatment for 2 h before the challenge did not affect the production of the prostanoid. These results are consistent with IL-1 β -induced hyperalgesia *via* the release of PGs (Ferreira *et al.*, 1988; Seibert *et al.*, 1994).

The results with murine macrophages are different from the rapid blockade by IL-4 of the release of PGE $_2$ (60 min) in human monocytes and synoviocytes (Seitz *et al.*, 1994; Endo *et al.*, 1996). This difference might be a reflection of differences in the cell types studied or might be a species difference, or both. The lack of effect of IL-4 on the hyperalgesic response to IL-8 (given either 12 h before or 12 + 2 h before the IL-8), reinforces the suggestion that the hyperalgesia induced by IL-8 is subsequent to the release of mediators other than cyclooxygenase products (Cunha *et al.*, 1991).

The finding that the hyperalgesic responses to carrageenin, bradykinin, and TNF α were enhanced by treatment of paws with two different MAbs to IL-4 strongly suggests that endogenous IL-4 has an important role in limiting inflammatory hyperalgesia. The failure of the MAbs to IL-4 to enhance

the hyperalgesic effects of IL-1 β and IL-8 suggests that the predominant effect of endogenous IL-4 in inflammatory hyperalgesia is to inhibit the release of cytokines, rather than the PGs which sensitize nociceptors (Ferreira & Lorenzetti, 1981; Nakamura & Ferreira, 1987).

IL-4 is released mainly by Th $_2$ lymphocytes and by mast cells (Lord & Lamb, 1996; Tunon de Lara *et al.*, 1994). To gain information about which cell types were releasing IL-4 during inflammatory hyperalgesia, the capacity of a MAb against IL-4 to enhance the hyperalgesic effect of bradykinin in athymic rats (deficient in T lymphocytes) and in rats depleted of mast cells (with compound 48/80, Di Rosa *et al.*, 1971) was investigated. Histopathological analysis confirmed the absence of the thymus (in the athymic animals) and the effectiveness of the depletion of mast cells after compound 48/80. The absence of the thymus was also confirmed using a splenocyte proliferation assay. The finding that the MAb to IL-4 potentiated the hyperalgesic effect of bradykinin in athymic rats but not in animals depleted of mast cells strongly suggests that mast cells were the source of the IL-4 that limited the inflammatory hyperalgesia. Supporting evidence for mast cells as the source of the IL-4 was obtained in the experiment with dextran, which disrupts mast cells (Nishida *et al.*, 1978; Nishida & Tomizawa, 1980). Thus, a MAb to IL-4 induced a hyperalgesic response to a substance, dextran, in (wild type) rats not previously sensitive to this agent.

In conclusion, the data described above provide evidence that IL-4, released by resident mast cells, limits the intensity and duration of inflammatory hyperalgesia. Consequently, a lack of IL-4 may contribute to the maintenance of chronic inflammatory pain. In the early phase of the hyperalgesic response, IL-4 appears to achieve its effects by inhibiting the production of hyperalgesic cytokines (e.g. TNF α and IL-1). In the later phase of the hyperalgesic response, the effect of IL-4 also may result also from inhibition of COX-2, since IL-4, given 12 h before IL-1 β , inhibited the hyperalgesic effect of IL-1 β and the release *in vitro* of PGE $_2$ by murine macrophages stimulated with LPS. These interpretations are consistent with data from preliminary experiments in our laboratory with the relatively selective COX-2 inhibitor meloxicam.

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Dual actions of the metabolic inhibitor, sodium azide on K_{ATP} channel currents in the rat CRI-G1 insulinoma cell line

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1 The effects of various inhibitors of the mitochondrial electron transport chain on the activity of ATP-sensitive K^+ channels were examined in the Cambridge rat insulinoma G1 (CRI-G1) cell line using a combination of whole cell and single channel recording techniques.

2 Whole cell current clamp recordings, with 5 mM ATP in the pipette, demonstrate that the mitochondrial uncoupler sodium azide (3 mM) rapidly hyperpolarizes CRI-G1 cells with a concomitant increase in K^+ conductance. This is due to activation of K_{ATP} channels as the sulphonylurea tolbutamide (100 μ M) completely reversed the actions of azide. Other inhibitors of the mitochondrial electron transport chain, rotenone (10 μ M) or oligomycin (2 μ M) did not hyperpolarize CRI-G1 cells or increase K^+ conductance.

3 In cell-attached recordings, bath application of 3 mM sodium azide (in the absence of glucose) resulted in a rapid increase in K_{ATP} channel activity, an action readily reversible by tolbutamide (100 μ M). Application of sodium azide (3 mM), in the presence of Mg-ATP, to the intracellular surface of excised inside-out patches also increased K_{ATP} channel activity, in a reversible manner.

4 In contrast, rotenone (10 μ M) or oligomycin (2 μ M) did not increase K_{ATP} channel activity in either cell-attached, in the absence of glucose, or inside-out membrane patch recordings.

5 Addition of sodium azide (3 mM) to the intracellular surface of inside-out membrane patches in the presence of Mg-free ATP or the non-hydrolysable analogue 5'-adenylylimidodiphosphate (AMP-PNP) inhibited, rather than increased, K_{ATP} channel activity.

6 In conclusion, sodium azide, but not rotenone or oligomycin, directly activates K_{ATP} channels in CRI-G1 insulin secreting cells. This action of azide is similar to that reported previously for diazoxide.

Keywords: K_{ATP} channels; azide; mitochondrial inhibitors; rotenone; oligomycin

Abbreviations: CRI-G1, Cambridge rat insulinoma G1; DMSO, Dimethyl sulphoxide; AMP-PNP, 5'-adenylylimidodiphosphate.

Introduction

It is well established that ATP-sensitive K^+ (K_{ATP}) channels play a key role in the insulin secretory mechanism of pancreatic beta cells (Ashcroft & Rorsman, 1991). Closure of these channels results in depolarization of the cell membrane and Ca^{2+} influx via voltage-gated Ca^{2+} channels which in turn triggers exocytotic release of insulin (Ashford, 1990). Thus agents that modulate the activity of K_{ATP} channels are important therapeutic tools in the treatment of hyperglycaemia and hypoglycaemia.

In pancreatic beta cells (Trube *et al.*, 1986; Dunne *et al.*, 1987) and insulin secreting cells (Sturgess *et al.*, 1988; Kozlowski *et al.*, 1989), the hyperglycaemic agent diazoxide activates K_{ATP} channels, resulting in suppression of insulin secretion. Diazoxide-induced activation of K_{ATP} channels requires the presence of Mg^{2+} and ATP (Sturgess *et al.*, 1988; Kozlowski *et al.*, 1989; Dunne, 1989), suggesting that a phosphorylation-dependent process may underly this action of diazoxide. The K_{ATP} channel of the pancreatic beta cell comprises of two proteins: the sulphonylurea receptor, SUR1 and an inwardly rectifying K^+ channel, Kir 6.2 (Inagaki *et al.*, 1995; Sakura *et al.*, 1995). Although the precise mode of action of diazoxide is not entirely clear it is thought to act directly on SUR1 (Inagaki *et al.*, 1996; Tucker *et al.*, 1997) rather than Kir 6.2. Recent studies also indicate that diazoxide may have actions in pancreatic beta cells, through inhibiting mitochon-

drial energy metabolism (Grimmsmann & Rustenbeck, 1998). However the functional significance of this, if any, in terms of K_{ATP} channel activity or stimulus secretion coupling is presently unknown.

In pancreatic beta cells, the activity of K_{ATP} channels is tightly regulated by the metabolic state of the cell, since a reduction in the cytosolic concentrations of ATP, by either an elevation in energy consumption or inhibition of energy metabolism, results in activation of K_{ATP} channels. Thus agents that interfere with ATP production via inhibition of mitochondrial energy metabolism, are commonly used to activate both native and cloned K_{ATP} channels (Ashcroft & Ashcroft, 1990). Indeed, metabolic inhibition by sodium azide (Misler *et al.*, 1989), cyanide (Misler *et al.*, 1986) and oligomycin (Niki *et al.*, 1989) has been reported to activate K_{ATP} channels in pancreatic beta cells and insulin secreting cells. Cyanide and rotenone are also reported to activate K_{ATP} channels in rat substantia nigra dopaminergic neurones (Murphy & Greenfield, 1991; Roper & Ashcroft, 1995) and cyanide activates K_{ATP} channels in cardiac myocytes (Takei & Noma, 1984; Ito *et al.*, 1991) and skeletal muscle (Allard *et al.*, 1995). Furthermore, in oocyte expression systems sodium azide is used routinely to activate K_{ATP} channel currents (Tucker *et al.*, 1997; Gribble *et al.*, 1997a). It is well known that sodium azide (Tsubaki & Yoshikawa, 1993) inhibits oxidative phosphorylation via inhibition of cytochrome oxidase, the final enzyme in the mitochondrial electron

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transport chain, thereby resulting in a rapid depletion of intracellular ATP. Indeed, the effects of sodium azide on K_{ATP} channels have been attributed to the resultant reduction in ATP levels (Gribble *et al.*, 1997a). In contrast, rotenone and oligomycin deplete intracellular ATP levels *via* inhibition of the mitochondrial flavoprotein NADH dehydrogenase (Ragan & Racker, 1973) and the mitochondrial F_1 -ATPase (Penefsky, 1985), respectively.

In view of the recent report that diazoxide may also act to inhibit mitochondrial metabolism, an action presumably distinct from its activation of K_{ATP} channels, we have re-examined the effects of sodium azide, rotenone and oligomycin on K_{ATP} channels using the rat CRI-G1 insulinoma cell line. We now demonstrate that sodium azide, but not rotenone or oligomycin directly activate K_{ATP} channel currents even in the presence of high concentrations of ATP, suggesting that inhibition of mitochondrial energy metabolism may not underlie this effect. Furthermore, in a manner similar to diazoxide (Sturgess *et al.*, 1988; Kozłowski *et al.*, 1989), sodium azide-induced K_{ATP} channel activation requires the presence of MgATP.

Methods

Cell culture

Cells from the insulin-secreting cell line CRI-G1 were grown in Dulbecco's modified Eagle's medium with sodium pyruvate and glucose, supplemented with 10% (v/v) foetal calf serum and 1% penicillin and streptomycin at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2 . Cells were passaged at 2–5 day intervals as described previously (Carrington *et al.*, 1986), plated onto 3.5 cm petri dishes (Falcon 3001) and used 1–4 days after plating.

Electrophysiological recording and analysis

Experiments were performed using whole cell current- and voltage-clamp recording configurations to monitor membrane potential and macroscopic currents, respectively and cell-attached and inside-out configurations to monitor single channel responses, as described previously (Harvey *et al.*, 1997). In voltage-clamp recordings the membrane potential was held at -50 mV and 10 mV steps of 100 ms duration were applied every 200 ms (range of voltages -120 mV to -30 mV). Current and voltage were measured using Axopatch 200B and List EPC-7 amplifiers, and currents evoked in response to the voltage-step protocol were analysed using pClamp 6.0 software (Axon Instruments, Foster City, CA, U.S.A). Single channel data were analysed for current amplitude (I) and average channel activity (Nf.Po) as described previously (Lee *et al.*, 1995). Changes in Nf.Po as a result of drug effects are expressed as a percentage of control. Current clamp and single channel data were recorded onto digital audio tapes and replayed for illustration on a Gould TA 240 chart recorder.

Recording electrodes were pulled from borosilicate glass capillaries and had resistances of 1–5 M Ω for whole cell recordings and 8–12 M Ω for single channel experiments when filled with electrolyte solution. The pipette solution for whole cell recordings comprised (mM): KCl 140, MgCl₂ 0.6, CaCl₂ 2.73, Mg-ATP 5.0, HEPES 10.0, EGTA 10, pH 7.2 (free Ca^{2+} of 100 nM), whereas for cell-attached and single channel recordings it contained (mM): KCl 140, CaCl₂ 1, MgCl₂ 1, HEPES 10, pH 7.2. The bath solution for whole cell and cell-

attached recordings comprised of normal saline (mM): NaCl 135, KCl 5, MgCl₂ 1, CaCl₂ 1, HEPES 10, pH 7.4, whereas for inside-out excised patches the bath solution contained either (mM): KCl 140, MgCl₂ 1, CaCl₂ 2, EGTA 10, HEPES 10, pH 7.2 (free Mg^{2+} of 0.6 mM and Ca^{2+} of <30 nM) or KCl 140, EDTA 10.0, CaCl₂ 4.6, HEPES 10, pH 7.2 (free Mg^{2+} and Ca^{2+} of <30 nM). The free Ca^{2+} and Mg^{2+} concentrations were calculated using the 'METLIG' programme (P. England and R. Denton, University of Bristol, U.K.). In order to calculate these values it was necessary to assume that the salts used to prepare the ' Mg^{2+} -free' solution were contaminated with the trace levels of Mg^{2+} indicated by the manufacturer. All solution changes were achieved by superfusing the bath with a gravity feed system at a rate of 10 ml min⁻¹ which allowed complete bath exchange within 2 min. All experiments were performed at room temperature (22–25°C).

Drugs

Sodium azide, rotenone, oligomycin, tolbutamide, Mg-AMP-PNP, K-ATP and Mg-ATP were obtained from Sigma, Poole, Dorset, U.K. Tolbutamide was made up as a 100 mM stock solution in dimethyl sulphoxide (DMSO). ATP (potassium and magnesium salts) was made up as a 100 mM stock solution in 10 mM HEPES at pH 7.2 and kept at -4°C until required, whereas Mg-AMP-PNP was stored at -4°C as a 10 mM stock solution in 10 mM HEPES.

Statistical analysis

All data are expressed as the mean $\pm$ s.e.mean and statistical analyses were performed using unpaired student's *t*-test (unless otherwise stated).

Results

Sodium azide activates K_{ATP} channels

Under current clamp conditions with 5 mM ATP in the pipette solution to maintain K_{ATP} channels in the closed state, the mean resting membrane potential of CRI-G1 cells was -37.5 ± 0.83 mV ($n=19$). Application of the metabolic inhibitor sodium azide (0.1–3 mM) under these conditions resulted in a rapid hyperpolarization of CRI-G1 cells to -66 ± 1.6 mV ($n=11$; Figure 1a). The hyperpolarization was initiated within 0.5–1.0 min of application (including a dead space time of 10–15 s), reached a maximum 2–3 min later and was maintained in the continued presence of the agent. Examination of the voltage-clamped macroscopic currents indicate that the azide-induced hyperpolarization was accompanied by an increase in the slope conductance from a control value of 0.59 ± 0.06 nS to 4.03 ± 1.02 nS following exposure to 3 mM sodium azide ($n=11$; Figure 1b). The mean reversal potential (obtained from the point of intersection of the current-voltage relationships) associated with this increase in conductance was -78 ± 0.64 mV ($n=11$) which is close to the calculated value for E_K of -84 mV under these conditions, indicating an increase in K^+ conductance. The azide-induced hyperpolarization and increased K^+ conductance were readily reversed on washout of this agent such that the membrane potential and slope conductance values following washout of azide were -38 ± 1.3 mV and 0.44 ± 0.09 nS ($n=3$). The sulphonylurea tolbutamide (100 μM) completely reversed the membrane hyperpolarization and increase in conductance to

pre-azide levels (-36 ± 1.8 mV and 0.39 ± 0.08 nS) indicating that these actions of azide are due to activation of K_{ATP} channels ($n=5$; Figure 1). Lower concentrations of sodium azide were also examined, 1 mM ($n=3$) and 0.3 mM ($n=2$) induced similar levels of hyperpolarization and increased slope conductance. The threshold concentration for azide action was approximately 0.1 mM, where two out of five cells responded as described above.

Effects of rotenone and oligomycin on whole cell currents and membrane potential

In order to determine whether the above effects of sodium azide on CRI-G1 cells were specific for this mitochondrial chain inhibitor, the actions of other mitochondrial inhibitors were examined. In particular, the effects of rotenone, a specific inhibitor of NADH dehydrogenase (Ragan & Racker, 1973) and oligomycin, which inhibits the mitochondrial F_1 -ATPase (Penefsky, 1985) on membrane potential and conductance were investigated. Under current clamp conditions with 5 mM ATP in the pipette solution, application of rotenone (10 μ M) for at least 15–20 min failed to effect the membrane potential of CRI-G1 insulinoma cells (Figure 2a). Thus the mean resting membrane potential values obtained in the absence and following 15 min exposure to rotenone were -36 ± 1.3 mV and -35 ± 1.9 mV, respectively ($n=5$; $P>0.05$). In addition, rotenone had no effect on the slope conductance (obtained from the current voltage relationship) of CRI-G1 cells. The mean slope conductance value obtained in control conditions was 0.63 ± 0.09 nS whereas in the presence of 10 μ M rotenone it was 0.65 ± 0.12 nS ($n=5$; $P>0.05$; Figure 2b).

Similarly, application of oligomycin (2 μ M) for at least 15–20 min, failed to effect either the membrane potential or slope conductance of CRI-G1 cells (Figure 2). Thus the membrane potential and slope conductance values obtained under control conditions were -35 ± 1.5 mV and 0.54 ± 0.02 nS, respectively, whereas following incubation with 2 μ M oligomycin for at least 20 min the values obtained were -36 ± 2.0 mV and 0.53 ± 0.05 nS, respectively ($n=3$; $P>0.05$; Figure 2c,d). The lack of effect of rotenone and oligomycin on the electrical

properties of CRI-G1 cells in the presence of excess intracellular ATP, is in keeping with their effects as metabolic inhibitors. However, it is unlikely that inhibition of mitochondrial energy metabolism *per se* underlies the azide-induced changes in membrane potential or conductance observed in CRI-G1 cells.

Sodium azide activates single K^+ channel currents

Identification of K_{ATP} channels as the molecular target for the acute actions of azide in these cells was obtained from cell-attached and excised inside-out single channel recordings. Application of sodium azide (3 mM) to the bath during cell-attached recordings induced activation of K_{ATP} channel currents in six out of seven recordings (Figure 3b). Analysis of total channel activity (over a 60 min period), 1–2 min after formation of the cell-attached configuration, resulted in a mean channel activity (Nf.Po) of 0.05 ± 0.02 and this increased to 0.22 ± 0.07 ($n=7$; $P<0.05$) 6–7 min after the addition of 3 mM sodium azide to the bath. Identical control experiments in the absence of sodium azide resulted in no K_{ATP} channel activation with mean channel activity values of 0.05 ± 0.02 and 0.04 ± 0.02 ($n=3$; $P>0.05$) obtained at equivalent time periods (Figure 3a). The azide-induced increase in channel activity was sustained for the duration of recordings, in the continued presence of azide and could be inhibited reversibly by tolbutamide (100 μ M; $n=4$; Figure 3b). Furthermore, following azide-induced increase in K^+ channel activity cell-attached, excision of the patch into the inside-out configuration allowed unequivocal identification of the channel as K_{ATP} by inhibition by either 0.1 mM AMP-PNP ($n=3$; data not illustrated) or 1 mM ATP ($n=3$). Thus these data are consistent with K_{ATP} channels as the molecular target for sodium azide-induced hyperpolarization of CRI-G1 cells.

Dual effects of sodium azide in excised patches

In excised inside-out patches recordings were made in symmetrical (140 KCl in pipette and bath) K^+ -containing solution and the membrane potential was held at +40 mV. In

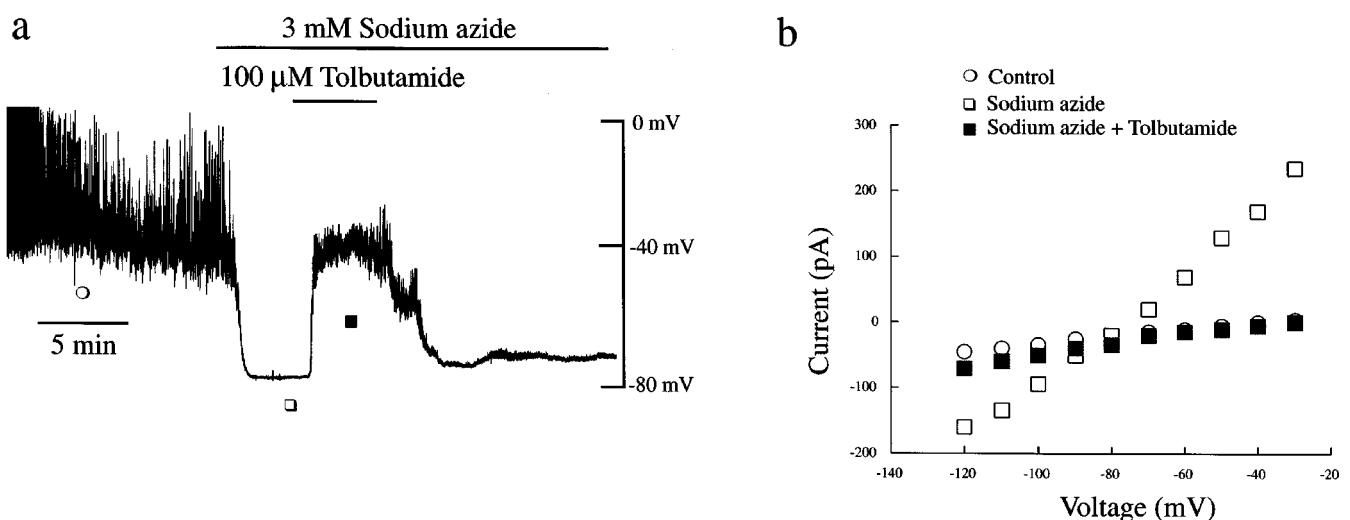


Figure 1 Sodium azide hyperpolarizes CRI-G1 cells *via* activation of K_{ATP} channel currents. (a) Whole cell current clamp trace of a CRI-G1 cell dialysed with 5 mM ATP. In this and subsequent figures, the trace begins approximately 5 min after obtaining the whole cell configuration. Application of sodium azide (3 mM) for the time indicated resulted in rapid hyperpolarization of the cell membrane from -38 mV to -78 mV, an action readily reversed by the sulphonylurea tolbutamide (100 μ M). (b) The current-voltage relations for the currents obtained at the specified points in (a): control ($\circ$); sodium azide ($\square$) and sodium azide and tolbutamide ($\blacksquare$). Sodium azide increased the membrane conductance relative to control and tolbutamide (100 μ M) reversed the azide-induced increase in conductance with a reversal potential of -80 mV.

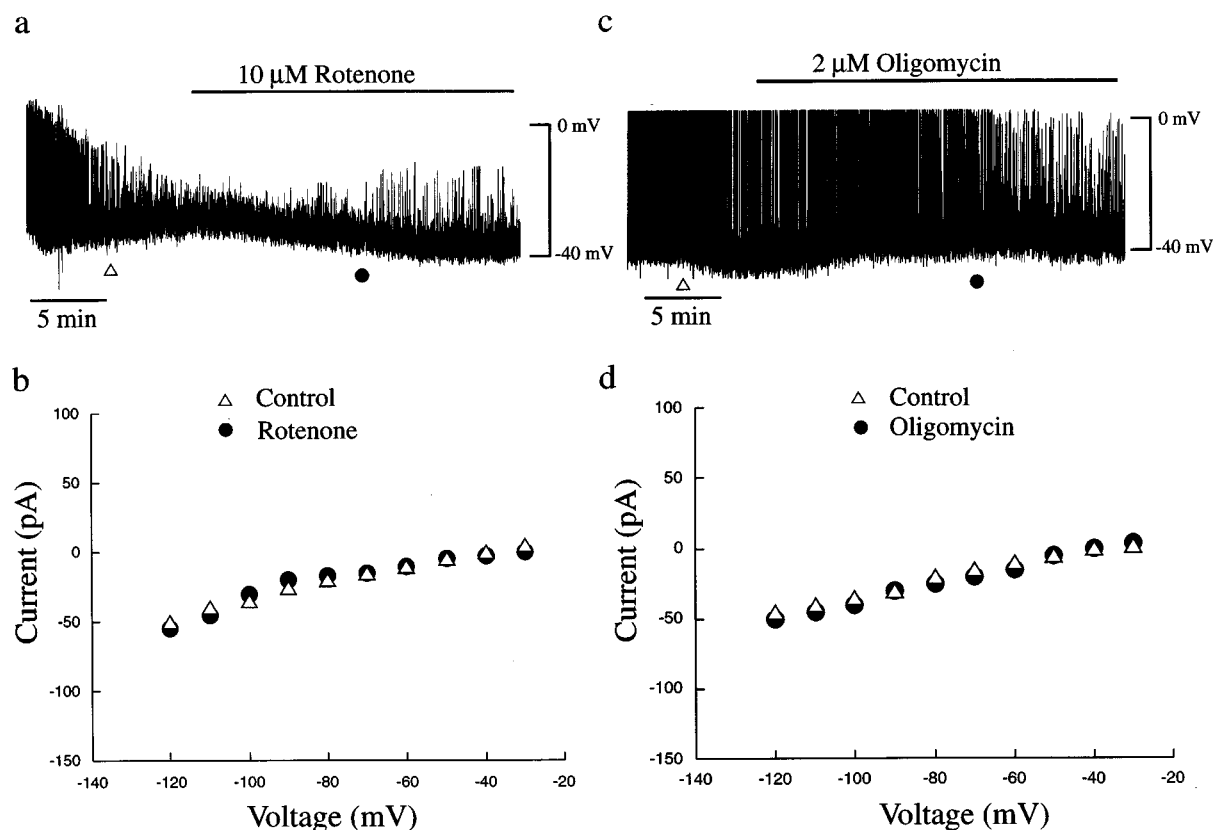


Figure 2 Effects of rotenone and oligomycin on membrane potential and whole cell currents. (a) The upper panel is a current clamp record of a cell dialysed with a 5 mM ATP-containing electrode solution. Application of rotenone (10 μ M) for the time indicated had no effect on the resting membrane potential of CRI-G1 cells. (b) Current-voltage relations for the voltage clamped macroscopic currents at the times specified in a: control (Δ) and 10 μ M rotenone ($\bullet$). Rotenone had no effect on the membrane conductance of CRI-G1 insulinoma cells. (c) Current clamp record of a CRI-G1 cell dialysed with a 5 mM ATP-containing electrode solution. Addition of oligomycin (2 μ M) for the time indicated had no effect on the resting membrane potential of the cell. (d) Plot of the current-voltage relations for the voltage clamped currents obtained in c; control (Δ) and oligomycin ($\bullet$). Oligomycin failed to affect the slope conductance of CRI-G1 cells.

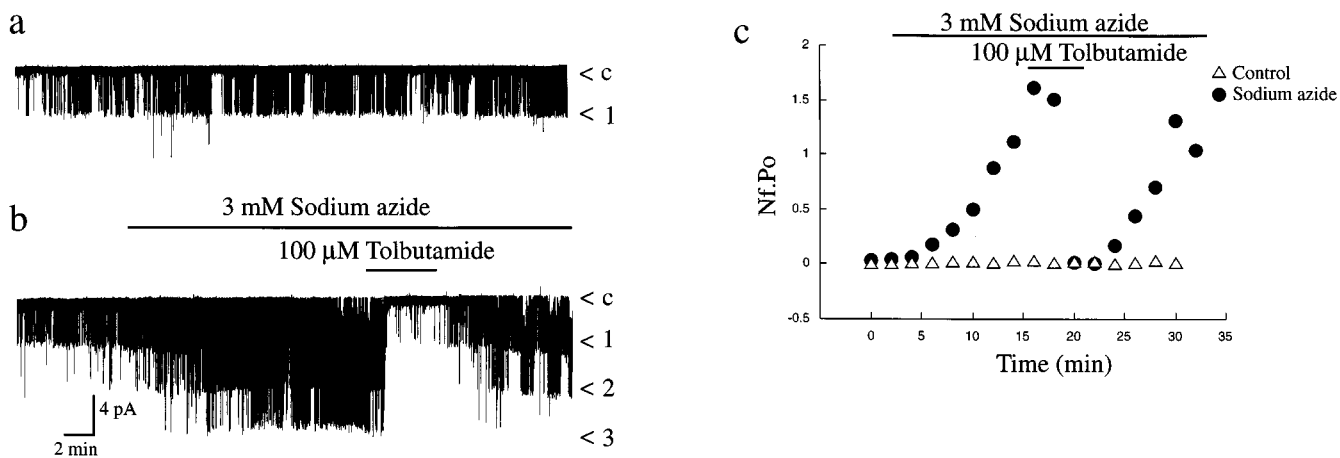


Figure 3 Sodium azide activates single potassium channels. (a) Cell attached recording from a single CRI-G1 cell, at 10 mV applied to the recording pipette. Continuous recording over a 20 min period showed that in the absence of any drugs there was no significant change in channel activity with time. Note that under these recording conditions channel openings are denoted as downward deflections (inward currents). In this and subsequent single channel recordings the symbol c refers to the closed state of the channel. (b) cell-attached recording of a CRI-G1 cell at 10 mV applied potential. Addition of sodium azide (3 mM) for the time indicated resulted in a rapid increase in channel activity, an action that was readily reversed by addition of tolbutamide (100 μ M). (c) Graph of channel activity (Nf.Po) expressed as a function of time obtained from the cell-attached patches in a (Δ) and b ($\bullet$).

the absence of MgATP in the bathing medium, application of sodium azide (3 mM) resulted in an inhibition of K_{ATP} channel activity on ten out of ten occasions (Figure 4a). This action of azide was reversible in only four of the ten patches. Run down of K_{ATP} channels during recordings is likely to underlie the lack of reversibility in the remaining six patches. The mean channel activity obtained in control conditions (in the absence of Mg^{2+}), in the presence of 3 mM sodium azide and on washout were 1.25 ± 0.29 , 0.58 ± 0.14 and 0.62 ± 0.19 , respectively ($n=10$). In contrast, in inside-out patches bathed in Mg^{2+} -containing medium, addition of MgATP (0.1 mM) resulted in a $77.8 \pm 5.4\%$ inhibition of K_{ATP} channel activity relative to control ($n=10$). Subsequent exposure to sodium azide (3 mM) in the continued presence of 0.1 mM MgATP resulted in an immediate (0.5–1 min) increase ($270 \pm 76\%$) in K_{ATP} channel activity ($n=10$; Figure 4b). The corresponding mean levels of channel activity in the presence of MgATP (0.1 mM) and following application of sodium azide were 0.16 ± 0.09 and 0.40 ± 0.17 , respectively ($n=10$; $P<0.05$). The azide-induced increase in channel activity was readily reversed on washout of azide, maintaining MgATP at 0.1 mM (0.03 ± 0.01 ; $n=4$). These data indicate that in the absence of MgATP, sodium azide inhibited K_{ATP} channel activity, whereas in the presence of MgATP sodium azide resulted in rapid activation of these channels. These findings parallel identical dual actions reported for the hyperglycaemic agent, diazoxide on K_{ATP} channels in this cell line (Kozlowski *et al.*, 1989).

In order to determine whether Mg^{2+} ions are a prerequisite for activation of K_{ATP} channels by sodium azide, the effects of sodium azide were examined in excised patches bathed in solution containing 0.1 mM ATP but in the absence of Mg^{2+} (by EDTA chelation and omission of $MgCl_2$). Under these conditions, application of 0.1 mM ATP resulted in $97.3 \pm 1.5\%$

inhibition of K_{ATP} channel activity ($n=6$, Figure 5), which was significantly greater ($P<0.05$) than that observed in the presence of MgATP as reported previously (Kozlowski *et al.*, 1989). Subsequent addition of sodium azide (3 mM) in the presence of 0.1 mM ATP failed to activate K_{ATP} channel activity ($n=6$). The values of Nf.Po obtained in the presence of 0.1 mM ATP, sodium azide and ATP, and following washout of sodium azide but with ATP still present were 0.063 ± 0.02 , 0.039 ± 0.008 and 0.016 ± 0.005 , respectively ($n=6$). These data are consistent with the absolute requirement of Mg^{2+} ions for sodium azide-induced activation of channel activity in the presence of ATP.

Rotenone and oligomycin do not activate single K_{ATP} channel currents

It is possible that in the whole cell configuration some intracellular component critical for the effects of rotenone and oligomycin may be dialysed from the cell interior. Thus in order to investigate this likelihood the effects of these mitochondrial inhibitors were also examined in the cell-attached recording mode. Application of rotenone (10 μ M; Figure 6a) or oligomycin (2 μ M; Figure 6b) to the bathing medium, in the absence of extracellular glucose, did not induce activation of K_{ATP} channel currents in cell-attached recordings. Thus, the mean channel activity obtained in control conditions were 0.16 ± 0.09 ($n=5$; rotenone) and 0.03 ± 0.01 ($n=8$; oligomycin) which were not significantly altered following at least 10–15 min exposure to rotenone (0.13 ± 0.05 ; $n=5$; Figure 6c) or oligomycin (0.03 ± 0.01 ; $n=8$; $P>0.05$; Figure 6c), respectively. Furthermore, addition of either rotenone (10 μ M; Figure 7) or oligomycin (2 μ M; data not illustrated) to the intracellular surface of excised inside-out membrane patches bathed in 0.1 mM MgATP failed to activate K_{ATP} channel currents. Thus

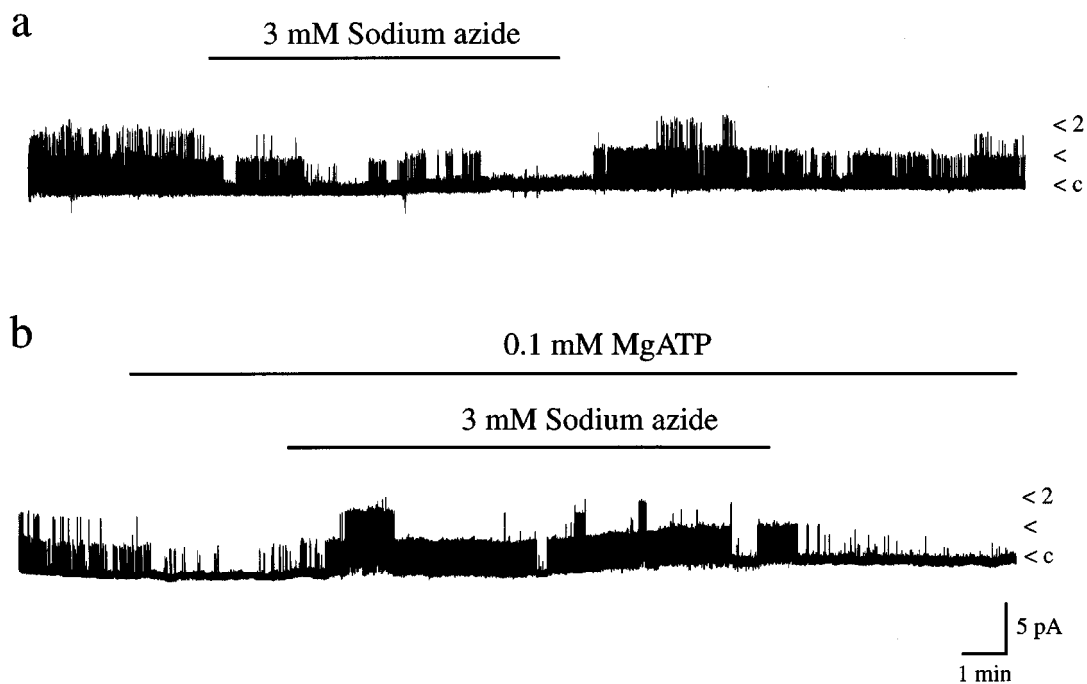


Figure 4 Effects of sodium azide on single channel currents. Continuous single channel currents recorded from inside-out membrane patches exposed to symmetrical 140 mM KCl at a membrane potential of +40 mV. Single channel openings are denoted by upward deflections. (a) Sodium azide (3 mM) inhibited K_{ATP} channels in the absence of Mg^{2+} in the bathing medium. This action of sodium azide was partially reversed on washout. The Nf.Po values obtained in control conditions, in the presence of sodium azide and following washout of azide were 1.06, 0.13 and 0.62, respectively. (b) In contrast, sodium azide (3 mM) activated K_{ATP} channels when 0.1 mM MgATP bathed the intracellular surface of inside out patches. Washout of azide partially reversed this action. The Nf.Po values were as follows: control 0.214; 0.1 mM MgATP 0.036; sodium azide and MgATP 0.600; wash 0.023.

the mean channel activity obtained in control patches (exposed to 0.1 mM MgATP) were 0.08 ± 0.04 (rotenone) and 0.09 ± 0.03 (oligomycin), whereas following application of these inhibitors the mean currents were 0.07 ± 0.04 ($n=4$; rotenone; $P>0.05$) and 0.04 ± 0.01 ($n=5$; oligomycin; $P>0.05$). These data clearly

demonstrate that, in cells exposed to zero glucose, rotenone or oligomycin, in contrast to sodium azide, have no effect on K_{ATP} channel activity, indicating that there is no correlation between their capacity to inhibit mitochondrial function and K_{ATP} channel activation.

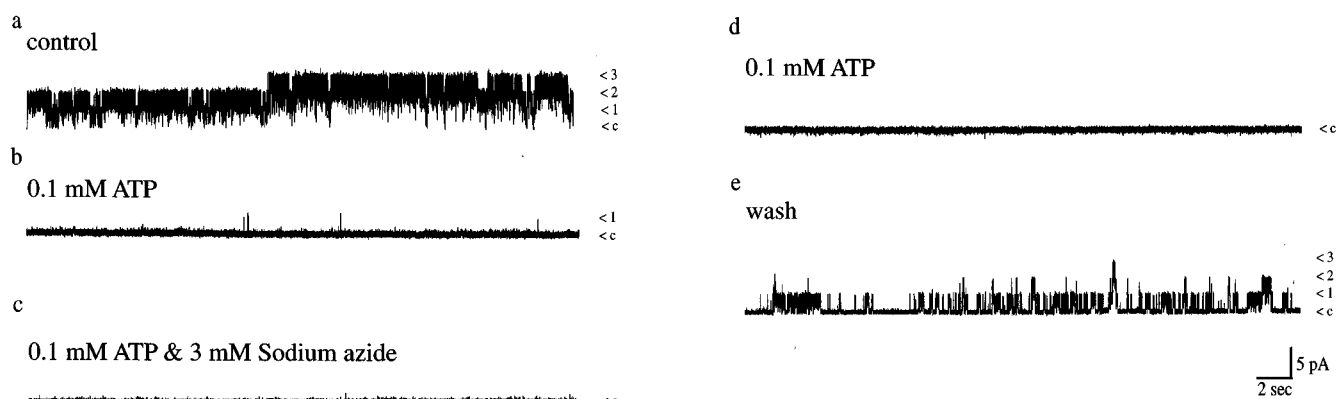


Figure 5 Sodium azide does not activate K_{ATP} channels in the absence of Mg^{2+} ions. Single channel currents recorded from an inside-out membrane patch exposed to symmetrical 140 KCl at a membrane potential of +40 mV. Single channel openings are denoted by upward deflections. Sodium azide (3 mM) inhibited, rather than activated K_{ATP} channels in patches bathed in 0.1 mM ATP and this was partially reversed on washout of azide. The Nf.Po values obtained in control conditions (a), in the presence of 0.1 mM ATP (b), in the combined presence of 0.1 mM ATP and sodium azide (3 mM; c), washout of azide (d) and washout of 0.1 mM ATP (e) were 2.25, 0.025, 0.024, 0.032 and 0.27, respectively.

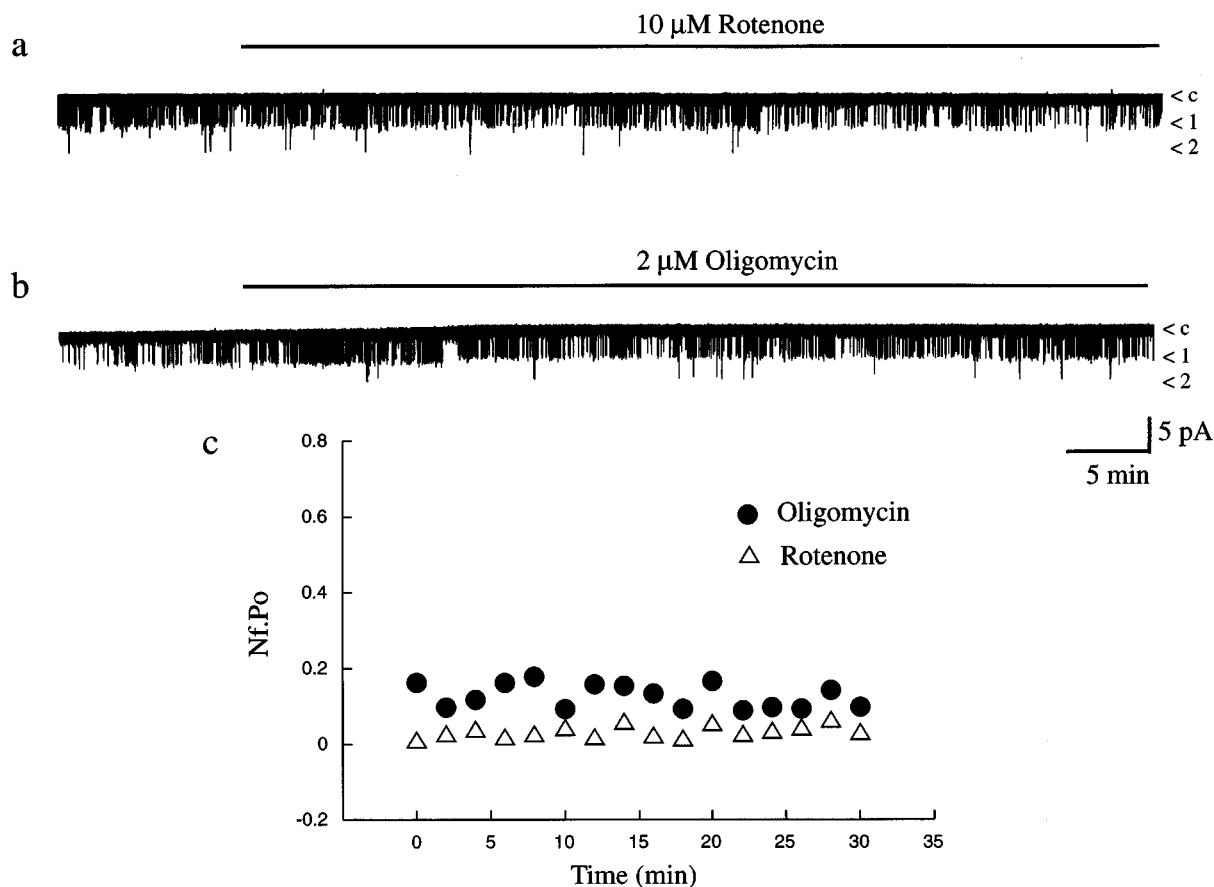


Figure 6 Rotenone and oligomycin do not activate K_{ATP} channels. Cell-attached recording from a single CRI-G1 cell at 10 mV applied potential. Application of rotenone (10 μ M; a) or oligomycin (2 μ M; b) for the time indicated failed to have any effect on channel activity over the time period of recording. (c) Graph of channel activity (Nf.Po) expressed as a function of time obtained from the cell attached patches in a (Δ) and b ($\bullet$).

AMP-PNP prevents sodium azide activation of K_{ATP} channels

The sodium azide-induced activation of K_{ATP} channels appears to be dependent upon the presence of MgATP, suggesting that

nucleotide hydrolysis or a phosphorylation dependent process is required for K_{ATP} channel activation. Therefore, the effects of the non-hydrolysable ATP analogue, Mg-AMP-PNP were investigated on azide-induced activation of K_{ATP} channels. In whole cell recordings, 5 mM Mg-ATP was substituted with

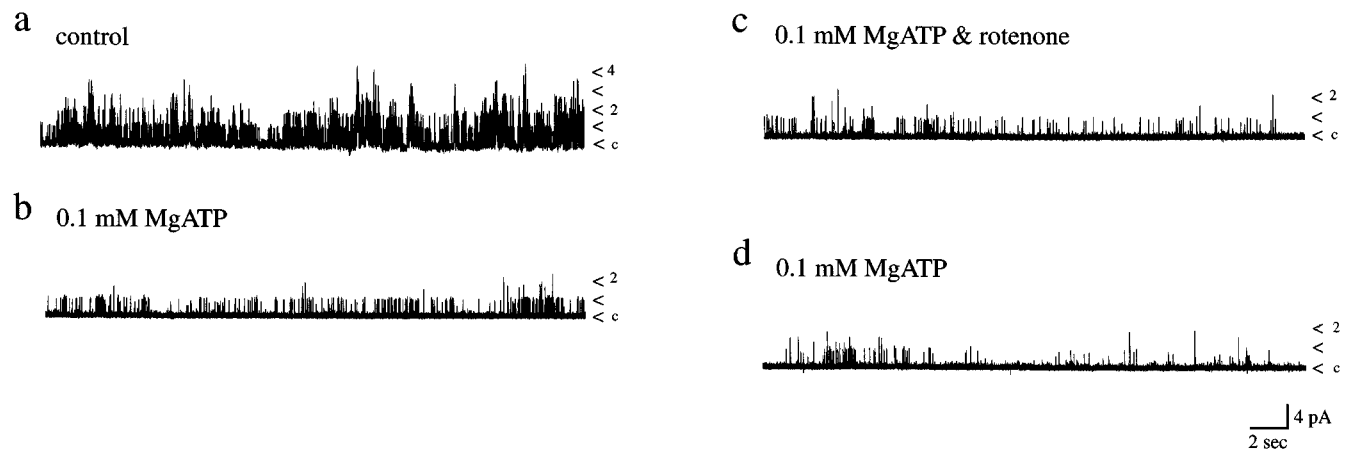


Figure 7 Effects of rotenone on single channel currents. Single channel currents recorded from inside-out membrane patches exposed to symmetrical 140 mM KCl at a membrane potential of +40 mV. Rotenone (10 μ M) failed to activate K_{ATP} channels in the presence of 0.1 mM MgATP. The $Nf.Po$ values obtained in control conditions, following application of 0.1 mM MgATP, in the combined presence of 0.1 mM MgATP and rotenone and following washout of rotenone were 0.265, 0.096, 0.072 and 0.045, respectively.

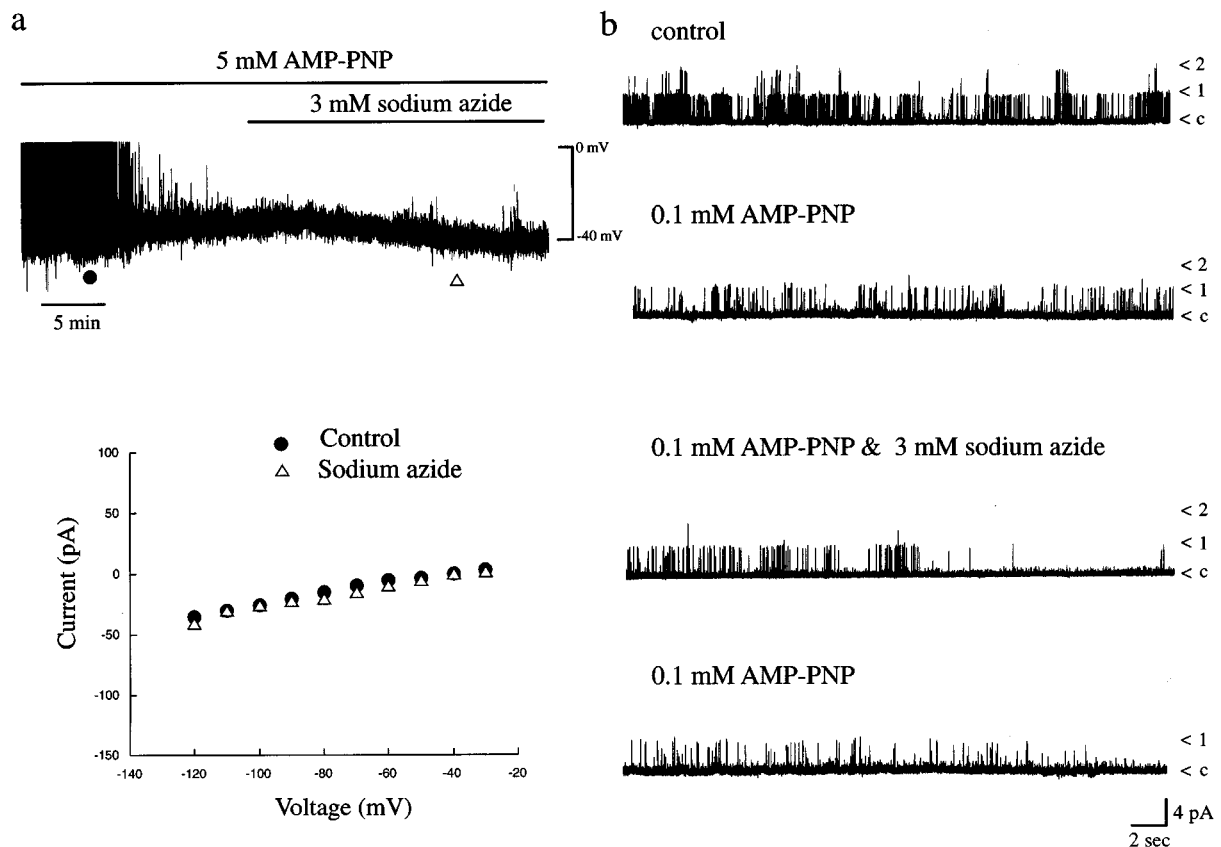


Figure 8 (a) Sodium azide activation of K_{ATP} channels is blocked by AMP-PNP. (a) The upper panel is a whole cell current clamp trace of cell dialysed with 5 mM AMP-PNP. Application of sodium azide (3 mM) for the time indicated failed to hyperpolarize CRI-G1 cells. The lower panel is a plot of the current-voltage relations for the voltage clamped currents obtained at the times specified in a: control (●) and sodium azide (△). Following dialysis with AMP-PNP, sodium azide failed to effect the membrane conductance of CRI-G1 cells. (b) Single channel currents recorded from an inside-out membrane patch exposed to symmetrical 140 mM KCl at a membrane potential of +40 mV. Following application of 0.1 mM AMP-PNP which itself inhibited K_{ATP} channels, subsequent addition of sodium azide (3 mM) failed to increase K_{ATP} channel activity. The $Nf.Po$ values were as follows: control 0.207; 0.1 mM AMP-PNP 0.087; 0.1 mM AMP-PNP and sodium azide 0.065; washout of azide 0.044.

5 mM Mg-AMP-PNP in the pipette solution and under current clamp recordings conditions, the mean resting membrane potential and slope conductance values were -32.4 ± 1.25 mV and 0.49 ± 0.04 nS, respectively ($n=7$; Figure 8a). After at least 10 min following formation of the whole cell configuration (to allow complete dialysis of Mg-AMP-PNP into the cell interior), sodium azide (3 mM) was applied to the bath. In contrast to its actions in control conditions (following dialysis with an electrode solution containing 5 mM Mg-ATP), azide failed to result in hyperpolarization of the membrane of CRI-G1 cells ($n=5$; Figure 8a). Thus the membrane potential and slope conductance values obtained after 5 min exposure to azide were -32.2 ± 2.3 mV and 0.44 ± 0.06 nS, respectively ($n=5$; $P>0.05$). However, on two separate occasions, Mg-AMP-PNP failed to prevent azide-induced hyperpolarization and increased K^+ conductance which may be due to insufficient dialysis of Mg-AMP-PNP.

In excised inside-out membrane patches, Mg-AMP-PNP was substituted for ATP in the bath solution and the effects of sodium azide examined (Figure 8b). In a manner similar to Mg-ATP, addition of Mg-AMP-PNP (0.1 mM) to the intracellular surface of the patch resulted in $71.5 \pm 1.3\%$ inhibition of K_{ATP} channel activity ($n=4$). Subsequent addition of sodium azide (3 mM) in the continued presence of Mg-AMP-PNP (0.1 mM) failed to increase the activity of K_{ATP} channel currents in four out of four patches (Figure 8b). The mean channel activity (Nf.Po) obtained following addition of 0.1 mM Mg-AMP-PNP, in the combined presence of Mg-AMP-PNP and sodium azide, and following washout of sodium azide were 0.048 ± 0.005 , 0.025 ± 0.007 and 0.008 ± 0.003 , respectively ($n=4$).

Discussion

The present study shows, using the whole cell recording configuration with 5 mM Mg-ATP in the electrode solution to maintain K_{ATP} channels closed, that the mitochondrial inhibitor sodium azide results in rapid hyperpolarization of CRI-G1 insulin-secreting cells, with an associated increase in K^+ conductance. This was due to activation of K_{ATP} channels as the sulphonylurea tolbutamide, at concentrations reported to inhibit maximally K_{ATP} channels in this cell line (Sturgess *et al.*, 1988; Lee *et al.*, 1994), completely reversed the effects of sodium azide. In contrast, rotenone and oligomycin, inhibitors of NADH dehydrogenase and F_1 -ATPase, respectively, failed to affect either the membrane potential or slope conductance of these cells, suggesting that acute inhibition of mitochondrial energy metabolism is unlikely to explain the azide-induced activation of K_{ATP} channel currents in CRI-G1 insulin-secreting cells.

Definitive identification of K_{ATP} channels as the molecular target for sodium azide-induced hyperpolarization was obtained from single channel studies. In cell-attached recordings K_{ATP} channels were opened by sodium azide when applied to the bath solution. Furthermore, in excised inside-out membrane patches bathed in a MgATP-containing solution, sodium azide induced a rapid increase in K_{ATP} channel activity that was readily reversed by the sulphonylurea tolbutamide. In contrast, rotenone and oligomycin both failed to increase the activity of K_{ATP} channels in cell-attached recordings and in excised inside-out patches in the presence of MgATP. The lack of effect of rotenone and oligomycin in excised patches in comparison to sodium azide under all recording conditions indicate that acute inhibition of mitochondrial function is unlikely to induce soluble mediators

(other than decreased ATP) capable of altering K_{ATP} channel function.

The failure of rotenone and oligomycin to activate K_{ATP} channels in cell-attached recordings in this study contrasts with previous reports showing that these agents activate K_{ATP} channels in insulin secreting cells (Niki *et al.*, 1989) and dopaminergic substantia nigra neurones (Roper & Ashcroft, 1995) within 5–7 min. This is perhaps surprising since rotenone and oligomycin are expected ultimately to cause depletion of intracellular ATP levels even though they inhibit mitochondrial function by distinct and different mechanisms. Thus inhibition of electron flow *via* the electron transport chain (by either sodium azide or rotenone) or inhibition of the ATP synthase (by oligomycin), ultimately results in depletion of intracellular ATP levels as the processes of electron transfer and ATP synthesis are obligatorily coupled in mitochondria (Senior, 1988).

Another possible explanation for the lack of effect of rotenone and oligomycin on cell-attached K_{ATP} channel activity is that these compounds are less effective at depleting intracellular levels of ATP than sodium azide. However, this seems unlikely as rotenone, oligomycin and sodium azide all produce comparable reductions in intracellular ATP levels (Niki *et al.*, 1989; Rustenbeck *et al.*, 1997; Gribble *et al.*, 1997a). It should be noted that recent evidence indicates that oligomycin fails to activate K_{ATP} channels in pancreatic beta cells even though it significantly reduces ATP production (Rustenbeck *et al.*, 1997). Alternatively the metabolic source and/or site of ATP production may be important in CRI-G1 insulinoma cells. Indeed, in guinea-pig cardiac myocytes (Weiss & Lamp, 1987) ATP produced by glycolysis is more effective than mitochondrial oxidative phosphorylation at inhibiting K_{ATP} channels, and in the present experiments there was a complete absence of glucose during cell-attached recordings. Regardless of these considerations it is clear that the effects of sodium azide observed in the present study are unrelated to inhibition of mitochondrial function and subsequent ATP depletion. Indeed the rapid reversibility of sodium azide-induced K_{ATP} channel activation in CRI-G1 cells is not consistent with the involvement of metabolic poisoning in this process. The ability of sodium azide to either inhibit or activate K_{ATP} channels, depending on the presence or absence of MgATP respectively adds further support to the notion that these actions of sodium azide are unrelated to mitochondrial function.

Previous studies have shown that the ability of diazoxide to activate K_{ATP} channels depends upon the presence and concentration of Mg-ATP in the intracellular environment (Trube *et al.*, 1986; Dunne *et al.*, 1987; Sturgess *et al.*, 1988). Thus K_{ATP} channels are not activated by diazoxide either in the absence of Mg-ATP or if high concentrations of Mg-ATP are present (Dunne *et al.*, 1987). Furthermore, a phosphorylation process is thought to underlie diazoxide-induced K_{ATP} channel activation as the presence of Mg^{2+} ions is required for activation (Sturgess *et al.*, 1988) and diazoxide is ineffective when Mg-ATP is replaced by the non hydrolysable ATP analogue, Mg-AMP-PNP (Kozlowski *et al.*, 1989; Dunne, 1989). Similarly, in this study sodium azide-induced K_{ATP} channel activation required the presence of intracellular Mg-ATP, such that sodium azide failed to activate K_{ATP} channels in inside-out patches in the absence of Mg-ATP. Mg^{2+} ions were also required for the actions of sodium azide, as activation of the channels was only observed when Mg-ATP-containing solution bathed the intracellular surface of inside-out patches. In addition, there was no activation of K_{ATP} channels by sodium azide when ATP (in the absence of Mg^{2+}

ions) or Mg-AMP-PNP was present to inhibit the K_{ATP} channels. Together these data indicate that, like diazoxide, ATP hydrolysis or a phosphorylation process is most likely required for K_{ATP} channel activation by sodium azide in CRI-G1 insulinoma cells, although we cannot rule out the possibility of a more direct interaction with a nucleotide binding site associated with the K_{ATP} channel complex (Babenko *et al.*, 1998).

In addition to K_{ATP} channel activation, the present data indicate that in the absence of Mg-ATP, sodium azide inhibits K_{ATP} channel activity in excised patches. Diazoxide is also reported to inhibit K_{ATP} channels under similar conditions in this cell line (Kozlowski *et al.*, 1989) and this has been attributed to diazoxide-induced enhancement of the rate of channel run down. However, further experiments are required to establish whether a change in the kinetics of K_{ATP} channel run down underlies this inhibitory action of sodium azide. Interestingly, sodium azide is also reported to inhibit cloned K_{ATP} channels in oocyte expression studies (Gribble *et al.*, 1997a), an action that has been attributed to a direct action of azide rather than some product of metabolic inhibition.

The characteristics of sodium azide-induced activation of K_{ATP} channels in CRI-G1 cells are identical, in most respects to those of diazoxide, suggesting that a similar mechanism or site of action may underlie the actions of these agents. For instance, the membrane hyperpolarization induced by sodium azide during whole cell recordings occurred over the same rapid time course observed with diazoxide (Trube *et al.*, 1986; Sturgess *et al.*, 1988). The time course of sodium azide-induced K_{ATP} channel activation in excised patches also parallels that of diazoxide. The inhibitory effects of sodium azide observed in this study and the requirement for MgATP for K_{ATP} channel activation are also characteristic effects of diazoxide in this cell line (Kozlowski *et al.*, 1989). Diazoxide is reported to evoke a small but significant depletion in ATP levels that has been attributed to inhibition of mitochondrial function (Grimms-

mann & Rustenbeck, 1998). However, in view of the lack of effect of rotenone and oligomycin in this study, diazoxide-induced activation of K_{ATP} channels in CRI-G1 cells is unlikely to be due to inhibition of mitochondrial energy metabolism.

Recent studies suggest that diazoxide may act directly on the sulphonylurea receptor, SUR1 (Inagaki *et al.*, 1996; Tucker *et al.*, 1997). In particular the nucleotide binding folds (NBF1 and NBF2) appear to be important for the stimulatory actions of diazoxide as mutations in these regions abolish diazoxide-induced activation of the cloned K_{ATP} channel (Nichols, 1996; Shyng *et al.*, 1997; Gribble *et al.*, 1997b). Thus even though sodium azide and diazoxide exhibit no similarities in structure, it is possible that sodium azide, like diazoxide acts directly on SUR1. However, in the absence of comparing the actions of sodium azide on Kir 6.2/SUR1 and the truncated form of Kir 6.2 (Tucker *et al.*, 1997) or on SUR1 with mutations at NBF2 and/or NBF1 heterologously expressed in oocytes, the precise site of sodium azide action is unclear. Inhibition of K_{ATP} channels by ATP is thought to occur at a site distinct from the stimulatory MgADP and diazoxide sites on SUR1 as mutations that block activation of K_{ATP} channels by these agents do not prevent the inhibitory actions of ATP (Gribble *et al.*, 1997b; Shyng *et al.*, 1997). The ability of AMP-PNP and ATP to inhibit K_{ATP} channels activated by sodium azide in the present study also supports the notion that the inhibitory and stimulatory nucleotide sites are distinct.

In conclusion, these data indicate that in CRI-G1 insulin secreting cells, sodium azide either activates or inhibits K_{ATP} channels depending on whether Mg-ATP is present. These actions of sodium azide are unlikely to be due to a fall in intracellular ATP levels as the Mg-ATP concentration was clamped during whole cell recording and as K_{ATP} activation was not evident with oligomycin or rotenone during cell-attached recordings. Consequently, this complication should be considered when using this agent for metabolic inhibition and subsequent activation of K_{ATP} channels.

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Enantioselective inhibition of the biotransformation and pharmacological actions of isoidide dinitrate by diphenyleneiodonium sulphate

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1 We have shown previously that the D- and L- enantiomers of isoidide dinitrate (D-IIDN and L-IIDN) exhibit a potency difference for relaxation and cyclic GMP accumulation in isolated rat aorta and that this is related to preferential biotransformation of the more potent enantiomer (D-IIDN). The objective of the current study was to examine the effect of the flavoprotein inhibitor, diphenyleneiodonium sulphate (DPI), on the enantioselectivity of IIDN action.

2 In isolated rat aortic strip preparations, exposure to 0.3 μM DPI resulted in a 3.6 fold increase in the EC_{50} value for D-IIDN-induced relaxation, but had no effect on L-IIDN-induced relaxation.

3 Incubation of aortic strips with 2 μM D- or L-IIDN for 5 min resulted in significantly more D-isoidide mononitrate formed (5.0 ± 1.5 pmol mg protein⁻¹) than L-isoidide mononitrate (2.1 ± 0.7 pmol mg protein⁻¹) and this difference was abolished by pretreatment of tissues with 0.3 μM DPI. DPI had no effect on glutathione *S*-transferase (GST) activity or GSH-dependent biotransformation of D- or L-IIDN in the 105,000 $\times g$ supernatant fraction of rat aorta.

4 Consistent with both the relaxation and biotransformation data, treatment of tissues with 0.3 μM DPI significantly inhibited D-IIDN-induced cyclic GMP accumulation, but had no effect on L-IIDN-induced cyclic GMP accumulation.

5 In the intact animal, 2 mg kg⁻¹ DPI significantly inhibited the pharmacokinetic and haemodynamic properties of D-IIDN, but had no effect L-IIDN.

6 These data suggest that the basis for the potency difference for relaxation by the two enantiomers is preferential biotransformation of D-IIDN to NO, by an enzyme that is inhibited by DPI. Given that DPI binds to and inhibits NADPH-cytochrome P450 reductase, the data are consistent with a role for the cytochromes P450-NADPH-cytochrome P450 reductase system in this enantioselective biotransformation process.

Keywords: Biotransformation; diphenyleneiodonium sulphate; glutathione *S*-transferase; glyceryl trinitrate; haemodynamics; isoidide dinitrate; NADPH-cytochrome P450 reductase; pharmacokinetics

Abbreviations: 1,2-GDN, glyceryl-1,2-dinitrate; 1,3-GDN, glyceryl-1,3-dinitrate; AUC, area under the curve; CDNB, 1-chloro-2,4-dinitrobenzene; DPI, diphenyleneiodonium sulphate; GSH, reduced glutathione; GST, glutathione *S*-transferase; GTN, glyceryl trinitrate; IDP, iodonium diphenyl; IIDN, isoidide dinitrate; IIMN, isoidide mononitrate; ISDN, isosorbide dinitrate; ISMN, isosorbide-2-mononitrate; MAP, mean arterial pressure; NO, nitric oxide.

Introduction

Since the introduction of glyceryl trinitrate (GTN) into clinical practice over a century ago, organic nitrates have become important clinical agents for the management of angina pectoris and congestive heart failure. Current hypotheses contend that organic nitrates are prodrugs, and that they require biotransformation to an active form (presumably nitric oxide (NO) or some derivative thereof) prior to initiating their pharmacological effects of increased cyclic GMP accumulation and vascular smooth muscle relaxation. Nitric oxide formation from GTN has been demonstrated in several vascular smooth muscle preparations (Chung & Fung, 1990; Feelisch & Kelm, 1991; Marks *et al.*, 1992; Kurz *et al.*, 1993). Vascular GTN biotransformation has been shown to be mediated by glutathione *S*-transferases (GSTs) (Tsuchida *et al.*, 1990; Nigam *et al.*, 1996) and cytochromes P450 (McDonald & Bennett, 1993). In addition, an unidentified microsomal

protein mediates NO formation from GTN (Chung *et al.*, 1992). However, the relative importance of these biotransformation systems in the formation of NO at pharmacologically relevant concentrations of organic nitrates is unclear.

It has been demonstrated that there is an enantioselective aspect to the organic nitrate response in isolated rat aorta, with the D-enantiomer of isoidide dinitrate (IIDN) being 10 fold more potent than L-IIDN for vascular relaxation and IIDN-induced cyclic GMP accumulation (Bennett *et al.*, 1986). This was associated with a greater rate of vascular biotransformation of the more potent enantiomer (Stewart *et al.*, 1989). When tissues were made tolerant to organic nitrates *in vitro* using high concentrations of GTN, enantioselectivity for relaxation by IIDN was lost (Bennett *et al.*, 1988). In a subsequent study, NADPH-dependent hepatic microsome mediated biotransformation of IIDN was shown to be selective for the D-enantiomer, thereby raising the possibility that the cytochromes P450 system was involved in the enantioselective biotransformation of IIDN (McDonald & Bennett, 1990). Finally, in a study of the haemodynamic effects D- and L-

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IIDN in conscious rats, the magnitude of both the initial and sustained decrease in mean arterial pressure (MAP) was greater for D-IIDN (Bennett *et al.*, 1991).

Recent reports from this laboratory have described inhibition of the biotransformation and pharmacological actions of GTN by the flavoprotein inhibitor diphenyleneiodonium sulphate (DPI) (McGuire *et al.*, 1994), and the *in vitro* inhibition of purified DNA expressed NADPH-cytochrome P450 reductase by DPI (McGuire *et al.*, 1998). This suggests that the basis for the inhibitory effect of DPI on GTN action may be inhibition of the metabolic activation of GTN mediated by the cytochromes P450-NADPH-cytochrome P450 reductase system. DPI has also been shown to inhibit a number of other flavin-containing enzymes such as the NADPH-oxidase system responsible for superoxide production in macrophages and neutrophils (Cross & Jones, 1986; Doussière & Vignais, 1992), xanthine oxidase (O'Donnell *et al.*, 1993) and macrophage NO synthase (Stuehr *et al.*, 1991).

Since the enantioselectivity of IIDN action appears to be related to enantioselective biotransformation, and since the inhibitory action of DPI may be due to inhibition of the cytochromes P450-NADPH-cytochrome P450 reductase system, it was of interest to determine whether DPI affected the enantioselective pharmacological properties of IIDN.

Methods

Drugs and solutions

Krebs' solution was composed of the following (mM): NaCl, 118; KCl, 4.74; MgSO₄, 1.18; KH₂PO₄, 1.18; CaCl₂ 2.5; NaHCO₃, 24.9; and glucose, 10. The solution was aerated with 95% O₂-5% CO₂ and maintained at 37°C. Diphenyleneiodonium sulphate (DPI) was purchased from Colour Your Enzyme (Kingston, Ontario, Canada). Stock solutions of DPI were prepared in distilled water. D-isoidide dinitrate (1,4:3,6-dianhydro-D-iditol 2,5-dinitrate, D-IIDN), L-isoidide dinitrate (1,4:3,6-dianhydro-L-iditol 2,5-dinitrate, L-IIDN), D-IIMN and L-IIMN were obtained from D.H. Stereochemical Consulting Ltd. (Vancouver, British Columbia, Canada). Stock solutions of IIDN and IIMN were prepared by extraction of organic nitrate-lactose powder (50% w/w) with ethanol. Further dilutions were made with the appropriate buffer solution. The concentrations of IIDN and IIMN in stock solutions were determined by a modification of the spectrophotometric method of Dean & Baun (1975) as described previously (Bennett *et al.*, 1988). Isosorbide dinitrate (ISDN) and isosorbide-2-mononitrate (ISMN) were gifts from Wyeth Ltd. (Toronto, Ontario, Canada). L-Phenylephrine hydrochloride, 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), β -NADPH, xanthine and xanthine oxidase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of at least reagent grade and were obtained from a variety of sources.

Relaxation studies

Thoracic aortic strips were prepared from 250–300 g male Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada) as described (Stewart *et al.*, 1989). Three aortic strip preparations were obtained from each animal. Tissues were contracted maximally with 10 μ M phenylephrine to ensure the viability of the preparation. After a 30 min washout period, the tissues were contracted submaximally with 0.1 μ M phenylephrine. Once the induced tone had stabilized, tissues

were exposed to diluent (control) or 0.3 μ M DPI (treated) and 10 min later cumulative concentration-response curves for D-IIDN (10 nM–0.1 mM) or L-IIDN (0.1 μ M–0.1 mM) were obtained.

Tissue biotransformation studies

Aortae were divided in half and placed into individual tubes containing 1 ml of Krebs' solution at 37°C aerated with 95% O₂-5% CO₂. Tissues were exposed to 0.1 μ M phenylephrine for 5 min and then to either diluent (control) or 0.3 μ M DPI for an additional 10 min. Both tissue segments were incubated with either 2 μ M D-IIDN or 2 μ M L-IIDN for 5 min and then frozen between liquid nitrogen precooled clamps. The frozen tissues were placed in extraction tubes and 50 pmol of ISDN was added as the internal standard. Each tissue was extracted four times with 1 ml isomeric hexanes to remove IIDN and ISDN and the extracts combined. Each tissue was then spiked with 10 pmol of ISMN as the internal standard, extracted three times with 1 ml of anhydrous diethyl ether to remove the IIMN and ISMN, and the combined diethyl ether extracts dried with anhydrous magnesium sulphate. The tissues were digested with 2N NaOH (1 ml) for 48 h and aortic protein levels determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

To determine the IIMN content of the Krebs' incubation medium, samples were first extracted five times with 2 ml isomeric hexanes to remove IIDN. Samples were spiked with 10 pmol ISMN as the internal standard, extracted three times with 2 ml anhydrous diethyl ether, and the combined diethyl ether extracts dried with anhydrous magnesium sulphate. Hexane or diethyl ether extracts were evaporated to dryness under a stream of nitrogen gas, the residue resuspended in 25 μ l benzene and frozen at –20°C until analysis the following day. To control for any IIMN formed artifactually during the incubation or present in the stock IIDN solution, 2 μ M D- or L-IIDN was incubated in Krebs' solution under the identical experimental conditions. The concentration of IIMN found in these samples was subtracted from the values obtained for the tissue samples, and ranged between 0.14 and 0.50% of the IIDN concentration. Organic nitrates were quantitated by megabore capillary column gas-liquid chromatography as described (McDonald & Bennett, 1990).

Cyclic GMP measurements

Each rat aorta was divided into four segments that were incubated individually in 5 ml of Krebs' solution at 37°C aerated with 95% O₂-5% CO₂. In some experiments, the endothelium was removed by gentle scraping with a scalpel blade. All tissues segments were exposed to 0.1 μ M phenylephrine for 5 min followed by either diluent (control) or 0.3 μ M DPI for an additional 10 min. Two tissue segments (one control and one DPI-treated) were incubated with 2 μ M D-IIDN or 10 μ M L-IIDN for 1 min while the other two tissues served as controls (basal and basal plus DPI). At the end of the incubation period, all tissue segments were frozen between liquid nitrogen precooled clamps. Tissues were homogenized in 1 ml of 6% trichloroacetic acid and centrifuged at low speed for 20 min. The supernatant fractions were extracted six times with 2 ml of water-saturated diethyl ether, acetylated (Harper & Brooker, 1975) and cyclic GMP was quantitated by radioimmunoassay (Steiner *et al.*, 1972). The pellet was digested in 2N NaOH for 48 h and aortic protein was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

Glutathione *S*-transferase activity/biotransformation

The 105,000 $\times$ *g* supernatant fraction of rat aorta was prepared as described (Nigam *et al.*, 1996) and GST activity determined by the spectrophotometric method of Habig *et al.* (1974) using 1 mM CDNB and 1 mM GSH as substrates. To assess the inhibition of GST activity by DPI, 20 μ g ml⁻¹ protein of rat aortic supernatant was incubated with 10 μ M DPI or diluent (control) and 1 mM NADPH for 10 min before measuring GST activity. One unit of GST activity is defined as the amount of enzyme catalyzing the conjugation of 1 μ mol CDNB per min at 25°C. To examine the inhibition of GST-dependent biotransformation of IIDN by DPI, samples of the 105,000 $\times$ *g* supernatant fraction of rat aorta (200 μ g ml⁻¹ protein) were pretreated for 15 min with 1 mM NADPH and either 10 μ M DPI or diluent (control). Samples were then incubated with 1 mM GSH and 2.0 μ M D- or L-IIDN for 60 min. Values for non-enzymatic denitration were determined by incubating D- or L-IIDN in the absence of protein and were subtracted from those obtained in the presence of aortic supernatant. The rates of non-enzymatic denitration of D- and L-IIDN were 0.15 ± 0.05 pmol min⁻¹ and 0.18 ± 0.07 pmol min⁻¹, respectively. IIMN was quantitated by megabore capillary column gas-liquid chromatography as described above. Protein content of aortic supernatant was determined by method of Bradford (1976) using bovine serum albumin as the standard.

Haemodynamic-pharmacokinetic studies

Two groups of male Sprague-Dawley rats (250–275 g) had catheters (PE-50 and 30-gauge Teflon fused to 0.02-inch inside diameter Tygon®) inserted into the abdominal vena cava and abdominal aorta, respectively. The animals were anaesthetized for surgery with a combination of ketamine (Rogarsetic® – 70 mg kg⁻¹) and xylazine (Rompun® – 5 mg kg⁻¹). The catheters were externalized between the scapulae, sutured into position, and filled with 50 IU ml⁻¹ heparin saline in order to help maintain patency during the ensuing 48 h post-surgery recovery period.

In one group of rats, continuous blood pressure measurements were made on conscious, unrestrained animals by connecting the aortic catheter to a Statham pressure transducer (P23D6, Grass Instruments, Quincy, MA, U.S.A.) coupled to a MacLab® data acquisition system for the recording of blood pressure and heart rate. An *in vivo* dose-response curve was done in order to compare the effects of a single dose of DPI (2 mg kg⁻¹) on varying doses of D- and L-IIDN. Animals were assigned at random to receive bolus i.v. doses of 0.2, 0.5, 1.0 and 2.0 mg kg⁻¹ D- or L-IIDN in saline through the venous catheter. The next dose of drug was administered when the blood pressure had stabilized at pre-injection control values. The control dose-response curve was followed by a 24 h washout period, after which a single 2 mg kg⁻¹ dose of DPI was administered. Fifteen minutes later, a second set of dose-response curve measurements were made using the protocol described above. Each animal received the same enantiomer of IIDN for both control and DPI-treated dose-response curves. Since DPI is an irreversible inhibitor, a second control dose response curve was performed in some animals following the 24 h washout period to control for any sequence effect of IIDN administration. There was no statistical difference between the control data obtained before or after the 24 h washout period ($P > 0.05$).

In a second group of rats, the pharmacokinetics after a bolus i.v. dose of D- or L-IIDN was compared in the presence

or absence of DPI (2.0 mg kg⁻¹). Animals were assigned at random to receive a bolus i.v. dose of 2.0 mg kg⁻¹ D- or L-IIDN in 0.5 ml saline through the venous catheter, followed by 0.2 ml saline to flush the remaining drug from the catheter. Blood samples (0.25 ml) were obtained at 2, 5, 10, 15, 20, 30, 45, 60 and 90 min. All blood samples obtained were immediately transferred to tubes containing 5 U heparin and centrifuged at 13,000 $\times$ *g* for 30 s. The plasma was separated from the erythrocytes and frozen at –20°C for later analysis. The red blood cells were collected and replaced at the end of the blood sampling protocol. After a 2-day washout period, a single i.v. dose of 2.0 mg kg⁻¹ DPI in 0.2 ml saline was administered. Fifteen minutes later, a bolus i.v. dose of 2.0 mg kg⁻¹ of the same enantiomer in 0.5 ml saline was administered through the venous catheter. The blood sampling protocol was repeated as described above. A second set of blood samples were taken in some animals following the 2-day washout period to control for any sequence effect of IIDN administration. There was no statistical difference between the control data obtained before or after the 2-day washout period ($P > 0.05$).

The frozen plasma samples obtained were analysed for levels of IIDN and IIMN. Upon thawing, 100 μ l aliquots of the plasma were placed in individual extraction tubes containing 5 μ l of 1.0 μ M ISDN as the internal standard. Each sample was extracted three times with 1 ml isomeric hexanes to remove IIDN and ISDN, the extracts combined and then evaporated to dryness under a stream of nitrogen gas. Each sample was then spiked with 5 μ l of 1.0 μ M ISMN as the internal standard. Each sample was extracted three times with 1 ml anhydrous diethyl ether to remove the IIMN and ISMN. The ether extract was dried with anhydrous magnesium sulphate and then evaporated to dryness under a stream of nitrogen gas. The residue was resuspended in 50 μ l of benzene and frozen at –20°C until analysis by megabore capillary column gas-liquid chromatography (Stewart *et al.*, 1989) as described previously. A standard curve was prepared by spiking 100 μ l aliquots of rat plasma with either IIDN or IIMN to produce final concentrations of 0.1–5.0 μ M.

Data analysis

All data are presented as the means $\pm$ standard deviation (s.d.). EC₅₀ values were determined from each concentration-response curve by interpolation. Pharmacokinetic data was analysed using the exponential curve-stripping program, ESTRIP (Brown & Manno, 1978), and pharmacokinetic parameters were obtained using the computer program, PKCALC (Shumaker, 1986). Data from all experiments was analysed by a one-way analysis of variance and Newman-Keuls *post hoc* test for multiple comparisons. The assumption of homogeneity of variance was tested in all cases using Bartlett's test. Due to inhomogeneity of variance, statistical analysis for the relaxation experiments was performed using logarithmically transformed data. A *P* value of 0.05 or less was considered statistically significant.

Results

Relaxation studies

The enantioselective relaxation of isolated rat aorta by D- and L-IIDN was reduced in the presence of 0.3 μ M DPI (Figure 1a and b). The inhibition of D-IIDN-induced relaxation by DPI was characterized by a parallel, rightward shift of the

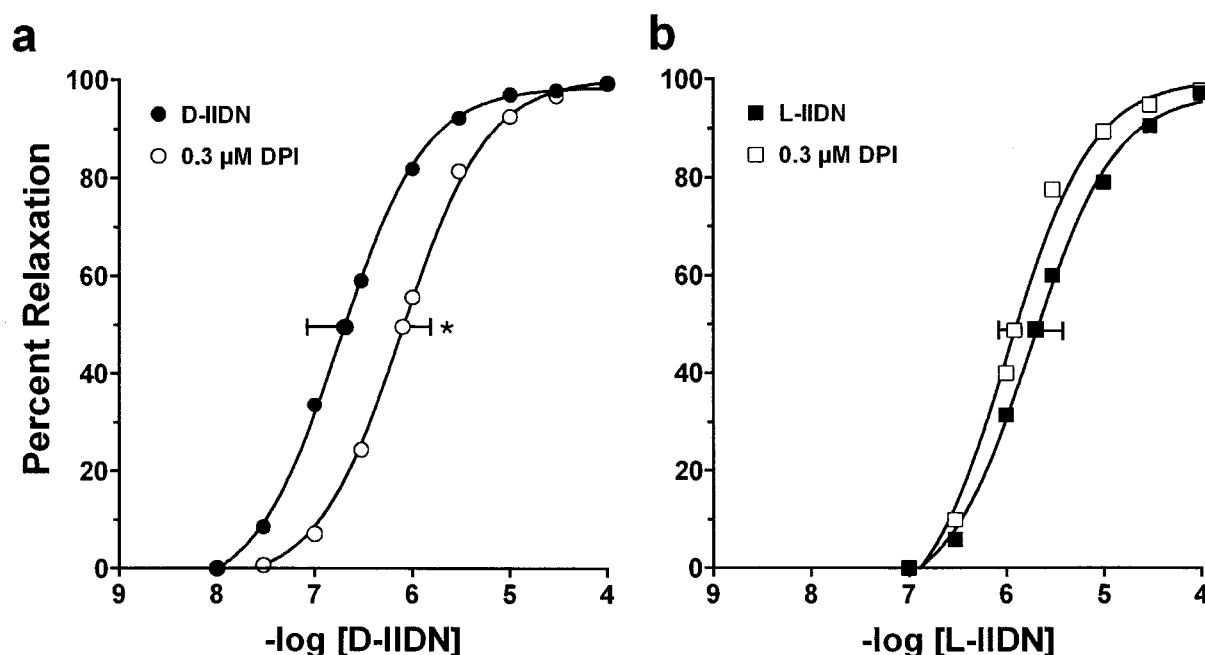


Figure 1 Effect of DPI on D- and L-IIDN-induced relaxation of isolated rat aorta. (a) The EC₅₀ values for D-IIDN-induced relaxation were: Control, $0.23 \pm 0.18 \mu\text{M}$; DPI, $0.83 \pm 0.47 \mu\text{M}$. *Indicates $P < 0.05$ vs control. (b) The EC₅₀ values for L-IIDN-induced relaxation were: Control, $2.39 \pm 1.35 \mu\text{M}$; DPI, $1.40 \pm 0.52 \mu\text{M}$ $P > 0.05$ vs control. Only the s.d. for the EC₅₀ value is shown ($n = 9$).

concentration-response curve. The EC₅₀ value for D-IIDN-induced relaxation was increased 3.6 fold in the presence of 0.3 μM DPI. In contrast, DPI did not inhibit L-IIDN-induced relaxation. In control experiments, the EC₅₀ values for D-IIDN- and L-IIDN-induced relaxation were significantly different, however, the EC₅₀ values for D-IIDN- and L-IIDN-induced relaxation in the presence of DPI were not different.

Tissue biotransformation studies

Aortic biotransformation was assessed at an IIDN concentration (2 μM) that exhibited a differential relaxation response and that was high enough to permit quantitation of parent drug and metabolites. The tissue concentration of the parent drug was not different (32.7 ± 12.14 and 24.1 ± 9.0 pmol mg protein⁻¹ for D- and L-IIDN, respectively. $P > 0.05$). Exposure of tissues to 0.3 μM DPI resulted in a significant decrease in D-IIMN formation, but had no effect on L-IIMN formation (Figure 2). IIMN formation from D- and L-IIDN in DPI-treated tissues was not different.

Cyclic GMP measurements

IIDN-induced cyclic GMP accumulation was assessed at an IIDN concentration that resulted in 60–80% relaxation of isolated rat aorta (2 μM D-IIDN and 10 μM L-IIDN) and cyclic GMP levels were assessed at the time of maximal cyclic GMP accumulation (1 min). In the endothelium-intact aorta, cyclic GMP accumulation was increased 2.0 ± 0.6 fold and 1.8 ± 0.4 fold above basal levels for D- and L-IIDN, respectively. In endothelium-denuded aorta, cyclic GMP accumulation was increased 4.0 ± 2.1 fold and 2.5 ± 1.4 fold above basal levels for D- and L-IIDN, respectively. Treatment of tissues with 0.3 μM DPI significantly inhibited D-IIDN-induced cyclic GMP accumulation in both the endothelium-intact and endothelium-denuded tissues, whereas L-IIDN-

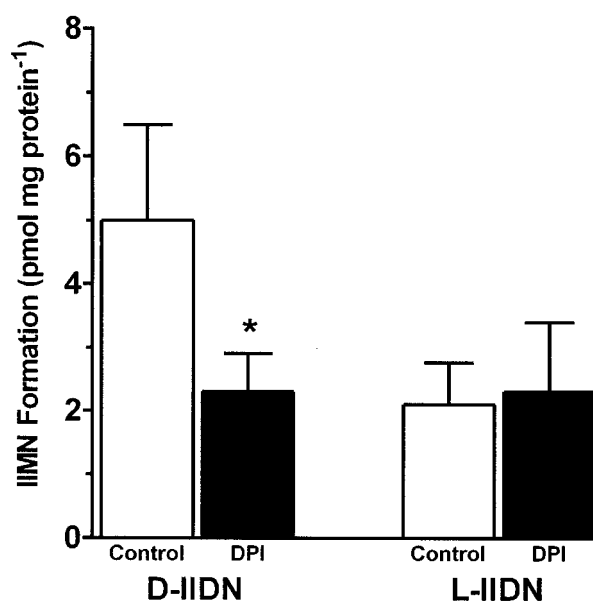


Figure 2 Effect of DPI on rat aortic biotransformation of IIDN. The total IIMN formed in both the aortic tissue and Krebs' medium during a 5 min incubation with 2 μM D- or L-IIDN was: D-IIDN control, 5.0 ± 1.5 pmol mg protein⁻¹; D-IIDN plus 0.3 μM DPI, 2.3 ± 0.6 pmol mg protein⁻¹; L-IIDN control, 2.1 ± 0.7 pmol mg protein⁻¹; L-IIDN plus 0.3 μM DPI, 2.3 ± 1.1 pmol mg protein⁻¹. *Indicates $P < 0.05$ vs control. Each value represents the means ± s.d. ($n = 6$).

induced cyclic GMP accumulation was not inhibited by 0.3 μM DPI (Figure 3). Decreased basal cyclic GMP accumulation in tissues treated with DPI was observed only in endothelium-intact tissues, suggesting an inhibitory effect of DPI on endothelial NO synthase resulting in decreased basal activity of smooth muscle guanylyl cyclase.

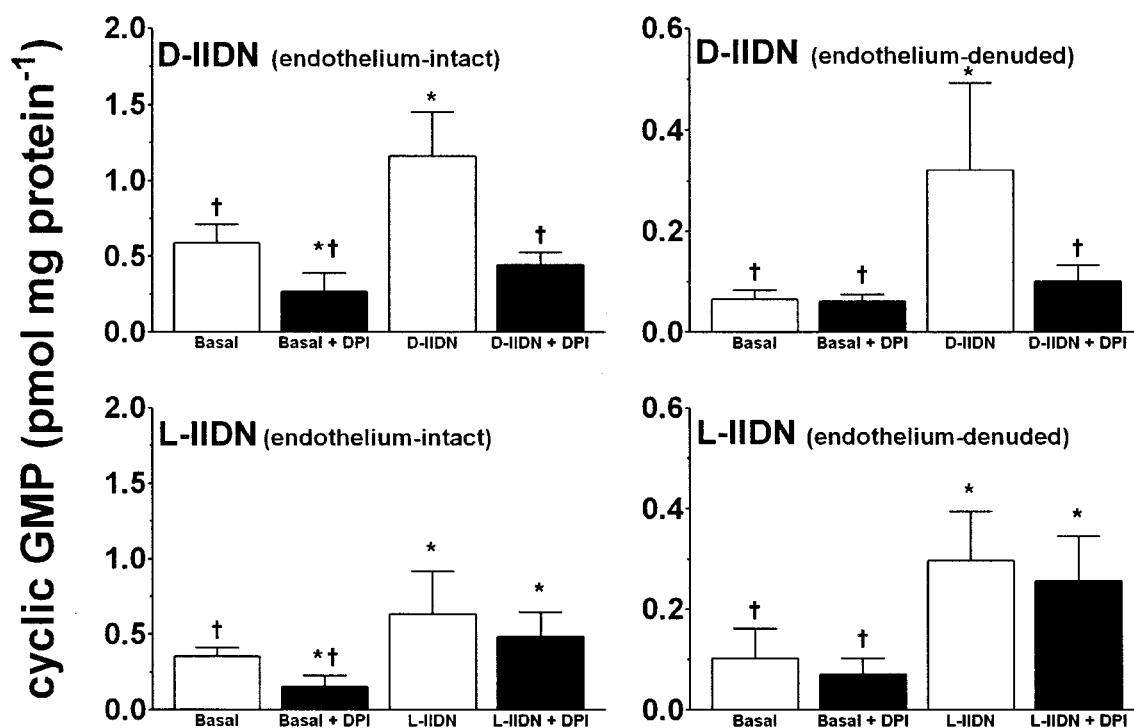


Figure 3 Effect of DPI on IIDN-induced cyclic GMP accumulation in isolated rat aorta. Tissue levels of cyclic GMP were determined after a 1 min incubation with 2 μ M D-IIDN or 10 μ M L-IIDN. *Indicates $P < 0.05$ versus Basal control and †indicates $P < 0.05$ versus IIDN control). Each value represents the means $\pm$ s.d., ($n = 7$, endothelium-intact; $n = 6$, endothelium-denuded).

Glutathione *S*-transferase activity/biotransformation

The GST activity in the 105,000 $\times$ g supernatant fraction of rat aorta was 0.20 ± 0.03 U mg protein⁻¹ and this was not significantly different after treatment of the supernatant fraction with 10 μ M DPI (0.19 ± 0.01 U mg protein⁻¹, $P > 0.05$, Student's *t*-test for unpaired data). Similarly, GSH-dependent biotransformation of D- and L-IIDN occurred in the 105,000 $\times$ g supernatant fraction of rat aorta, and this was not affected by treatment of the samples with 10 μ M DPI. The control levels of GSH-dependent biotransformation of D- and L-IIDN were 1.94 ± 0.23 pmol min⁻¹ mg protein⁻¹ ($n = 7$) and 1.78 ± 0.22 pmol min⁻¹ mg protein⁻¹ ($n = 5$), respectively.

Haemodynamic-pharmacokinetic studies

In the intact rat model, the haemodynamic study investigating the decrease in MAP after bolus i.v. doses of IIDN of 0.2, 0.5, 1.0 and 2.0 mg kg⁻¹ demonstrated that 2 mg kg⁻¹ DPI significantly inhibited the decrease in MAP by D-IIDN at doses of 0.5 mg kg⁻¹ or greater and eliminated the potency advantage of D-IIDN such that in the presence of DPI both D- and L-IIDN were equipotent vasodilators (Figure 4). DPI did not affect the decrease in MAP by any dose of L-IIDN. The absolute pressure values before DPI treatment were: D-IIDN, 89.9 ± 12.2 mmHg and L-IIDN, 90.0 ± 8.7 mmHg. The absolute pressure values after DPI treatment were: D-IIDN, 79.2 ± 6.8 mmHg and L-IIDN, 75.6 ± 8.2 mmHg. These values were not significantly different ($P > 0.05$). Although the data in Figure 4 is expressed as the percentage decrease in MAP, the same results are obtained when absolute decreases in MAP are used (data not shown). The pharmacokinetic results following a bolus i.v. dose of 2 mg kg⁻¹ IIDN indicated that 2 mg kg⁻¹ DPI significantly increased the plasma levels of D-IIDN at 5, 10, 15, 20, 30, 45, 60 and 90 min while having no effect on

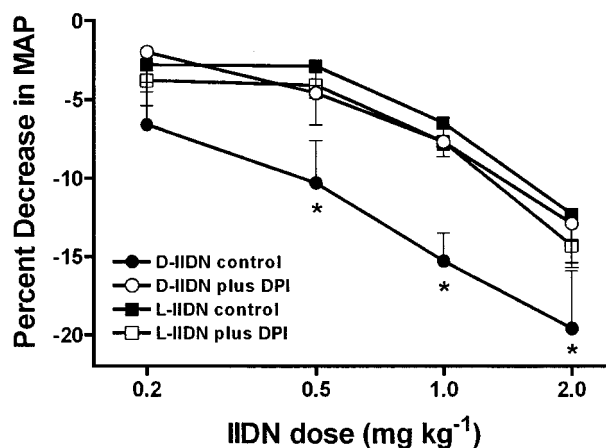


Figure 4 Effect of DPI on the per cent decrease in MAP caused by IIDN. The maximum decrease in MAP was measured in conscious rats following bolus i.v. doses of IIDN. A bolus i.v. dose of 2 mg kg⁻¹ DPI significantly inhibited the decrease in MAP caused by D-IIDN at all doses except 0.2 mg kg⁻¹. *Indicates $P < 0.001$. D-IIDN control vs D-IIDN plus DPI. DPI had no effect on the decrease in MAP caused by L-IIDN ($P > 0.05$, L-IIDN vs L-IIDN plus DPI). (D-IIDN, $n = 4$; L-IIDN, $n = 5$).

plasma levels of L-IIDN (Figure 5). Evaluation of these data also indicated that the area under the curve (AUC) was significantly increased from control for D-IIDN (58.7 ± 12.2 μ M min vs treated, 151.8 ± 16.6 μ M min) while L-IIDN was unaffected (46.2 ± 7.9 μ M min vs treated, 57.5 ± 4.6 μ M min) ($P < 0.001$). DPI also significantly extended the elimination half-life of D-IIDN from a control value of 15.3 ± 2.4 min to 32.0 ± 6.6 min, while the control and treated values for L-IIDN were not different at 17.2 ± 3.3 min and 19.6 ± 1.6 min, respectively ($P < 0.01$).

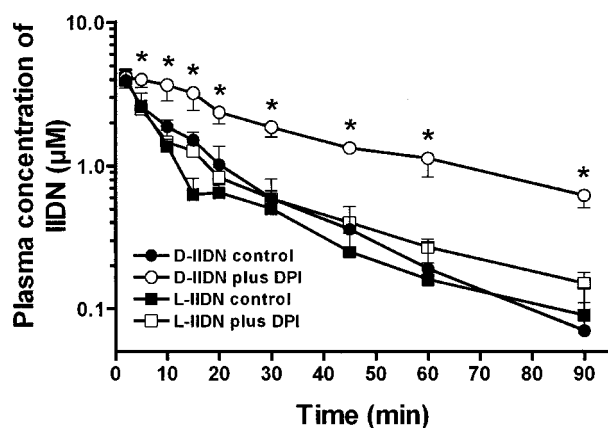


Figure 5 Effect of DPI on the plasma levels of IIDN. In the intact animal model, plasma levels of IIDN were measured following a bolus i.v. dose of 2 mg kg^{-1} IIDN. A bolus i.v. dose of 2 mg kg^{-1} DPI significantly increased the plasma levels of D-IIDN. *Indicates $P < 0.001$. D-IIDN control vs D-IIDN plus DPI. DPI had no effect on plasma levels of L-IIDN ($P > 0.05$, L-IIDN vs L-IIDN plus DPI) ($n = 4$).

Discussion

The major finding of this study was that DPI abolished the enantioselective differences between the D- and L-enantiomers of IIDN in both the intact tissue preparation and in the intact animal *via* an inhibitory effect on the biotransformation and pharmacological actions of D-IIDN. DPI caused a parallel, rightward shift in the concentration-response curve for D-IIDN and increased the EC_{50} value for D-IIDN-induced relaxation, but had no effect on L-IIDN-induced relaxation. Vascular biotransformation of D-IIDN to its mononitrate metabolite was significantly inhibited by DPI, but DPI had no effect on L-IIDN formation. In both endothelium-intact and endothelium-denuded rat aorta, DPI markedly inhibited D-IIDN-induced cyclic GMP accumulation, but had no effect on L-IIDN-induced cyclic GMP accumulation. *In vivo*, DPI altered both the pharmacokinetic and haemodynamic properties of D-IIDN, but those of L-IIDN were not affected. Previous studies have suggested that DPI inhibited the biotransformation and pharmacological actions of GTN *via* an effect on the cytochromes P450-NADPH-cytochrome P450 reductase system (McGuire *et al.*, 1994, 1998). The data reported herein suggest that the enantioselective biotransformation of D-IIDN to NO is mediated by an enzyme that is inhibited by DPI.

The biotransformation of organic nitrates can result in the formation of inorganic nitrite anion (NO_2^-) or NO. The formation of NO_2^- predominates, but NO_2^- is not active at concentrations that could be formed from pharmacologically relevant concentrations of organic nitrates. This non-productive formation of NO_2^- from organic nitrates has been referred to as 'clearance-based' biotransformation to distinguish it from 'mechanism-based' biotransformation—the biotransformation of organic nitrates that results in the formation of activators of guanylyl cyclase. Since the greater potency of D-IIDN for vascular relaxation is associated with a greater rate of biotransformation, it would appear that mechanism-based biotransformation is the basis for enantioselectivity in vascular relaxation by these compounds. This component of the biotransformation of D-IIDN was abolished after treatment with DPI, suggesting that this compound inhibits an enzyme that mediates mechanism-based biotransformation of D-IIDN to NO.

DPI and other diaryliodonium compounds have been shown to inhibit a number of flavin-containing enzymes, including NADPH-oxidase (Cross & Jones, 1986; Doussière & Vignais, 1992), NO synthase (Stuehr *et al.*, 1991) and xanthine oxidase (O'Donnell *et al.*, 1993). The inhibition of bovine and rat hepatic NADPH-cytochrome P450 reductase by DPI has been reported (Doussière & Vignais, 1992; McGuire *et al.*, 1998), and Tew (1993) has demonstrated the inhibition of rat hepatic NADPH-cytochrome P450 reductase by iodonium diphenyl (IDP), an analogue of DPI. DPI and IDP are thought to bind to the reduced redox center of the flavoprotein to form a reversible enzyme-inhibitor complex that is subsequently converted to an irreversible enzyme-inhibitor complex (O'Donnell *et al.*, 1993; Tew, 1993). With respect to the cytochromes P450-NADPH-cytochrome P450 reductase system, sites of covalent binding of DPI may include the flavin or haeme moieties or adjacent amino acids near the active site. In this regard, incubation of rat aortic microsomes with ^{125}I -labelled DPI resulted in the labelling of two proteins (79 kDa and 50 kDa), which would be consistent with the binding of DPI to both NADPH-cytochrome P450 reductase and cytochromes P450 (McGuire & Bennett, 1996). The results of the current study suggest that it is an enzyme inhibited by DPI that is the basis for the potency difference between the two enantiomers of IIDN. Possible candidates for inhibition by DPI include both the NADPH-cytochrome P450 reductase and cytochromes P450 since both can mediate the biotransformation of organic nitrates (McDonald & Bennett, 1990, 1993; McGuire *et al.*, 1998). Although biotransformation of organic nitrates by other flavoproteins has not been reported, evidence to exclude the involvement of several potential DPI targets exists. In a previous study by McGuire *et al.* (1994), the inhibitory effect of DPI on GTN relaxation, biotransformation and cyclic GMP accumulation was the same in endothelium-intact and endothelium-denuded aorta, suggesting that endothelial NO synthase was not involved in the bioactivation of GTN. Under the same experimental conditions, acetylcholine-induced relaxation in endothelium-intact tissues was significantly inhibited by DPI. In a study by McDonald & Bennett (1993), it was demonstrated that the aortic microsomal biotransformation of GTN could be inhibited greater than 90% by treatment of microsomes with carbon monoxide. Since microsomal flavin-containing monooxygenase (FMO) activity is unaffected by carbon monoxide (Stefek *et al.*, 1989; Eriksson & Bostrom, 1988), this would exclude a role for FMO in organic nitrate biotransformation. NADPH-oxidase activity is assayed as cytochrome *c* reduction that is inhibitable by superoxide dismutase (the O_2^- generated by the enzyme is responsible for the reduction of cytochrome *c*). The NADPH-dependent cytochrome *c* reductase activity of aortic microsomes is decreased by about 20% by superoxide dismutase (unpublished observations) and therefore it is possible that NADPH-oxidase could contribute to microsomal organic nitrate biotransformation. We have performed experiments to assess the role of xanthine oxidase in organic nitrate biotransformation. Under anaerobic conditions only, incubation of purified xanthine oxidase and xanthine with GTN, D- or L-IIDN resulted in biotransformation that was inhibited by DPI, indicating that this flavoprotein can biotransform organic nitrates (data not shown). To assess whether xanthine oxidase contributed to vascular organic nitrate biotransformation, the $105,000 \times g$ supernatant fraction of rat aorta was incubated with GTN, D- or L-IIDN and added xanthine (1 mM). Organic nitrate biotransformation was not observed under anaerobic conditions and was not affected by the addition of xanthine, suggesting that vascular

xanthine oxidase does not contribute to organic nitrate biotransformation.

To determine if the findings in the intact tissue preparation translated into significant alterations in the enantioselective haemodynamic-pharmacokinetic properties of IIDN that have been observed in the intact animal (Bennett *et al.*, 1991), rats were treated with 2 mg kg⁻¹ DPI *in vivo*. DPI clearly abolished the enantioselective differences between D- and L-IIDN and results showed that inhibition of the *in vivo* biotransformation of D-IIDN related to a decrease in its haemodynamic effects (Figures 4 and 5). Biotransformation of D-IIDN, which is thought to be necessary for its vasodilator activity, was inhibited in the presence of DPI as indicated by an increase in both plasma levels of the parent compound as well as the AUC and elimination half-life. None of the haemodynamic or pharmacokinetic parameters measured for L-IIDN were inhibited by DPI. In effect, there were no differences between the haemodynamic properties of D- and L-IIDN in the presence of DPI, thereby confirming the results obtained in the intact tissue preparation. In the whole animal, IIDN undergoes both mechanism-based and clearance-based biotransformation. Based on the elevated plasma levels of D-IIDN following DPI administration, DPI must also inhibit the clearance-based biotransformation of D-IIDN. Considering the haemodynamic data, where DPI inhibited the D-IIDN-induced decrease in MAP, one might argue that there should have been an increased MAP lowering with DPI because there were significantly higher plasma levels of the parent compound available for mechanism-based biotransformation. However, the haemodynamic data confirms that DPI also inhibited the mechanism-based biotransformation of D-IIDN since the D-IIDN-induced decrease in MAP was less than in control animals. Due to the fact that the pharmacokinetic data are based on whole body arterial blood samples, the increase in plasma levels of D-IIDN reflects the combined effect of DPI on both mechanism- and clearance-based biotransformation. Separating the relative contribution of the mechanism- and clearance-based biotransformation from the overall IIDN biotransformation was not feasible in this system. It was also evident that DPI did not have an effect on the clearance-based biotransformation of L-IIDN meaning that clearance must also be an enantioselective process. Taken together, there is strong evidence that DPI inhibits an enzyme or enzyme system that gives D-IIDN the enantioselective potency advantage over L-IIDN that has been observed both *in vitro* and *in vivo*.

Although DPI inhibited the enantioselectivity of IIDN action, D-IIDN-induced relaxation still occurred in the presence of DPI, and the responses to L-IIDN were not altered by DPI. This suggests that other biotransformation systems can mediate NO formation from organic nitrates, assuming that the cytochromes P450 system is the target for

the inhibitory actions of DPI and that it is effectively inhibited at the concentration of DPI employed. An alternative interpretation would be that NO- and cyclic GMP-independent mechanisms for relaxation by organic nitrates exist (although none have been reported). Other vascular enzymes reported to mediate organic nitrate biotransformation are the GSTs (Tsuchida *et al.*, 1990; Nigam *et al.*, 1996) and an unidentified microsomal protein that mediates NO formation from GTN (Chung *et al.*, 1992). The GSTs from several different tissues and species exhibit enantioselectivity for the conjugation of glutathione with a variety of substrates (Mannervik & Danielson, 1988). However, the rate of biotransformation of D- and L-IIDN in the 105,000 × g supernatant fraction of rat aorta was not different. Furthermore, DPI did not inhibit GST activity in the 105,000 × g supernatant fraction of rat aorta nor did it inhibit GSH-dependent biotransformation of D- and L-IIDN, suggesting that the enantioselective component of biotransformation is not mediated by the GSTs. Whether the GSTs are responsible for the non-enantioselective biotransformation of IIDN to NO in intact cells remains to be determined.

In conclusion, we found that the flavoprotein inhibitor, DPI, inhibited D-IIDN-induced relaxation and cyclic GMP accumulation, and vascular D-IIDN biotransformation, but had no effect on the biotransformation and pharmacological actions of L-IIDN. These results were confirmed in the intact rat where DPI inhibited the blood pressure response to D-IIDN and altered the pharmacokinetic properties of D-IIDN, but had no effect on L-IIDN. DPI had no effect on the GST activity or the GSH-dependent biotransformation of D- or L-IIDN in the 105,000 × g supernatant fraction of rat aorta. A specific enzyme system responsible for the enantioselective differences between D- and L-IIDN and that is inhibited by DPI was not identified in the current study. However, the accumulated evidence for the cytochromes P450 system as a target for the inhibitory effects of DPI on GTN action (McGuire *et al.*, 1994, 1998; McGuire & Bennett, 1996) would make this system a likely candidate. Since the enantioselective relaxation response of IIDN is abolished in organic nitrate-tolerant tissues (Bennett *et al.*, 1988), the enzymes targeted by DPI may also be targets for the tolerance-inducing effects of organic nitrates.

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Homologous regulation of the $\alpha 2C$ -adrenoceptor subtype in human hepatocarcinoma, HepG2

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1 Previous studies of the regulation of the $\alpha 2C$ -adrenoceptor in OK and in transfected cells have led to discrepant conclusions. In the present work, we examined the homologous regulation of the human $\alpha 2C$ -adrenoceptor in the hepatocarcinoma cell-line, HepG2; a model which expresses this subtype spontaneously.

2 Short-period treatment of the cells with UK14304 provoked neither a diminution of the potency of the $\alpha 2$ -agonist to inhibit forskolin-induced cyclic AMP-accumulation nor a change in the degree of receptor coupling to G-proteins.

3 Long-period exposure to UK14304 resulted in a large reduction of [³H]MK912 binding sites (55% decrease). The action of UK14304 was dose-dependent ($EC_{50} = 190 \pm 45$ nM), rapid ($t_{1/2} = 4.2$ h) and reversible. Receptor down-regulation was also observed with clonidine or (–)adrenaline (38 and 36% decrease, respectively) and was blocked by the addition of $\alpha 2$ -antagonists.

4 Conversely to that observed with $\alpha 2$ -agonists, treatment of the cells with RX821002 or yohimbine alone, but not with phentolamine, promoted a significant increase of the receptor expression.

5 The observed alterations of receptor density are not the reflection of changes at the $\alpha 2C4$ mRNA level. Estimation of the receptor protein turnover and measurement of its half-life demonstrated that down-regulation by $\alpha 2$ -agonists and up-regulation by $\alpha 2$ -antagonists, with inverse-agonist efficacy, are respectively the consequence of increased and decreased rate of receptor degradation.

6 In conclusion, our data show that $\alpha 2C$ -adrenoceptor does not undergo desensitization but is down-regulated in HepG2. The lack of desensitization agrees with previous results obtained in cells transfected with the $\alpha 2C4$ gene, but not with observations made in OK cells. Inversely, down-regulation fits with results obtained in OK but not in transfected cells. The reasons for these discrepancies are discussed. Our results also demonstrated that certain $\alpha 2$ -antagonists behave as inverse agonist on the HepG2 model and thus provide for the first time evidence of inverse efficacy of antagonists on a cellular model expressing physiological level of a wild-type $\alpha 2$ -adrenoceptor.

Keywords: $\alpha 2C$ -adrenoceptor; down-regulation; desensitization; HepG2 cell

Abbreviations: β ARK, β -adrenergic receptor kinase; DMEM, Dulbecco's Modified Eagle's Medium; EDTA, ethylenediaminetetraacetic acid; GPCR, G-protein-coupled receptor; MK912, 2s, 12bs-1',3' dimethylspiro (1,3,4,5',6,6',7,12b-octahydro-2H-benzo[b]furo [2,3-a] quinazoline)-2,4'-pyrimidin-2'one; PBS, phosphate buffered saline; RX821002, 2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline; UK14304, 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline

Introduction

Many observations made both *in vivo* and *in vitro* have demonstrated that sustained stimulation of G-protein-coupled receptors (GPCRs) generally results in a subsequent attenuation of their triggered biological responses. The magnitude and the determinants of this adaptive process appear to vary with the type of GPCR considered; but according to our knowledge of the most well-defined models, it is now clear that the emergence of refractoriness is the consequence of the combination of a set of timely-operated phenomena including desensitization, internalization and down-regulation of the receptor (for review see Böhm *et al.*, 1997).

Receptor desensitization occurs within seconds to minutes following exposure to the agonist. It corresponds to a

functional uncoupling of the receptor from its G-proteins and it is primarily due to receptor phosphorylation by protein kinases belonging either to the second-messenger protein kinase family (heterologous desensitization) or to G-protein-coupled receptor kinases (homologous desensitization). Receptor internalization, on the other hand, occurs within minutes to hours following agonist exposure and may contribute to further attenuation of the cellular receptivity by translocating surface receptors to an intracellular compartment where physical interactions with the agonist are no longer possible. The precise mechanisms involved in this process are not fully elucidated yet, but it is considered that the predominant pathway is *via* the clathrin-coated pits. Finally, down-regulation is characterized by a net decrease in receptor number and is only observed after prolonged exposure to agonist for hours to days. The molecular mechanisms responsible for this phenomenon largely depend on the type of GPCR considered and the diminution of receptor number reflects enhanced degradation and/or altered synthesis of the receptor polypeptide.

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The effects of catecholamines on cyclic AMP production are mediated through interactions with $\alpha 2$ - and β -adrenoceptors which are negatively and positively coupled to adenylyl cyclase, respectively. While most of our present understanding of the determinants of GPCR regulation derives from extensive studies of the β -adrenoceptor system (Collins *et al.*, 1991) far less is known about $\alpha 2$ -adrenoceptors. The $\alpha 2$ -adrenoceptors are currently classified into three pharmacological subtypes, namely: $\alpha 2A$, $\alpha 2B$ and $\alpha 2C$ (Bylund *et al.*, 1994). The genes encoding these subtypes have been cloned in different species and were respectively termed $\alpha 2C10$, $\alpha 2C2$ and $\alpha 2C4$ in human (Kobilka *et al.*, 1987; Regan *et al.*, 1988; Lomasney *et al.*, 1990). According to the results obtained on transfected cells, a major divergence between $\alpha 2$ -adrenoceptor subtypes lies in their ability to undergo desensitization and down-regulation. Studies carried out on cells transfected with the human genes indicated that exposure to adrenaline resulted in a rapid attenuation of $\alpha 2$ -agonist-induced inhibition of adenylyl cyclase in cells expressing the $\alpha 2A$ - or the $\alpha 2B$ -subtype, while no desensitization was observed with the $\alpha 2C$ (Eason & Liggett, 1992; Kurose & Lefkowitz, 1994). Moreover, long term exposure to the catecholamine provoked a down-regulation of the human $\alpha 2A$ - or $\alpha 2B$ -subtype, but not of the $\alpha 2C$ (Eason & Liggett, 1992). On the other hand, experiments performed on the OK cell-line (a model which spontaneously expresses an $\alpha 2C$ -adrenoceptor pharmacologically identical to human, but from opossum origin) demonstrated that treatment with noradrenaline results in a rapid decrease in the potency of $\alpha 2$ -agonists to inhibit PTH-induced cyclic AMP production (Jones *et al.*, 1990). Furthermore, long-term exposure to the neurotransmitter induces a significant reduction in receptor number (Shreve *et al.*, 1991; Pleus *et al.*, 1993) indicating that both desensitization and down-regulation of $\alpha 2C$ -adrenoceptor subtype occurs in these cells.

In contrast to $\alpha 2A$ -adrenoceptor, which largely benefited of the existence of cell-lines such as HT29 (Paris *et al.*, 1987) or HEL (McKernan *et al.*, 1988), the study of the regulation of the human $\alpha 2C$ -adrenoceptor has been so far restricted to transfected cells because of the lack of model constitutively expressing this subtype. The recent discovery of the human hepatocarcinoma HepG2 as exhibiting $\alpha 2C$ -adrenoceptors negatively coupled to adenylyl cyclase (Schaak *et al.*, 1997a) allows now to investigate the regulation of this subtype in a cell-line natively expressing this receptor subtype.

The present study carried out on HepG2 shows that $\alpha 2C$ -adrenoceptor does not undergo rapid desensitization but is largely down-regulated in response to long-term exposure to $\alpha 2$ -agonists. Conversely cell treatment with certain $\alpha 2$ -antagonists resulted in an increase of receptor density. According to estimation of receptor protein turnover and to measurement of its half-life, the changes induced by agonists and antagonists are respectively due to increased and decreased rate of the receptor degradation. These observations bring new insights onto the mechanisms of homologous regulation of $\alpha 2C$ -adrenoceptors.

Methods

Cell culture and treatments

The human hepatocarcinoma HepG2 was cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 25 mM glucose, 100 $\mu\text{g ml}^{-1}$ streptomycin, 100 IU ml^{-1} penicillin, 2 mM glutamine and 10% foetal calf serum. Unless

otherwise specified, all treatments were performed on confluent attached cells and in the absence of FCS. At zero time of the treatment, the hormone or drug to be tested was added to the culture from frozen stock solution. At the indicated time, the medium was removed and the cell-layers rinsed twice with phosphate buffered saline (PBS). According to their intended use, cells were either frozen at -80°C until analysis (binding experiments, RNA extraction) or isolated in PBS containing 0.5 mM ethylenediaminetetraacetic acid (EDTA) (measurement of intracellular cyclic AMP).

Generation and synthesis of $\alpha 2C4$ and β -actin riboprobes

The probe for detection of $\alpha 2C4$ mRNAs was obtained by subcloning a 370 bp fragment (*SmaI*-*MaeIII*) corresponding to nucleotides 1014–1382 of the $\alpha 2C4$ coding region into pBluescriptII KS+ (pKS+, Stratagene, La Jolla, CA, U.S.A.). The specificity of this probe has been previously assessed (Valet *et al.*, 1993). The β -actin probe was obtained by PCR and TA-cloning into the *EcoRV* site of pKS+, the amplified fragment (236 bp) corresponds to nucleotides 415–650 of the cDNA (exon 3). For synthesis of the radiolabelled probes, the two plasmids were cut with the appropriated restriction enzyme and antisense RNAs were synthesized in the presence of [^{32}P]UTP using the T3 RNA polymerase (Promega).

RNA preparation and RNase protection assays (RPA)

Cellular RNAs were isolated using the guanidium isothiocyanate/phenol-chloroform extraction method (Chomczynski *et al.*, 1987). The integrity of the preparations was assessed by agarose gel electrophoresis and the RNA concentrations were measured by u.v. spectrophotometry. RPA were performed as previously described (Schaak *et al.*, 1997a). Lyophilised RNAs (100 μg) were taken in 30 μl of hybridization buffer (80% deionized formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM Pipes, pH 6.7) containing an excess of ^{32}P -labelled riboprobe. The samples were heated to 95°C for 5 min and then immediately placed at 55°C for 14 h. Non-hybridized probe was eliminated by the addition of 0.3 ml of TEN buffer (10 mM Tris-HCl, 5 mM EDTA, 300 mM NaCl, pH 7.5) containing RNases A (40 $\mu\text{g ml}^{-1}$) and T_1 (2 $\mu\text{g ml}^{-1}$). After 2 h at 37°C , digestion was stopped by the addition of 5 μl of proteinase K (10 mg ml^{-1}) and samples were further incubated for 15 min at 37°C . Carrier tRNA (10 μg) in 0.3 ml of solution D (4 M guanidium thiocyanate, 25 mM sodium citrate, 0.1 M 2-mercaptoethanol and 0.5% sarkosyl, pH 7.0) were then added and protected hybrids precipitated with isopropyl alcohol. After washing with 70% ethanol, RNA pellets were dissolved in 10 μl of sample buffer (97% deionized formamide, 0.1% SDS, 10 mM Tris-HCl, pH 7.0) and loaded onto a 5% acrylamide/7 M urea gel. The amounts of protected radiolabelled probe were quantified using a Phosphor-Imager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Receptor quantification

The number of $\alpha 2$ -adrenoceptors was measured on crude membrane preparations using the selective $\alpha 2$ -adrenergic antagonist [^3H]MK912 (2s, 12bs-1',3' dimethylspiro (1,3,4,5',6,6',7,12b-octahydro-2H-benzo[b]furo [2,3-a] quinoxaline)-2,4'-pyrimidin-2'one) (Pettibone *et al.*, 1989). Frozen cells were homogenized in 25 ml of TE buffer (50 mM Tris-HCl, 5 mM EDTA, pH 7.5) and centrifuged at $39,000 \times g$ for 10 min. The particulate fraction was washed in TE buffer and the final crude membrane pellet was taken up in the

appropriate volume of TM buffer (50 mM Tris-HCl, 0.5 mM MgCl_2 , pH 7.5). The protein concentration was determined using the Coomassie blue method (Bradford, 1976). Total binding was measured by incubating 100 μl of cell membrane preparation with the radioligand in a total volume of 400 μl of TM buffer. After a 45 min period of incubation at 25°C, bound radioactivity was separated from free by filtration through GF/C Whatman filters using a Millipore Manifold Sampling unit. Filters were rapidly washed with ice-cold TM buffer and membrane-bound radioactivity was determined by liquid spectrometry. Specific binding was defined as the difference between total and non-specific binding measured in the presence of 10^{-5} M phentolamine. For saturation studies, the final concentrations of [^3H]MK912 ranged from 0.04–3 nM.

Determination of intracellular cyclic AMP content

Cells were detached in PBS containing 0.5 mM EDTA and collected by gentle centrifugation ($900 \times g$, 5 min, 4°C). The pellet was suspended in DMEM buffered with 10 mM HEPES (pH 7.4). Aliquots of the cell suspension were incubated in a 200 μl final volume of HEPES-buffered DMEM containing 0.2 mM 3-isobutyl-1-methylxanthine and the indicated concentration of the drug to be tested. After 15 min at 37°C, the reaction was stopped by adding 1.8 ml of methanol/formic acid (95/5, v/v). The cell lysate was centrifuged ($3000 \times g$, 10 min, 4°C) and aliquots of the supernatant were evaporated. The dry samples were taken in acetate buffer containing 0.1% NaN_3 and their cyclic AMP content was determined by radio-immunological assay (Steiner *et al.*, 1972).

Drugs and chemicals

[^3H]MK912 (79 Ci mmol^{-1}) was from New England Nuclear (Boston, MA, U.S.A.) and [α - ^{32}P]UTP from ICN (Costa Mesa, CA, U.S.A.). Phentolamine and clonidine were donated by Ciba-Geigy (Basel, Switzerland) and Boehringer-Ingelheim (Ingelheim, Germany). UK14304 (5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline) and RX821002 (2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline) were gifts from Pfizer (Sandwich, U.K.) and Reckitt and Colman Laboratories (Kingston-upon-Hull, U.K.). RNase A, RNase T_1 and proteinase K were purchased from Boehringer Mannheim (Meylan, France). Yohimbine, (–)adrenaline, benextramine, forskolin, concanavalin A, cycloheximide and all other chemicals were from Sigma (St. Louis, MI, U.S.A.). Foetal calf serum was purchased from Gibco-BRL (Cergy Pontoise, France). Radio-immunoassay kits for cyclic AMP determination were from Immunotech (Luminy, France).

Analysis of data

Binding data were analysed using the EBDA-LIGAND computer programs (McPherson, 1985). All data are expressed as means $\pm$ s.e.mean and statistical analysis was performed by Student's *t*-test. Statistical differences for *P* less than 0.05 and 0.001 were indicated with asterisks (* and ***, respectively).

The kinetics of receptor recovery after benextramine treatment were fitted according to a model which validity was already verified for other receptors (Neve & Molinoff, 1986). This model assumes that within each group (untreated and treated cells) (i) the rate of receptor synthesis remains constant during the period of receptor recovery and (ii) the rate of receptor degradation is proportional to its concentration. The equation which fits this model is $[R_t] = V/k(1 - e^{-Kt})$, where R_t

is the receptor density at discrete time *t*, *V* its rate of synthesis and *k* its disappearance rate constant. When *t* tends to ∞ , then $[R_t]$ approaches V/k and represents receptor density at steady state $[R_{ss}]$. Therefore the equation can be written $[R_t] = [R_{ss}](1 - e^{-Kt})$. Its logarithmic transformation ($\ln [R_{ss}] / [R_{ss} - R_t] = kt$) gives straight line and allows to calculate *k*.

Results

Effect of short-term agonist-exposure on $\alpha 2$ C-adrenoceptor responsiveness

HepG2 cells were demonstrated to express $\alpha 2$ C-adrenoceptors negatively coupled to adenylyl cyclase very recently (Schaak *et al.*, 1997a) and no information is available yet about the mechanisms regulating this receptor subtype in this model. Previous studies of $\alpha 2$ C-adrenoceptor desensitization in OK cells (Jones *et al.*, 1990; Shreve *et al.*, 1991) and in transfected cells having led to divergent conclusions (Eason & Liggett, 1992; Kurose & Lefkowitz, 1994), we thought of interest to examine first whether the functional efficacy of HepG2 receptor underwent desensitization with prior exposure to $\alpha 2$ -agonist. To do so, HepG2 cell-layers were incubated with 10 μM UK14304 (full agonist). After a 30 min period of treatment, cells were extensively washed in PBS, rapidly isolated in cold and assayed for $\alpha 2$ -adrenoceptor mediated inhibition of forskolin-induced cyclic AMP accumulation. Results from this experiment, which are depicted in Figure 1a, indicated that neither basal nor forskolin-stimulated cyclic AMP levels were affected by the pretreatment. Maximal inhibition of cyclic AMP accumulation by UK14304 reached similar extend ($56 \pm 4\%$ in control and $51 \pm 5\%$ in pretreated cells). Dose-response curves were superimposable and no shift in the potency of UK14304 was observed. Computer-assisted analysis of the data indicated that EC_{50} values for UK14304 to inhibit cyclic AMP accumulation were not significantly different (28 ± 4 and 53 ± 15 nM in control and pretreated-cells, respectively). Strictly similar results were obtained after a 15 min period of pretreatment with UK14304 (not shown), indicating that the $\alpha 2$ C-adrenoceptor did not undergo desensitization in HepG2 cells.

This conclusion was further confirmed by the study of the inhibition of the binding of the radiolabelled antagonist [^3H]MK912 by UK14304. The inhibition curves obtained on washed membranes prepared from control and from agonist-treated cells are depicted in Figure 1b. Analysis of the data according to a two-site inhibition model allowed to calculate that the percentage of high-affinity agonist binding sites was $53 \pm 6\%$ in control cells and $58 \pm 8\%$ in UK14304-pretreated cells (mean $\pm$ s.e.mean, *n* = 3). Exposure to the agonist provoked a slight rightward shift of the inhibition curve resulting in an small increase (4 fold) of both K_iH and K_iL values. However, the ratio of K_i values of UK14304 for low- and high-affinity state ($K_iL : K_iH$) was unchanged (106 ± 55 in control versus 80 ± 29 in treated-cells, mean $\pm$ s.e.mean, *n* = 3) indicating that the degree of receptor coupling to G_i -proteins was unaffected by the exposure to the agonist.

Effect of long-term exposure to $\alpha 2$ -adrenergic compounds on $\alpha 2$ -adrenoceptor density

As determined by saturation binding isotherms with [^3H]MK912, the density of $\alpha 2$ -adrenoceptors in HepG2 cells cultured in the presence of 10% FCS was 55.7 ± 3.7 femtomoles mg^{-1} of membrane protein (*n* = 17). In contrast

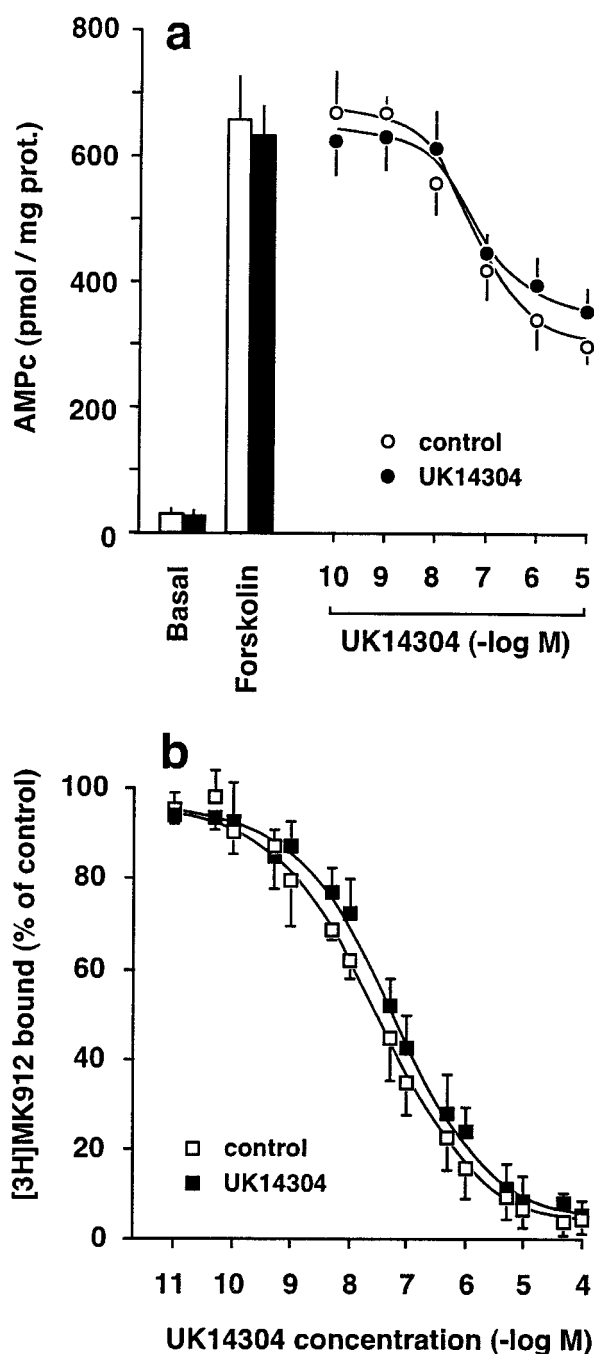


Figure 1 Effect of short-term exposure to UK14304 on the functional efficacy and degree of coupling of the $\alpha 2C$ -adrenoceptor. HepG2 cell layers were pre-incubated without or with 10 μM UK14304 for 30 min and were extensively washed in cold PBS. (a) Control (○) and agonist-treated cells (●) were detached in cold PBS containing 0.5 mM EDTA. After mild centrifugation at 4°C, cells were suspended and incubated 15 min at 37°C in 200 μl of HEPES-buffer DMEM containing either vehicle (basal) or 10 μM forskolin or 10 μM forskolin plus indicated concentrations of UK14304. Concentration of cyclic AMP was measured as described in the 'Methods' section. Results are expressed as picomoles of cyclic AMP mg^{-1} of cellular protein and are means $\pm$ s.e.mean ($n=6$). (b) Cell-layers from control (□) or agonist-treated (■) dishes were rapidly rinsed and frozen. Membranes were then prepared as described in the Methods section and incubated in the presence of 0.8 nM [^3H]MK912 and increasing concentrations of UK14304. The amount of specifically bound radioligand was determined using 10^{-5} M phentolamine to estimate non-specific binding. The amounts of bound [^3H]MK912 are expressed as per cent of control and the presented data are the mean $\pm$ S.D. from three independent experiments. Analysis of the inhibition curves according to a two-site model indicated that the per cent of receptor under high-affinity state for agonist was $53 \pm 6\%$ and

to that previously observed for $\alpha 2A$ -adrenoceptor subtype in HT29 cells (Devedjian *et al.*, 1991), a 48 h period of serum deprivation did not provoke an increase in receptor number. All further treatments were however carried out in serum-deprived medium in order to avoid any interference of FCS with the tested compounds. Under such culture conditions, a 48 h exposure to UK14304 (10 μM) induced a large reduction (55%) of receptor number (Figure 2a). No alteration of receptor affinity was observed, the dissociation constant for [^3H]MK912 being unchanged by the treatments (0.08 ± 0.03 nM in control versus 0.09 ± 0.02 nM in treated cells). As shown in the Figure 2b, exposure to 10 μM (–)adrenaline or to 10 μM clonidine resulted also in a significant decrease of receptor expression (38 and 36% decrease, respectively). The effect of 10 μM UK14304 was prevented by the prior addition of 100 μM RX821002 ($\alpha 2$ -antagonist), thus demonstrating that receptor occupancy by the agonist is necessary for receptor down-regulation to occur. A blockade of UK14304-induced down-regulation was also observed with 100 μM yohimbine or 100 μM phentolamine (not shown). It is remarkable that treatment with solely RX821002 or yohimbine, but not phentolamine, resulted in a significant increase in receptor number, indicating that certain $\alpha 2$ -antagonists have the potency to up-regulate the receptor and behave as inverse agonists in this regard.

The dose-dependence and the time-course of the receptor down-regulation are depicted in Figure 3a and b. The decrease in receptor expression provoked by UK14304 was dose dependent ($EC_{50} = 190 \pm 45$ nM) and reached its maximum (55% diminution) at 5 μM concentration of agonist. On the other hand, the decline of $\alpha 2$ -adrenoceptor number induced by UK14304 was rather rapid and was completed within 12 h following exposure. The process was consistent with a mono-exponential model with a half-life of disappearance of 4.2 h. Finally, when HepG2 cells exposed to UK14304 for 12 h were washed and then further incubated in drug-free medium, $\alpha 2$ -adrenoceptors were observed to recover up to control levels within 24 h following agonist removal.

Measurement of $\alpha 2C4$ mRNA levels

Previous studies of the molecular mechanisms accounting for $\beta 2$ -adrenoceptor down-regulation have established that the diminution in receptor number induced by β -agonists is the reflection of decreased levels of receptor mRNA. This change results from an accelerated rate of mRNA degradation (Böhm *et al.*, 1997) which is the consequence of the binding of a 35 kD protein to AUUUA pentamers located in the 3'UTR of the $\beta 2$ -adrenoceptor transcript (Port *et al.*, 1992; Tholanikunnel *et al.*, 1995). Such a sequence being present in the 3'UTR of the $\alpha 2C4$ mRNA (Schaak *et al.*, 1997b), we thus wondered whether such a mechanism also occurred in HepG2 cells. HepG2 cells were incubated or not in the presence of the different $\alpha 2$ -adrenergic drugs and the amounts of $\alpha 2C4$ mRNA were measured by RPA using a ^{32}P -labelled $\alpha 2C4$ antisense RNA. β -actin was used as an internal standard to normalize the quantification. A typical autoradiogram is presented in Figure 4. Analysis of the data obtained from cells treated for different periods of time (6, 12, 24 and 48 h) showed that neither $\alpha 2$ -agonists (UK14304,

$58 \pm 8\%$ in control and UK14304-treated cells respectively. K_d and K_i values for UK14304 were respectively 0.41 ± 0.17 nM and 43 ± 8 nM in control cells and 1.70 ± 0.25 nM and 161 ± 67 nM and agonist-treated cells (mean $\pm$ s.e.mean, $n=3$).

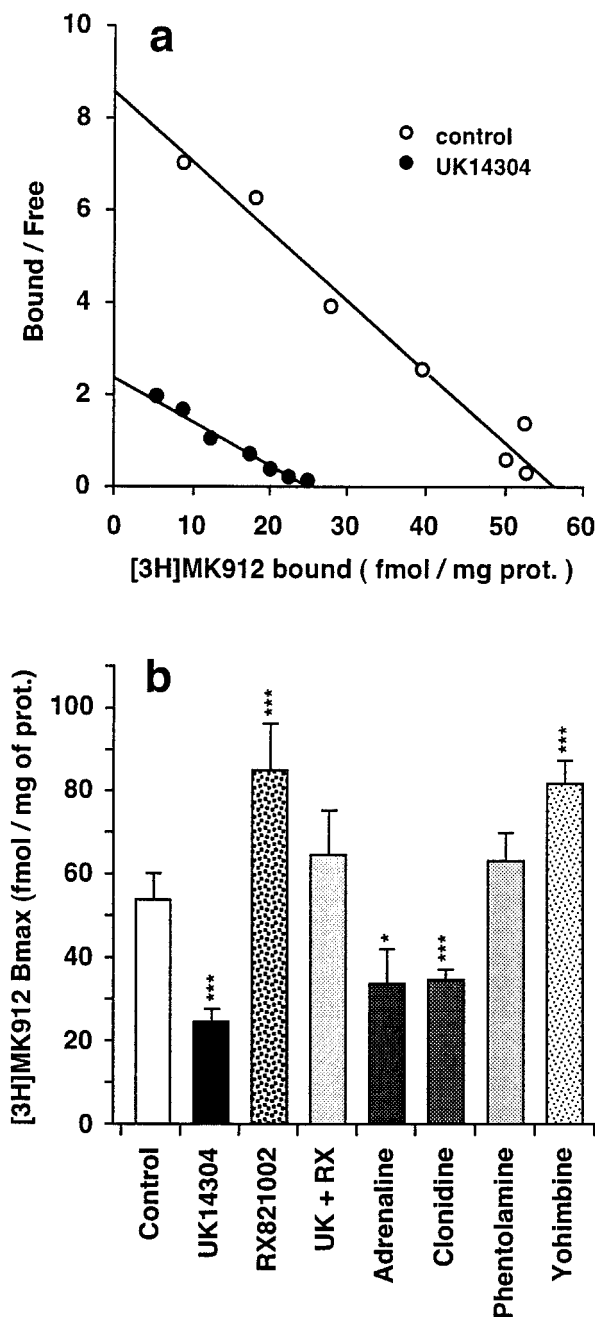


Figure 2 Effect of long-term exposure to $\alpha 2$ -adrenergic compounds on $\alpha 2C$ -adrenoceptor density. (a) HepG2 cells were maintained for 48 h in serum-free DMEM containing either 10 μM UK14304 (●) or not (○). Membranes were prepared as described in the Methods section and incubated in the presence of various concentrations of [^3H]MK912. The amount of specifically bound radioligand was determined using 10 $^{-5}$ M phentolamine to estimate non-specific binding. The data from a typical experiment are displayed as a Scatchard plot. Each point is the mean of determination in duplicate. (b) HepG2 cells were exposed for 48 h to various $\alpha 2$ -adrenergic compounds. The tested drugs were 10 μM UK14304, 100 μM RX821002, 10 μM UK14304 plus 100 μM RX821002 (UK + RX), 10 μM (–)adrenaline, 10 μM clonidine, 100 μM phentolamine or 100 μM yohimbine. Membranes were prepared and assayed for their capacity to bind [^3H]MK912. The maximum number of binding sites expressed as femtomoles mg $^{-1}$ of proteins was calculated by analysis of the radioligand saturation isotherms according to a one-component model. Reported values are the means $\pm$ s.e. mean from four determinations (***) and * indicate values significantly different from control at $P < 0.001$ and $P < 0.05$, respectively).

(–)adrenaline, clonidine) nor the $\alpha 2$ -antagonist (RX821002) affected the steady state levels of the $\alpha 2C4$ mRNAs.

UK14304 increases and RX821002 decreases the rate of $\alpha 2C$ -adrenoceptor degradation

The decrease in receptor density induced by UK14304 being rather rapid and not ascribable to a change in the amount of $\alpha 2C4$ mRNA, we hypothesized that the effect of the agonist results from a modification of the receptor turnover. The half-life of the receptor protein and the rate of its degradation were thus examined to investigate in this way. As previously done for the $\alpha 2A$ -adrenoceptor in HT29 cells (Paris *et al.*, 1987), the half-life of the $\alpha 2C$ -adrenoceptor was first determined by following the recovery of the binding capacity of the HepG2 cells after blockade of pre-existing receptors with the irreversible ligand, benextramine. This alkylating agent having never been used on $\alpha 2C$ -adrenoceptors, preliminary experiments were carried out to assess its apparent affinity for this receptor subtype and to verify the irreversibility of the blockade. The apparent affinity of benextramine for the $\alpha 2C$ -adrenoceptor was estimated by determining its potency to inhibit [^3H]MK912 binding to HepG2 cell membrane preparations. The inhibition curves obtained with benextramine and with the selective $\alpha 2$ -antagonist RX821002 are shown in Figure 5a.

Analysis of the data according to a one-site inhibition model indicated that the value of EC_{50} for benextramine (49 ± 5 nM) was close to that obtained for the specific $\alpha 2$ -antagonist, RX821002 (17 ± 1 nM), demonstrating this compound had a rather good affinity for the $\alpha 2C$ -adrenoceptor. A benextramine concentration of 2 μM was used in all further experiments on intact cells. As shown in Figure 5b, the treatment of HepG2 cells with such a dose of benextramine resulted in a total abolition of the capacity of cell membrane to bind [^3H]MK912. As expected, this effect was not observed with the reversible antagonist RX821002. It was moreover prevented by the prior addition of an excess of RX821002, indicating that the irreversible ligand docks in the receptor binding pocket. Given these preliminary data, a series of culture dishes were treated with benextramine 20 min at 37°C. Cells were then extensively washed in drug-free DMEM and allowed to recover their receptors over a period up to 3 days. The measurement of the reappearance of [^3H]MK912 binding sites demonstrated that receptor recovery was a mono-exponential process (Figure 6a). The restoration was rapid (90% of the steady state level of the receptor being recovered within 24 h) and was exclusively due to receptor neosynthesis since it was totally abolished by the addition of cycloheximide (50 μg ml $^{-1}$) into the culture medium (not shown). The fitting of the kinetic of receptor reappearance according to the model described in the Methods section (Figure 6b), allowed to determine the rate constant of the receptor degradation ($k_{cont} = 0.063$ h $^{-1}$). From this value, it can be calculated that the rate constant for receptor synthesis was 3.65 ± 0.5 fmol mg $^{-1}$ prot h $^{-1}$ and that the receptor half-life was 11 h, under basal conditions. Results from the parallel study of the receptor turnover on UK14304-treated cells yielded a significantly higher value for the rate constant of the receptor degradation ($k_{uk} = 0.112$ h $^{-1}$). According to this value, the rate constant for receptor synthesis was unchanged by the treatment (3.36 ± 0.6 fmol mg $^{-1}$ prot h $^{-1}$). The receptor half-life was by contrast reduced to 6.2 h, thus suggesting that the decrease in receptor density by UK14304 was the consequence of an accelerated receptor degradation. Such a conclusion was further confirmed by the direct estimation of the kinetics of

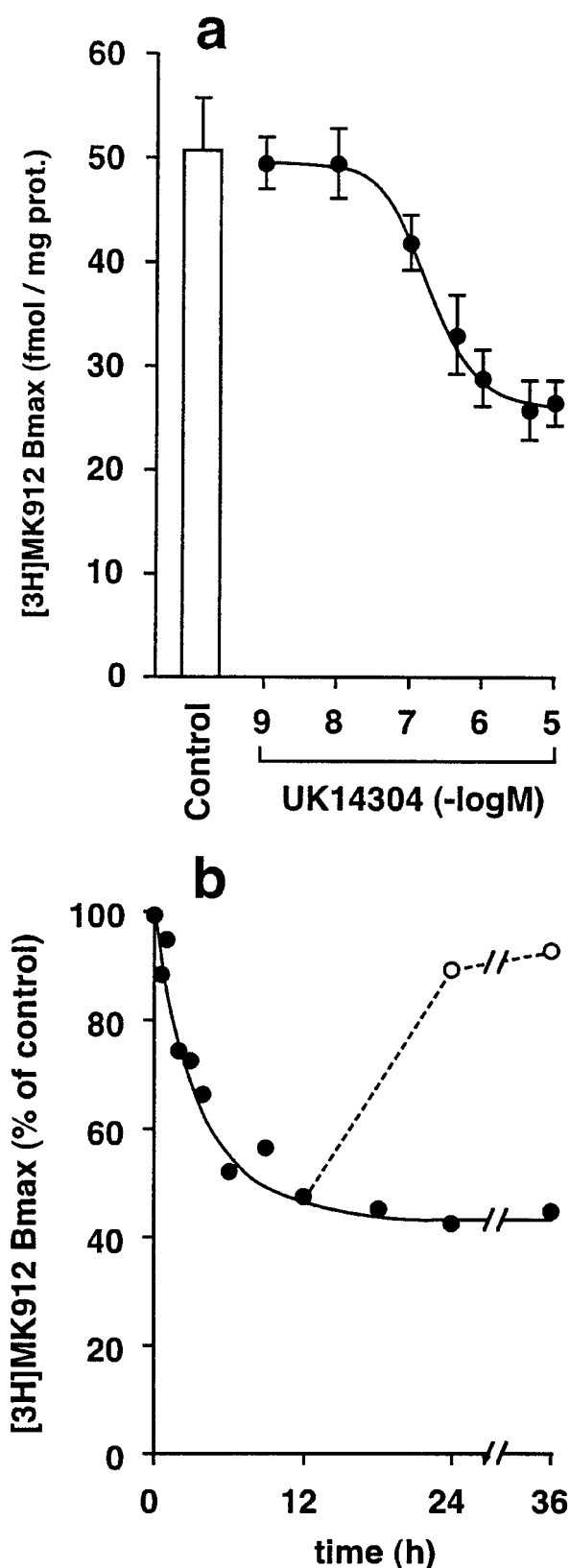


Figure 3 Dose-dependence, kinetics and reversibility of $\alpha 2C$ -adrenoceptor down-regulation induced by UK14304. (a) HepG2 cells were exposed for 48 h to final concentrations of UK14304 ranging from 1 nM to 10 μ M. Membranes were prepared and assayed for their capacity to bind [^{3} H]MK912 as described in the legend of Figure 2. Each data point represents the mean $\pm$ s.e. mean from three experiments. Analysis of the dose-response indicated that half-maximal down-regulation of the receptor was achieved at 190 ± 45 nM UK14304. (b) HepG2 cells were incubated with 10 μ M UK14304. Cells were harvested at the indicated times and membrane

receptor degradation in the presence of an inhibitor of protein synthesis (Figure 7). Indeed, the measurement of the decay of [^{3} H]MK912 binding sites in the presence of cycloheximide (50 μ g ml $^{-1}$) yielded values for receptor half-life of 13.4 h and 6.9 h in control and UK14304-treated cells, respectively. Inversely to UK14304, RX821002 increased receptor stability ($t_{1/2}$ = 20.2 h) demonstrating that the up-regulation provoked by antagonists was primarily due to a decrease in the rate of receptor degradation. No significant modification of receptor half-life was observed in cells treated with phentolamine (not shown).

Studies carried out on cells transfected with cDNA encoding rat or mouse $\alpha 2C$ -adrenoceptors have demonstrated that this subtype has both membranous and intracellular localization. The subcellular distribution of $\alpha 2C$ -adrenoceptor was not examined in HepG2, but we questioned whether receptor down-regulation requires sequestration into an internal compartment to occur. To address this, HepG2 were treated for 6 h with 10 μ M UK14304 under conditions where sequestration was blocked. As shown in the Table 1, blockade of sequestration by 0.25 mg ml $^{-1}$ concanavalin A or 0.4 M sucrose completely prevented receptor down-regulation.

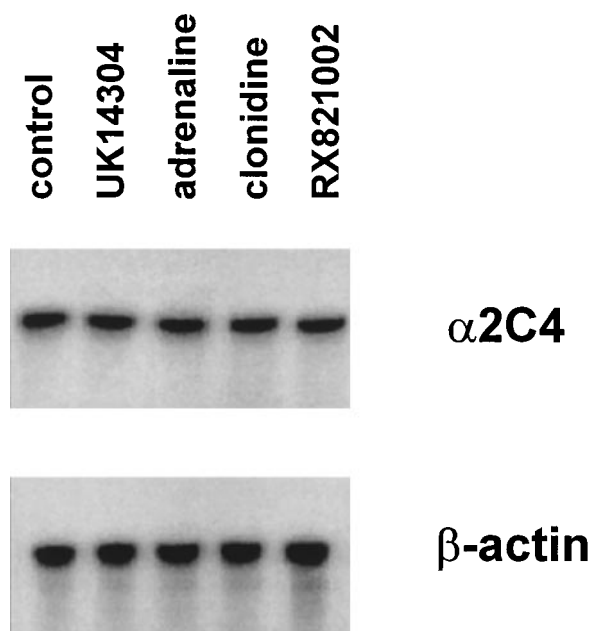


Figure 4 Effect of $\alpha 2$ -adrenergic compounds on $\alpha 2C4$ mRNA level. Cellular RNAs prepared from HepG2 cells maintained for 12 h under different culture conditions were hybridized with labelled antisense riboprobe for $\alpha 2C4$ or β -actin. The samples were digested with a mixture of RNases and the resistant hybrids were separated by electrophoresis. The treatments tested were the following: none (control), 10 μ M UK14304, 10 μ M (–)adrenaline, 10 μ M clonidine or 100 μ M RX821002. A representative autoradiogram is shown. Analysis of four different experiments by direct quantification of the radioactive bands using a Phosphor-Imager indicated that none of the tested drugs provoked a significant change in the amount of $\alpha 2C4$ mRNA.

preparations assayed for their capacity to bind [^{3} H]MK912 as described in the legend of Figure 2. After a 12 h period of exposure to UK14304, cells were extensively washed and then replaced in agonist-free medium (○). The number of [^{3} H]MK912 binding sites is expressed as per cent of control (untreated-cells). Data represent the mean of two experiments.

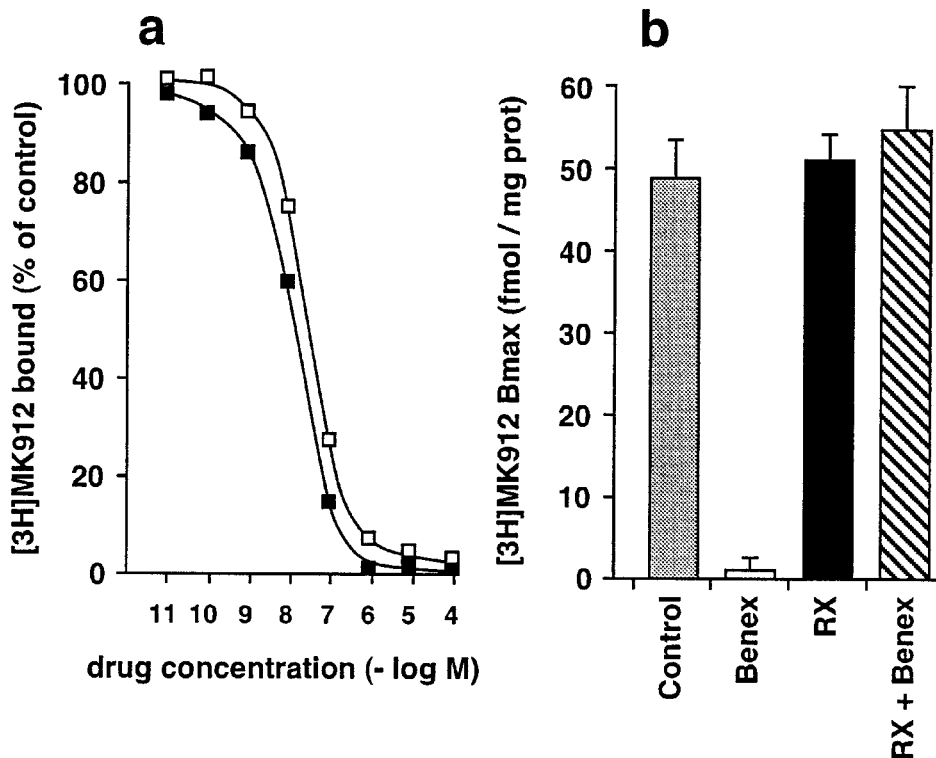


Figure 5 Irreversible effect of benextramine on $\alpha 2C$ -adrenoceptors. (a) Membranes prepared from HepG2 cells were incubated in the presence of 0.5 nM [^{3} H]MK912 and increasing concentrations of benextramine (□) or RX821002 (■). According to Hill coefficient values not significantly different from unity, inhibition curves were fitted to a one-site model. The calculated EC_{50} values were 49 ± 5 nM for benextramine and 17 ± 1 nM for RX821002. (b) HepG2 cells were incubated for 20 min at 37°C in the presence of either 2 μ M benextramine (Benex), 20 μ M RX821002 (RX) or 2 μ M benextramine plus 20 μ M RX821002 (RX + Benex). Cells were extensively washed, membranes were prepared and $\alpha 2$ -adrenoceptors were quantified by analysis of [^{3} H]MK912 saturation isotherms.

Discussion

The molecular mechanisms underlying the regulation of the expression of $\alpha 2$ -adrenoceptors of the $\alpha 2C$ -subtype are still unclear. While experiments on transfected cells showed that human $\alpha 2C$ -adrenoceptor is refractory to short-term desensitization (Eason & Liggett, 1992; Kurose & Lefkowitz, 1994) and long-term down-regulation (Eason & Liggett, 1992), other studies, carried out with the opossum kidney-derived cell-line OK, suggested that both processes occurred (Jones *et al.*, 1990; Shreve *et al.*, 1991). The objective of the present work was therefore to examine the regulation of the $\alpha 2C$ -adrenoceptor in HepG2, a human cell-line which spontaneously expresses this receptor subtype.

The experiments, carried out on this model, show that short-term exposure to $\alpha 2$ -agonist results neither in an attenuation of receptor functionality nor in an alteration of its degree of coupling to G_i -proteins, thus demonstrating that $\alpha 2C$ -adrenoceptor does not undergo rapid desensitization in HepG2. In this respect, our results agree with what previously found in COS or CHO cells transfected with the $\alpha 2C4$ gene, but do not fit with observations made on OK cells. According to previous studies on purified recombinant $\alpha 2$ -adrenoceptors or on transfected cells (Kurose & Lefkowitz, 1994; Jewell-Motz & Liggett, 1996), the failure of the human $\alpha 2C$ -adrenoceptor to undergo desensitization is due to the fact that this receptor subtype is not a substrate for G protein-coupled receptor kinases. The reasons for the discrepancy between the results obtained in HepG2 and OK are not fully elucidated. The most likely explanation is that OK cells express

a receptor which amino acid sequence exhibits large divergence with the human $\alpha 2C$ -adrenoceptor (Blaxall *et al.*, 1994). Major differences between the two polypeptides are found in the third intracellular loop, a region which appears important in receptor phosphorylation and desensitization. In regard to this latter point, it is noteworthy that the third intracellular loop of the OK receptor contains an EESSTS motif (amino acids 306–312) which resembles the EESSSS sequence that is phosphorylated by β -adrenergic receptor kinase (β ARK) in the human $\alpha 2A$ -adrenoceptor (amino acids 293–299) (Eason *et al.*, 1995). Such a motif is absent from all other $\alpha 2C$ -adrenoceptors cloned so far from other species. The possibility therefore exists that OK $\alpha 2C$ -adrenoceptor represents an exception in that it undergoes desensitization because it is a substrate for β ARK. This hypothesis would merit examination.

In contrast to what found in transfected cells where human $\alpha 2C$ -adrenoceptor was refractory to down-regulation (Eason & Liggett, 1992), our current results demonstrate that long-lasting exposure of HepG2 cells to $\alpha 2$ -agonists induces a marked decrease in receptor number. HepG2 being from human origin, differences in the primary structure of the receptors cannot be suspected here. CHO cells used in the above-cited study expressed approximately 20 fold higher levels of $\alpha 2C$ -adrenoceptors than HepG2, furthermore a large proportion of these receptors was found in an intracellular compartment, a situation that might not fully reflect the physiological reality. Alternatively, the extent of down-regulation could depend of the cell-type. Study of other human cell-lines expressing $\alpha 2C$ -subtype endogenously, such

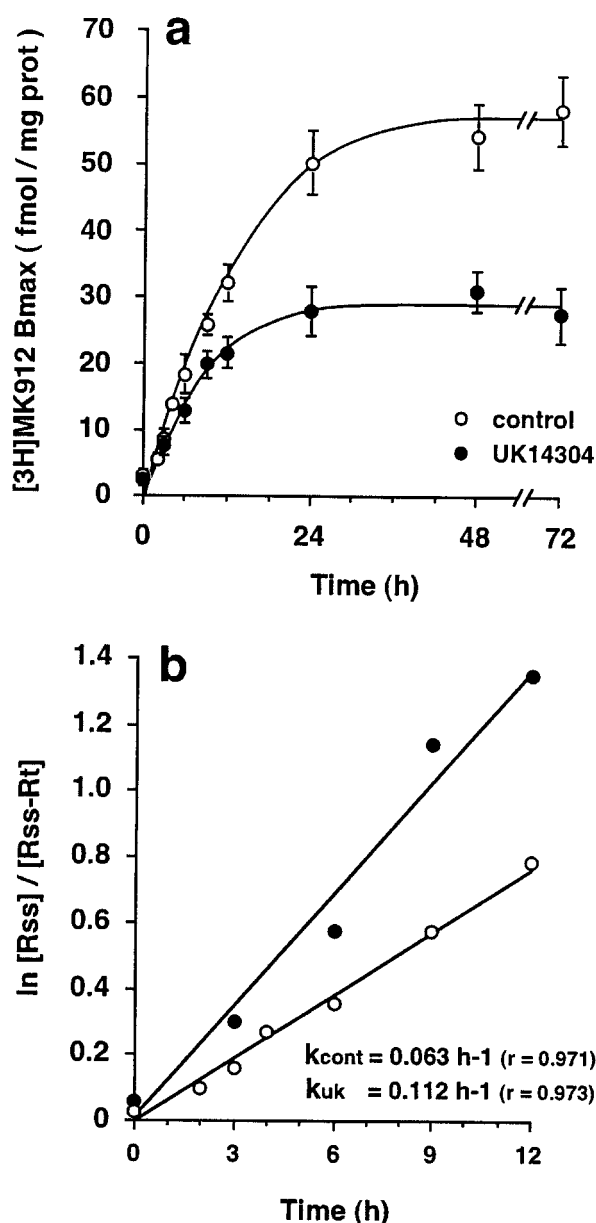


Figure 6 Kinetics of the α_2C -adrenoceptor reappearance after irreversible blockade with benextramine. (a) A series of culture dishes was incubated for 20 min at 37°C in the presence of 2 μ M benextramine. Cells layers were then extensively washed and finally replaced either in DMEM (control, ○) or in DMEM containing 10 μ M UK14304 (UK14304, ●). Cell dishes were collected at the indicated times, membranes were prepared and assayed for their capacity to bind [^{3} H]MK912 as described in the legend of Figure 2. Data represent the means $\pm$ s.e.mean from three experiments. (b) Semi-logarithmic plot of the kinetics of α_2 -adrenoceptor recovery. The kinetics of receptor recovery in control (○) or in UK14304-treated cells (●) were plotted according to the equation $\ln [R_{ss}] / [R_{ss} - R_t] = kt$, in which R_{ss} is the number of receptors at steady state and R_t the number of receptors at discrete time. The slopes of the plots which represent the rate constant for receptor degradation (k) were determined by linear regression analysis.

as SK-N-MC or Y79 (Schaak *et al.*, 1997a; Gleason & Hieble, 1992), should bring a beginning of answer to this latter point. Conversely to that observed for desensitization, the characteristics of receptor down-regulation in HepG2 are fairly similar to those in OK (Pleus *et al.*, 1993). The maximal extent of receptor decrease after 24 h exposure to 10 μ M UK14304 in

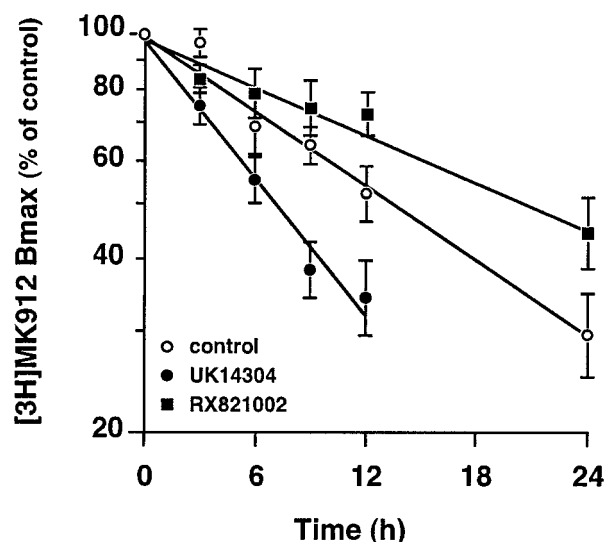


Figure 7 Estimation of the rate of receptor degradation in the presence of cycloheximide. HepG2 cells were pre-incubated for 12 h in the presence of 10 μ M UK14304 (●), 100 μ M RX821002 (■) or not (○). Cycloheximide (50 μ g ml $^{-1}$) was then added into the culture medium and cells were harvested at the indicated times. Membranes were prepared and assayed for their capacity to bind [^{3} H]MK912 as described in the legend of Figure 2. Binding site number is expressed as per cent of the corresponding control (untreated with cycloheximide). Each data point represents the mean $\pm$ s.e.mean from three experiments.

HepG2 cells (55%) is very similar to that provoked by 30 μ M noradrenaline in OK cells (58%). The mechanisms accounting for this phenomenon were not investigated in OK cells. According to the present study, down-regulation of α_2C -adrenoceptor was not due to a reduction of α_2C4 mRNA levels nor to a diminution of receptor synthesis, but to an approximately 2 fold increase in its rate of degradation. Such a process was recently demonstrated to also account for down-regulation of human α_2A - and rat α_2B -adrenoceptor in HT29 and transfected-CHO cells (Heck & Bylund, 1997). Our work did not investigate the mechanisms leading to accelerated degradation of α_2C -adrenoceptor. However, increased degradation is not the consequence of change in cyclic AMP level because α_2 -agonists have no significant effect on basal cyclic AMP concentration in HepG2. Moreover, down-regulation is abolished when HepG2 are exposed to concanavalin A or when 0.4 M sucrose is added to the culture medium indicating that internalization is a prerequisite to α_2C -adrenoceptor degradation. The receptor being not 'phosphorylable', it is likely that the internalization process is independent of β -arrestin. As for β_2 -adrenoceptor (Zhang *et al.*, 1996), further studies, including transfection of HepG2 cells with dominant negative forms of this protein and of dynamin, should afford more information on the exact route of α_2C -adrenoceptor processing.

Interestingly, exposure of HepG2 cells to RX821002 or yohimbine provoked a significant rise in the receptor expression which is primarily the consequence of an attenuated rate of receptor degradation. Our experiments being carried out in serum-free conditions and serum deprivation having no consequence on receptor density, there is no concern these effects might reflect blockade of the action of catecholamines or catecholamine metabolites present in FCS. Such a possibility is also eliminated by the observation that phentolamine, which is able to block the receptor down-

Table 1 Effects of concanavalin A and sucrose on receptor down-regulation

	$[^3\text{H}]\text{MK912 } B_{\text{max}}$ (fmol mg ⁻¹ prot.) vehicle	+ 10 μM UK14304	Down-regulation (%)
Control	46.1 $\pm$ 7	26.7 $\pm$ 1.2	40 $\pm$ 4
Conc. A	33.6 $\pm$ 3.4	30.8 $\pm$ 2.3	4 $\pm$ 3
Sucrose	41.8 $\pm$ 5.3	46.9 $\pm$ 5.3	no

HepG2 cells were exposed for 6 h to 10 μM UK14304 in DMEM containing concanavalin A 0.25 mg ml⁻¹ (conc. A) or sucrose 0.4 M (sucrose). Membranes were prepared and assayed for their capacity to bind [^3H]MK912. The maximum number of binding sites expressed as femtomoles mg⁻¹ of proteins was calculated by analysis of the radioligand saturation isotherms according to a one-component model. Reported values are the means $\pm$ s.e.mean from four determinations. Extent of receptor down-regulation promoted by UK14304 is expressed in per cent.

regulation induced by UK14304, does not affect by itself receptor number and thus behaves as a neutral antagonist on this system. Up-regulation by $\alpha 2$ -antagonist has never been reported for any of the $\alpha 2$ -adrenoceptor subtypes; however antagonist-induced augmentation of receptor expression was already observed for other GPCRs including $\alpha 1$ - and β -adrenoceptors. Increase in receptor expression following exposure to antagonists was for instance observed in NG108-15 cells and in Rat1 fibroblasts permanently transfected with the wild-type human $\beta 2$ -adrenoceptor (Lee *et al.*, 1997) and with a constitutively active mutant (CAM) of the hamster $\alpha 1\text{B}$ -adrenoceptor (MacEwan & Miligan, 1996). Because solely β -antagonists with inverse agonist property are efficient on the wild-type $\beta 2$ -adrenoceptor whereas all $\alpha 1$ -antagonists are efficient on the CAM $\alpha 1\text{B}$ -adrenoceptor, it is generally believed that up-regulation of GPCR by antagonists is the special feature of inverse-agonists acting on receptors displaying agonist-independent coupling to G-protein (Milligan & Bond,

1997). Whether antagonist-induced up-regulation of HepG2 $\alpha 2\text{C}$ -adrenoceptor was dependent of its coupling to G_i-proteins was not assayed in the present work, but future studies should address this issue.

Altogether the data provided in this manuscript show that human $\alpha 2\text{C}$ -adrenoceptor is refractory to rapid desensitization but is down-regulated in response to long-term exposure to $\alpha 2$ -agonists. They furthermore demonstrated that certain $\alpha 2$ -antagonists behave as inverse agonist on the HepG2 model. To our knowledge this report is the first evidence of inverse efficacy of antagonists on a cell expressing physiological level of a wild-type $\alpha 2$ -adrenoceptor.

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Proliferative effects of cholecystokinin in GH₃ pituitary cells mediated by CCK₂ receptors and potentiated by insulin

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1 Proliferative effects of CCK peptides have been examined in rat anterior pituitary GH₃ cells, which express CCK₂ receptors.

2 CCK-8s, gastrin(1-17) and its glycine-extended precursor G(1-17)-Gly, previously reported to cause proliferation *via* putative novel sites on AR4-2J and Swiss 3T3 cells, elicited significant dose dependent increases of similar magnitude in [³H]thymidine incorporation over 3 days in serum-free medium of 39 ± 10% (*P* < 0.01, *n* = 20), 37 ± 8% (*P* < 0.01, *n* = 27) and 41 ± 6% (*P* < 0.01, *n* = 36) respectively.

3 CCK-8s and gastrin potentially stimulated mitogenesis (EC₅₀ values 0.12 nM and 3.0 nM respectively), whilst G-Gly displayed similar efficacy but markedly lower potency. L-365,260 consistently blocked each peptide. The CCK₂ receptor affinity of G-Gly in GH₃ cells was 1.09 μM (1.01;1.17, *n* = 6) and 5.53 μM (3.71;5.99, *n* = 4) in guinea-pig cortex.

4 1 μM G-Gly weakly stimulated Ca²⁺ increase, eliciting a 104 ± 21% increase over basal Ca²⁺ levels, and was blocked by 1 μM L-365,260 whilst CCK-8s (100 nM) produced a much larger Ca²⁺ response (331 ± 14%).

5 Insulin dose dependently enhanced proliferative effects of CCK-8s with a maximal leftwards shift of the CCK-8s curve at 100 ng ml⁻¹ (17 nM) (EC₅₀ decreased 500 fold, from 0.1 nM to 0.2 pM; *P* < 0.0001). 10 μg ml⁻¹ insulin was supramaximal reducing the EC₅₀ to 5 pM (*P* = 0.027) whilst 1 ng ml⁻¹ insulin was ineffective. Insulin weakly displaced [¹²⁵I]BHCCCK binding to GH₃ CCK₂ receptors (IC₅₀ 3.6 μM).

6 Results are consistent with mediation of G-Gly effects *via* CCK₂ receptors in GH₃ cells and reinforce the role of CCK₂ receptors in control of cell growth. Effects of insulin in enhancing CCK proliferative potency may suggest that CCK₂ and insulin receptors converge on common intracellular targets and indicates that mitogenic stimuli are influenced by the combination of extracellular factors present.

Keywords: CCK; glycine-extended gastrin; proliferation; CCK₂ receptor; insulin; mitogenic pathways; L-365,260

Abbreviations: [¹²⁵I]BHCCCK, [¹²⁵I]Bolton Hunter labelled sulphated CCK-8; CCK-8s, sulphated CCK octapeptide; EGTA, ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid; G-Gly, glycine-extended gastrin 1-17; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid; SDS, sodium dodecyl sulphate

Introduction

Despite extensive hybridization screening in multiple species and tissues under both high and low stringency conditions, molecular studies identify only two members of the cholecystokinin receptor family, CCK₁ (formerly CCK-A) and CCK₂ (formerly CCK-B/gastrin) receptors (classification decreed by the IUPHAR Committee on Receptor Nomenclature and Drug Classification XIth International Congress of Pharmacology, Munich, July 1998), although these studies do not rule out the existence of less closely related members (Wank *et al.*, 1994a). Suggestions of further receptor heterogeneity have arisen from a number of pharmacological and biochemical studies in the literature. Both CCK₁ and CCK₂ receptors can occur in truncated form (Poirot *et al.*, 1994; Miyake, 1995) and the human CCK₂ receptor gene can be alternatively spliced in exon 4 to produce two isoforms (Song *et al.*, 1993) differing by five amino acids in the third cytoplasmic loop. Despite similar signal transduction and trophic responses to CCK-8s and gastrin (Ito *et al.*, 1994) the two isoforms exhibit subtle differences in antagonist binding

affinity and agonist efficacy for stimulating phospholipase C (Wank *et al.*, 1994b).

Differences in species and tissue-specific posttranslational processing, receptor coupling, different affinity states, alternative splicing and the occurrence of truncated isoforms may all contribute to this apparent heterogeneity. The immediate precursor of bioactive amidated gastrin is a glycine-extended form (G-Gly) (Daugherty & Yamada, 1989), which is present at high levels in some malignant tissues such as Zollinger-Ellison tumours and colon cancers, is co-secreted with gastrin from antral G cells, and may achieve equivalent plasma concentrations to amidated gastrin (Sawada & Dickinson, 1997). Potent proliferative effects were recently reported with G-Gly which were mediated *via* a putative novel site on AR4-2J cells, occurring maximally at 0.1 nM, a concentration four orders of magnitude below its CCK₂ affinity, which were not blocked by the antagonist L-365,260 (Seva *et al.*, 1994; Todisco *et al.*, 1995). Novel gastrin receptors were also proposed to mediate mitogenic effects of gastrin and G-Gly in CCK receptor-lacking Swiss 3T3 cells (Singh *et al.*, 1995).

Growth-promoting effects of brain and gut peptides, CCK and gastrin, have been widely documented in a variety of tissues and cells of peripheral origin, including gastric,

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pancreatic and lung cancer cell lines (Crawley & Corwin, 1994; Taniguchi *et al.*, 1994). There is less information, however, regarding proliferative effects in neuronal or pituitary tissue. We therefore examined the effects of these peptides on mitogenesis in the GH₃ pituitary tumour cell line, previously shown to express a single population of functionally coupled CCK₂ receptors which stimulate intracellular Ca²⁺ mobilization and polyphosphoinositide turnover (Kuwahara *et al.*, 1993; Smith *et al.*, 1994).

Methods

Materials

The GH₃ cell line was obtained from the American Type Culture Collection (ATCC). Human gastrin 1-17, C-terminally extended with glycine (G-Gly), was custom synthesized by Genosys (Cambridge, U.K.) and normal amidated gastrin was also obtained from this source. Sulphated CCK octapeptide (CCK-8s) was obtained from Bachem. All peptides were made up as 1 mM stocks in 0.1% NH₄OH. [¹²⁵I]Bolton Hunter-labelled CCK-8s (BHCKK) (specific activity 2000 Ci mmol⁻¹) was supplied by Amersham (Buckinghamshire, U.K.) and [methyl-³H]thymidine (specific activity 83 Ci mmol⁻¹) was supplied by NEN (Hertfordshire, U.K.). Fura-2 acetoxymethyl ester (fura-2-AM) from Calbiochem was dissolved in DMSO as a 1 mM stock. Rat interleukin-2, bovine insulin, lavendustin A and wortmannin were all obtained from Sigma (Poole, Dorset, U.K.).

[¹²⁵I]BHCKK displacement

Radioligand binding was performed as previously described in whole cell suspensions and guinea-pig cortical and rat pancreatic membranes (Smith *et al.*, 1994). Displacement of 50 pM [¹²⁵I]BHCKK binding was measured for 90 min at room temperature in 20 mM HEPES buffer at pH 6.5 containing 150 mM NaCl, 5 mM MgCl₂, 1 mM EGTA and 0.025% bacitracin. Specific binding was defined with 1 μM CCK-8s. Results were expressed as percentage inhibition of specific [¹²⁵I]BHCKK binding and curves were fitted by non-linear least squares regression analysis using RS1 (BBN Research Systems, Cambridge, MA, U.S.A.).

Intracellular calcium increase

Intracellular calcium increase was measured at 37°C in GH₃ cells loaded with 5 μM fura-2 as previously described (Smith *et al.*, 1994). Measurements were made in 20 mM HEPES/Krebs buffer at 37°C on a Perkin Elmer LS50 fluorimeter. Intracellular calcium concentrations were calculated using the Perkin Elmer Intracellular Biochemistry software package after calibration of the sample to determine R_{min} and R_{max}.

Cell culture and proliferation measurements

GH₃ cells between passage 13 and 49 were seeded at between 6 and 9 × 10⁴ cells ml⁻¹ into 24-well plates coated with poly-L-lysine and allowed to attach for a period of 24 h in normal serum-containing medium (1:1 DMEM/Hams F10 + 15% horse serum). Initially, a range of seeding densities were examined and the degree of confluence assessed after 24 h in serum-containing medium. An initial confluence of approximately 40% before the change to serum-free conditions was found to be optimal for proliferation measurements. This

allowed room for proliferative increases without appreciable cell loss through upwards growth and cells budding off into the medium, as the density increased towards confluence. A change to serum-free conditions was achieved over 24 h with two changes of medium and peptides added at the end of this period in fresh serum-free medium for a further 72 h. Antagonists were added 1 h prior to agonists. 1 μCi [³H]thymidine was added to each well (final volume 1 ml per well) for the last 24 h of cell growth. At the end of the 72 h treatment, medium containing compounds was aspirated and the cells were washed before lysing with 0.5 ml of 1% SDS. Thymidine uptake was then determined by scintillation counting. Manual cell counting using Trypan blue exclusion to assess cell viability was performed in parallel to validate the thymidine uptake results.

Data analysis

Proliferative responses were assessed by performing analysis of variance followed by *post hoc* testing to determine significance. The maximal observed effects of each peptide, and of L-365,260 alone on basal cell growth, were tested to assess the degree of significance from control by one-way analysis of variance followed by Dunnett's Multiple Comparison Test using Prism version 2.0b (Graphpad Software Inc.). [³H]thymidine uptake results and manual cell counting were both assessed in this manner. Full peptide concentration response curves in the presence and absence of 1 μM L-365,260 were analysed using a two-way analysis of variance, so that the effects of the different peptides, the antagonist L-365,260, and any interactions could be assessed together. The analysis was performed using Genstat 5 release 4.1 (Numerical Algorithms Group, Oxford) and the responses were only compared where the same concentrations of peptide were used to ensure that the analysis was valid. The significance of shift of the CCK concentration response curve in the presence of each dose of insulin was assessed by performing unpaired two-tail *t*-tests on the log EC₅₀ ± s.e.mean of log EC₅₀ in the presence and absence of insulin using Prism.

Results

Binding studies

[¹²⁵I]BHCKK-8s bound dose dependently and saturably to GH₃ cells with a dissociation constant of 85 ± 4 pM and a maximal binding capacity of 23 ± 6 fmol mg⁻¹ protein (*n* = 3). These studies were conducted on cells of passage numbers between 5 and 20. Corresponding measurements in guinea-pig cortex (from 450–550 g animals) yielded a dissociation constant of 54 ± 1 pM and B_{max} of 91 ± 33 fmol mg⁻¹ protein (*n* = 3). However, a final saturation study with [¹²⁵I]BHCKK on cells of passage 49 at the end of the series of proliferation experiments indicated that receptor expression had increased with passage number and B_{max} was now 152 fmol mg⁻¹ protein.

Compounds used in proliferation studies were tested to determine their affinity at CCK₂ receptors on GH₃ cells, and compared to their affinity at guinea-pig cortical CCK₂ receptors and rat pancreatic CCK₁ receptors. Results are shown in Table 1 and mean curves for displacement of [¹²⁵I]BHCKK binding from GH₃ cells by CCK agonists and antagonists are illustrated in Figure 1.

Binding studies were also performed to see whether insulin had any affinity for CCK₂ receptors. Insulin weakly displaced

[¹²⁵I]BHCCCK binding from GH₃ cells with an IC₅₀ of 3.63 μ M (2.97;5.98, *n*=3). The displacement curves all had low Hill slopes, with the shape of the curve suggestive of two components.

Intracellular calcium increase

Stimulation of intracellular calcium increase was measured as an index of agonist activity at GH₃ CCK₂ receptors for CCK-8s and G-Gly (Figure 2). Resting intracellular calcium concentration at the beginning of experiments was 148 $\pm$ 14 nM (*n*=13). 100 nM CCK-8s produced a 331 $\pm$ 14% increase (*n*=3; *P*<0.0001) in intracellular calcium concentration (EC₅₀ determined to be 5.2 nM in previous studies, Smith *et al.*,

1994). G-Gly weakly stimulated intracellular calcium increase with the maximum concentration of G-Gly tested (1 μ M) producing a 104 $\pm$ 21% (*n*=4; *P*=0.0004) increase in prestimulated calcium levels which was inhibited by a 3 min preincubation with 1 μ M L-365,260 (26 $\pm$ 2%). At the lower dose of 100 nM, G-Gly elicited a much reduced calcium response, representing a 23 $\pm$ 5% increase over basal levels (*n*=3).

Cell proliferation

CCK peptides elicited small but significant dose dependent increases in GH₃ cell growth after 3 day periods in the absence of serum as determined by [³H]thymidine incorporation.

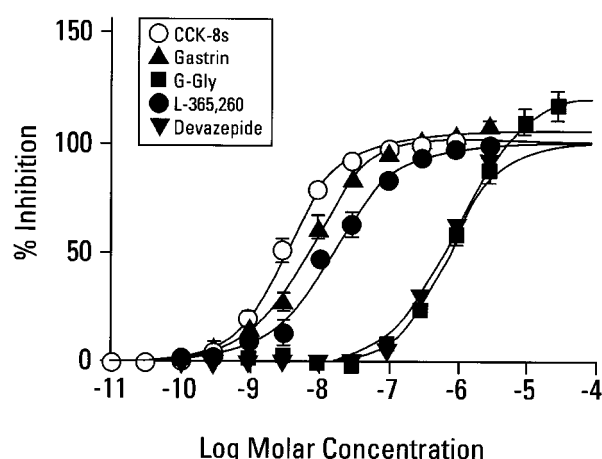


Figure 1 Mean displacement curves are shown for inhibition of 50 pM [¹²⁵I]BHCCCK binding to whole GH₃ cells for the peptides CCK-8s, gastrin and G-Gly, and the nonpeptide CCK receptor antagonists L-365,260 and devazepide.

Table 1 Binding profile of CCK receptor agonists and antagonists used in proliferation studies

Compound	GH ₃ cells CCK ₂ IC ₅₀ (nM)	Brain CCK ₂ IC ₅₀ (nM)	Pancreas CCK ₁ IC ₅₀ (nM)
CCK-8s	2.6 (1.9;3.6)	0.34 (0.3;0.39)	0.18 (0.12;0.28)
Gastrin	8.1 (7.2;9.0)	15 (12;17)	1270 (1160;1390)
G-Gly	1090 (1010;1170)	5530 (3710;5990)	71600 (62400;82200)
L-365,260	25 (17;37)	8.5 (6.5;11.2)	740 (585;925)
Devazepide	780 (630;970)	150 (120;170)	0.10 (0.05;0.23)
Insulin	3630 (2970;5980)	n.d.	n.d.

Affinities for displacement of agonist [¹²⁵I]BHCCCK binding are shown in nM for cortical and pancreatic membranes and GH₃ whole cell suspensions. Results are expressed as the geometric mean of the IC₅₀ from at least four independent experiments, with low and high errors of the mean in parentheses. n.d., not determined.

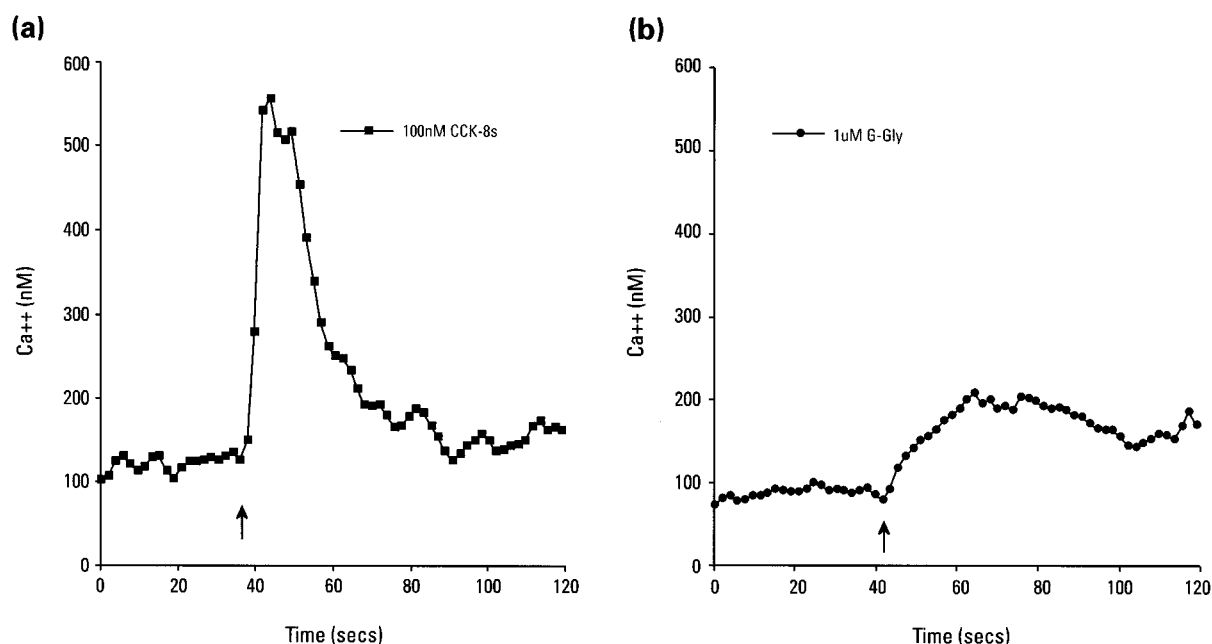


Figure 2 Intracellular calcium increase was stimulated in GH₃ cells in response to both GCK-8s (a) and G-Gly (b), although the size of the response differed between the two agonists despite the fact that the same concentrations elicited equivalent proliferative responses. Arrows denote point of agonist addition.

CCK-8s, gastrin(1-17) and G(1-17)-Gly all produced similar maximal increases over control growth of $39 \pm 10\%$ ($P < 0.01$, $n = 20$), $37 \pm 8\%$ ($P < 0.01$, $n = 27$) and $41 \pm 6\%$ ($P < 0.01$, $n = 36$) respectively (Figure 3) (P values denoting significance of increase over control cell growth determined by Dunnett's Multiple Comparison Test following one-way analysis of variance). CCK-8s responses were maximal at 100 nM with an EC_{50} of 0.12 nM (0.06;0.23, $n = 9$) (low and high errors of geometric mean) (Figure 3a). Gastrin also potently and dose dependently stimulated GH₃ cell proliferation with an EC_{50} of 3.0 nM (1.8;5.3, $n = 7$) and maximal effects at 1 μ M (Figure 3b). G-Gly dose dependently stimulated GH₃ cell growth with

similar efficacy to CCK-8s and amidated gastrin ($41 \pm 6\%$, $n = 36$; $P < 0.001$) but markedly lower potency (Figure 3c). The maximum increase in cell proliferation was observed at the highest concentration of G-Gly tested (1 μ M), slight stimulation occurred at 100 nM ($24 \pm 11\%$ increase, $n = 7$) and no effects were observable at lower doses. However, limited amounts of G-Gly peptide prevented testing at higher concentrations, so it is not clear how close the curve was to attaining its plateau. In a number of experiments, medium was changed and CCK-8s was refreshed daily to counter possible peptide degradation. However, proliferative effects were no larger than those seen with the standard 3-day treatment. In

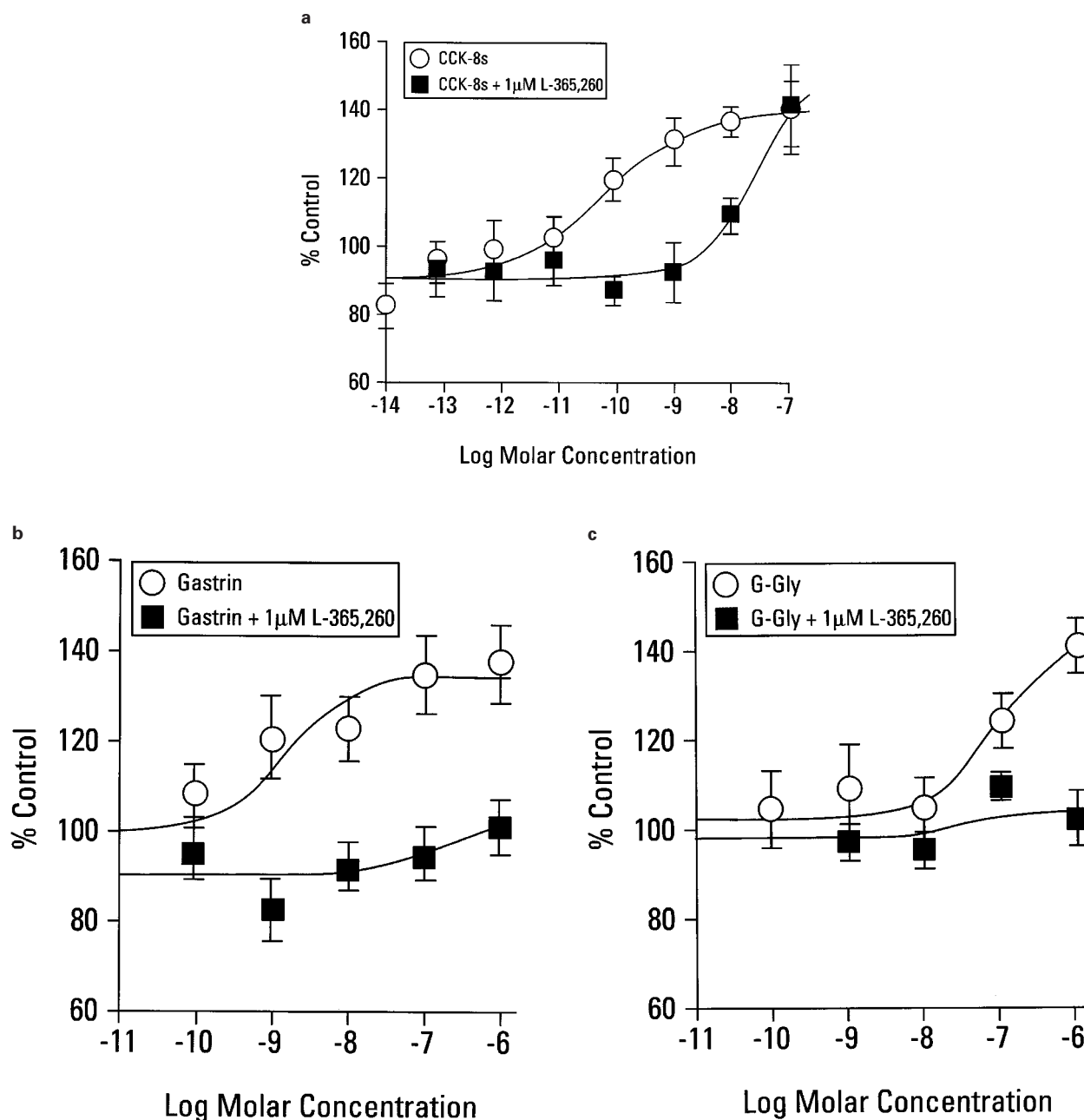


Figure 3 Effects of three CCK peptide agonists on GH₃ cell proliferation over 3 day periods. (a) CCK-8s potently and dose dependently increased cell growth with EC_{50} of 0.12 nM and maximal effects between 10 and 100 nM. Responses were specifically blocked in the presence of 1 μ M of the competitive CCK₂ receptor antagonist L-365,260. (b) gastrin1-17 also stimulated proliferation in a concentration dependent manner but was an order of magnitude less potent than CCK-8s, with an EC_{50} of 3.0 nM and maximal effects between 100 nM and 1 μ M. Responses to gastrin were specifically inhibited by 1 μ M L-365,260. (c) G-Gly dose dependently stimulated GH₃ cell growth with similar maximal efficacy to CCK-8s and amidated gastrin but markedly lower potency. Maximum proliferative effects were observed at the highest tested dose (1 μ M) and slight stimulation at 100 nM, with no effects at lower concentrations. Proliferative effects of G-Gly were antagonized by 1 μ M L-365,260.

control wells, exposed only to serum-free medium, daily replacement of medium resulted in a reduction in control counts from 2371 ± 133 – 2030 ± 76 c.p.m. ($n=4$), a decrease of 14%.

Interleukin-2 is a potent mitogen for GH₃ cells (Arzt *et al.*, 1993), and activates various non-receptor tyrosine kinases such as p56^{lck} and other Src family kinases, Jak and Syk kinases, resulting in induction of *c-fos/c-jun*, *c-myc* and *bcl-2* genes (Miyazaki & Taniguchi, 1996). Interleukin-2 was routinely included as a positive control and elicited a $252 \pm 33\%$ ($n=18$) increase in cell proliferation at a concentration of 20 units ml⁻¹.

Antagonism of cell proliferation

The CCK curve was shifted to the right in the presence of 1 μ M of the competitive CCK₂ receptor antagonist L-365,260, with the EC₅₀ for the CCK-8s-stimulated response decreasing over 200 fold from 0.12–25 nM (Figure 3a). The mean curve for CCK alone was more shallow in character with a Hill slope of 0.64. A CCK₁ receptor-selective dose of the antagonist devazepide (10 nM) was ineffective against stimulation of GH₃ cell proliferation by 10 nM CCK-8s ($35 \pm 10\%$ increase over control, $n=4$). 10 nM devazepide is a concentration that would be expected to fully block CCK₁ receptors but to have no antagonistic effects at CCK₂ sites (see Figure 1 and Table 1). Responses to gastrin were also blocked by 1 μ M L-365,260 (Figure 3b), a concentration of the antagonist which fully inhibits binding of [¹²⁵I]BHCKK to CCK₂ receptors on these cells (Figure 1). L-365,260 also antagonised the proliferative effects of G-Gly with the response to 1 μ M G-Gly being fully blocked in the presence of the antagonist from 44 ± 14 – $6 \pm 7\%$ increase over control ($n=4$) (Figure 3c). Two-way analysis of variance for full peptide concentration response curves in the presence and absence of antagonist showed a statistically significant difference for each peptide over control ($P<0.001$). L-365,260 consistently blocked peptide responses ($P<0.001$) and antagonism did not differ with the nature of the peptide.

When L-365,260 was examined alone in the absence of CCK, a small reduction in control responses was observed at 1 μ M from 100 ± 1 – $89 \pm 4\%$ but in parallel control cultures treated with the solvent, 0.1% DMSO, there was no significant effect. However, under stringent analysis (one-way analysis of variance followed by Dunnett's Multiple Comparison Test) this effect was not significant ($P>0.05$).

Experiments where cell counting was performed in parallel to the thymidine uptake protocol generated data consistent with that described above. Cell counts for control wells, exposed only to serum-free medium, were $9.7 \pm 0.9 \times 10^4$ cells well⁻¹ ($n=15$) rising to $14.4 \pm 0.9 \times 10^4$ cells well⁻¹ ($n=17$) in the presence of 10 nM CCK-8s, an increase of 49% with respect to control ($P<0.01$). When 10 nM CCK-8s was tested in the presence of 1 μ M L-365,260, proliferation was significantly blocked at $7.1 \pm 0.6 \times 10^4$ cells well⁻¹ ($n=3$) ($P<0.01$). Although the antagonist appeared to inhibit CCK-8s to below control levels, this was not a statistically significant difference ($P>0.05$; one-way analysis of variance followed by Tukey's Multiple Comparison Test).

Effects of insulin on control and CCK-stimulated GH₃ cell growth

The effect of insulin on GH₃ cell growth was examined using three different insulin concentrations: 1 ng ml⁻¹ (0.17 nM), 100 ng ml⁻¹ (17 nM) and 10 μ g ml⁻¹ (1.7 μ M). In the absence of added CCK, insulin elicited a small dose dependent effect on

cell proliferation of $89 \pm 5\%$ ($n=6$), $120 \pm 11\%$ ($n=9$), and $148 \pm 15\%$ ($n=13$) (expressed as per cent of serum-free control), at 1 ng, 100 ng and 10 μ g ml⁻¹ respectively. However, when the response to CCK-8s in combination with insulin was measured, some interesting differences became apparent compared to the effects of CCK alone. The dose response curve to CCK-8s was maintained in the presence of insulin but the maximal effects of CCK-8s, when expressed relative to normal control, were enhanced in the presence of 100 ng ml⁻¹ and 10 μ g ml⁻¹ insulin (Figure 4b,c). In contrast, 1 ng ml⁻¹ insulin had no significant effect on the response to CCK (Figure 4a).

In addition to this increase in the apparent maximum, the CCK-8s concentration response curve was shifted to the left, appearing more potent in the presence of 100 ng ml⁻¹ and 10 μ g ml⁻¹ insulin. CCK concentration response curves in the presence and absence of each dose of insulin are illustrated in Figure 4a–c, where all data are expressed as a percentage of normal serum-free control. The degree of shift of the CCK-8s dose response curve was a function of the concentration of insulin. No significant shift was observed with the lowest insulin concentration of 1 ng ml⁻¹ (Figure 4a); the EC₅₀ for CCK-8s alone was 0.1 nM and in the presence of insulin 0.05 nM (log EC₅₀ $\pm$ s.e.mean values were respectively -10.00 ± 0.12 ($n=7$) and -10.27 ± 0.12 ($n=7$); $P=0.136$). At 100 ng ml⁻¹ insulin the shift was very pronounced with the EC₅₀ decreasing over 500 fold from 0.1 nM to 0.2 pM (Figure 4b); the curve attained a maximum between 0.1 and 1 nM and at higher concentrations of CCK the response then progressively declined (log EC₅₀ $\pm$ s.e.mean values respectively -10.00 ± 0.12 ($n=7$) and -12.71 ± 0.31 ($n=6$); $P<0.0001$). The highest dose of insulin tested (10 μ g ml⁻¹) appeared to be supramaximal, with the shift of the CCK curve attenuated in comparison to 100 ng ml⁻¹, decreasing only 20 fold from 0.1 nM to 5 pM (Figure 4c) (log EC₅₀ $\pm$ s.e.mean values respectively -10.00 ± 0.12 ($n=7$) and -11.28 ± 0.45 ($n=9$); $P=0.027$). The apparent leftwards shift was not merely a result of the CCK curve moving upwards due to the raised baseline in the presence of insulin because when the (CCK + insulin) data were corrected for the effects of insulin alone by expressing them relative to controls with insulin rather than normal control, the CCK curve was still shifted to the left with 100 ng ml⁻¹ and 10 μ g ml⁻¹ insulin.

Again, cell counting was used to confirm that the increased proliferation observed with insulin alone was due to an increase in cell number and not merely in cell mass. In the presence of the maximal concentration of insulin (10 μ g ml⁻¹), the number of cells increased from 9.7 ± 0.9 ($n=15$) to 13.8 ± 1.1 ($n=13$) $\times 10^4$ cells well⁻¹, 143% of control ($P<0.01$). Some morphological changes were noted in insulin-treated cells, with the normally rounded cells becoming more angular in shape.

Discussion

This study demonstrates that CCK, gastrin, and G-Gly act as proliferative agents for pituitary GH₃ cells. The proliferative potencies of all three peptides were consistent with their CCK₂ receptor binding affinities, with CCK-8s being the most potent agonist, and each peptide response was blocked by the CCK₂ receptor antagonist L-365,260 indicating that the effects were mediated *via* G-protein coupled CCK₂ receptors. In contrast to studies in AR4-2J cells and Swiss 3T3 fibroblasts (Seva *et al.*, 1994; Todisco *et al.*, 1995; Singh *et al.*, 1995), G-Gly only promoted GH₃ cell proliferation at micromolar concentrations

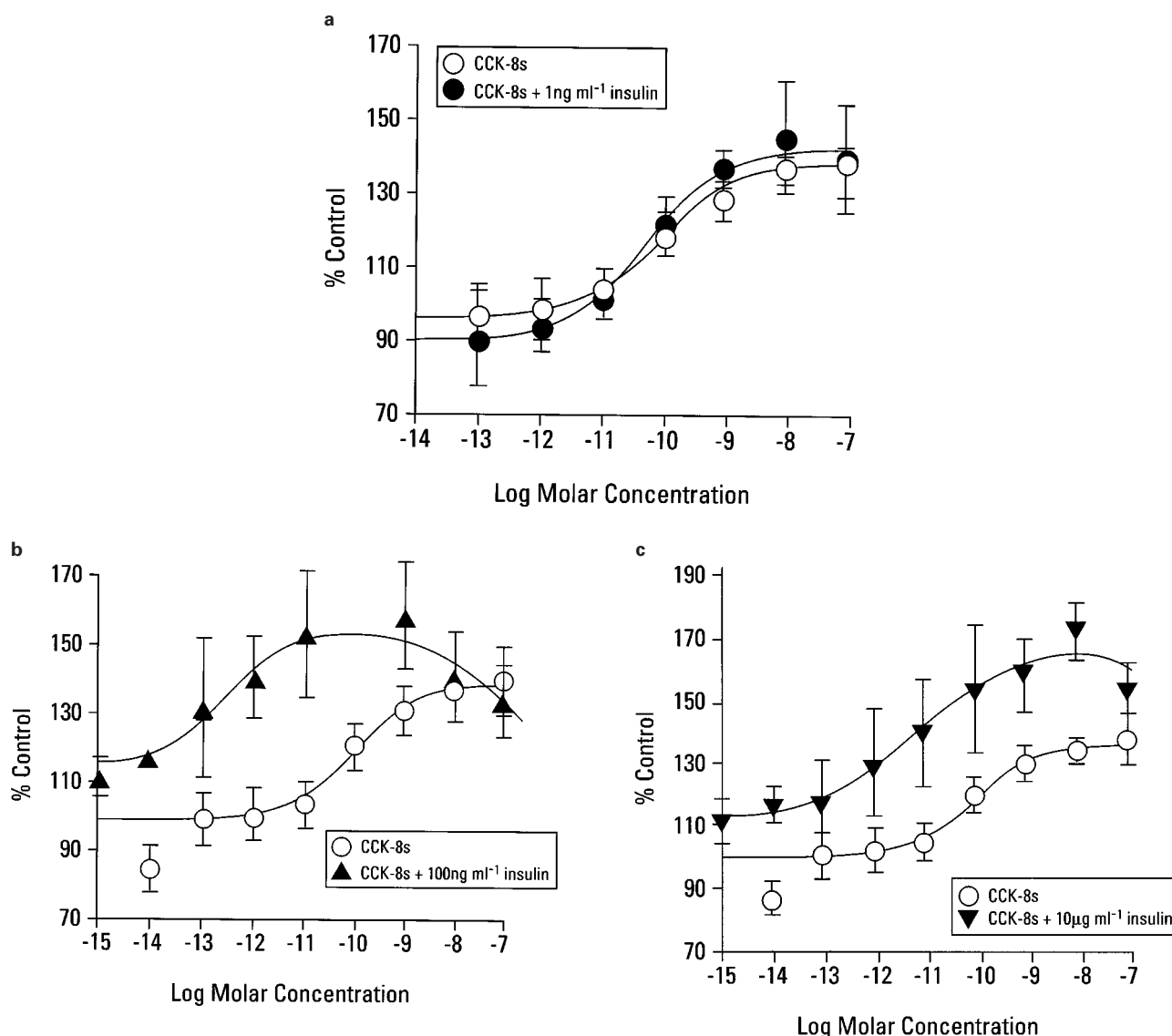


Figure 4 The effect of insulin on the proliferative concentration response curve to CCK-8s was examined using three different insulin concentrations. All data points were expressed as a percentage of normal serum-free control and CCK concentration response curves are shown in the presence and absence of each dose of insulin. (a) no significant shift was observed with 1 ng ml⁻¹ insulin (0.17 nM); (b) the effects of CCK were maximally enhanced in the presence of 100 ng ml⁻¹ insulin (17 nM) with the CCK curve significantly shifted to the left; and (c) 10 µg ml⁻¹ insulin (1.7 µM) also produced a leftwards shift of the CCK curve but the shift was less pronounced than that seen with 100 ng ml⁻¹.

compared to nanomolar effects of amidated gastrin and CCK-8s. However, these concentrations are consistent in its affinity for CCK₂ receptors on GH₃ cells.

Both CCK-8s and G-Gly stimulated intracellular calcium increase. This is in contrast to reports by other authors that G-Gly had no effect on intracellular calcium (Todisco *et al.*, 1995; Sawada *et al.*, 1997). However, whilst CCK-8s potentially stimulated intracellular calcium increase with an EC₅₀ of 5.2 nM, effects of G-Gly were observed only at micromolar concentrations. Thus the concentrations at which G-Gly promoted proliferation correlated well with those at which it stimulated intracellular calcium increase. The efficacy of all three peptides in promoting cell proliferation was similar, with the highest concentration of G-Gly tested producing similar maximal effects to CCK-8s and gastrin. Limited peptide prevented testing G-Gly above 1 µM and dose response curves for calcium increase and cell proliferation could not be extended in the present study to determine their absolute maxima. However, it is interesting to note that 1 µM G-Gly,

which elicited a significantly smaller calcium response approximately one third the size of CCK-8s, produced proliferative increases of similar magnitude to CCK-8s and gastrin. Perhaps only partial occupancy or minimal coupling with an agonist such as G-Gly, together with intracellular amplification, is sufficient to trigger mitogenesis.

L-365,260 produced a small depression of control proliferation responses when applied alone and there was a small decrease in control counts when serum-free medium was replaced daily, although this could also have resulted from cell loss during medium changes. Xu *et al.* (1996) recently showed that GH₃ cells produce an endogenous CCK peptide which influences growth, measuring immunoreactive CCK/gastrin of 0.25 fmol 10⁵ cells⁻¹ well⁻¹ in GH₃ cell extract and 0.59 fmol well⁻¹ released into serum-free medium over 3 days.

Time-courses chosen for thymidine experiments are dictated by cell growth rate and GH₃ cells multiply up to 4 fold over 7 days in full serum (ATCC catalogue). Therefore the test growth period of 3 days was approximately equivalent to

normal cell doubling time. It was difficult to examine longer growth periods because the cells did not tolerate serum-free medium well for extended periods. Other authors, examining effects of hormones and growth factors, have used GH₃ cell growth periods of 24 h (Webster *et al.*, 1991; Constantinescu, 1993) and 72 h (Black *et al.*, 1990; Andries *et al.*, 1994). A few authors examined longer growth periods of 6 days (Yajima & Saito, 1984; Brunet *et al.*, 1981), and 7 and 14 days (Xu *et al.*, 1996). However, in the latter studies, authors used a hormone-supplemented medium to maintain cells and prevent detachment (Brunet *et al.*, 1981; Yajima & Saito, 1984; Andries *et al.*, 1994; Xu *et al.*, 1996; Murayama *et al.*, 1996), with 5–10 µg ml⁻¹ insulin and 5 µg ml⁻¹ transferrin frequently being used for this purpose. Where [³H]thymidine incorporation is used to assess cell growth, [³H]thymidine incubation periods vary with cell type, although Freshney (1994) recommends incubations limited to 24 h or less to avoid [³H]-induced DNA radiolysis. Two studies in GH₃ cells used 24 h 1 µCi ml⁻¹ [³H]thymidine incubations (Webster *et al.*, 1991; Constantinescu, 1993), whilst another used a 72 h incubation (Black *et al.*, 1990). Based on these considerations, a 24 h [³H]thymidine exposure was selected in the present study.

Interestingly, there was an interaction between CCK and insulin, with insulin dose dependently enhancing the proliferative potency of CCK-8s. The affinity of insulin for CCK₂ receptors was 3.6 µM but the concentration of insulin producing a maximal shift of the CCK dose response curve was 100 ng ml⁻¹ or 17 nM. It therefore seems unlikely that insulin was having a direct effect *via* CCK₂ receptors. Insulin can also act with subnanomolar affinity *via* insulin receptors and with 0.2 µM affinity *via* IGF-I receptors, both expressed by GH₃ cells (Rosenfeld *et al.*, 1985). This may suggest that signalling pathways stimulated by CCK and insulin converge on common intracellular targets. CCK₂ receptors transmit mitogenic signals *via* cytosolic tyrosine kinase cascades in mouse NIH3T3 fibroblasts, stimulating MAP kinase and focal adhesion kinase (Taniguchi *et al.*, 1994). In AR4-2J cells, 10 nM gastrin stimulated tyrosine phosphorylation of Shc adaptor protein isoforms which formed complexes with Grb-2 and Sos and activated MAP kinase (Seva *et al.*, 1996); gastrin also stimulated tyrosine phosphorylation of the cytoplasmic

docking protein insulin receptor substrate-1 (IRS-1) (Nishiya-ma & Wands, 1992) and its association with PI 3-kinase and Grb-2 (Kowalski-Chauvel *et al.*, 1996). Thus IRS-1 may represent a potential point of convergence for insulin and CCK₂ receptors. The specific tyrosine kinases involved in CCK₂-mediated IRS-1 phosphorylation remain to be identified but evidence suggests G protein receptors activate Src family kinases (Wan *et al.*, 1996). CCK also phosphorylates Shc isoforms at pancreatic CCK₁ receptors (Dabrowski, 1996).

An earlier study of effects of TRH on GH₃ cell proliferation reported a 16% increase in cell number in serum-free hormone-supplemented medium (10 µg ml⁻¹ insulin, 5 µg ml⁻¹ transferrin, 0.5 ng ml⁻¹ parathyroid hormone) compared to Ham's F12 alone in which there was no proliferation (Brunet *et al.*, 1981). Interestingly, transfer of cells into this supplemented medium caused increased responsiveness to TRH and a decrease in the number of high-affinity TRH binding sites, suggesting that the potentiation occurred at a step distal to the initial binding. A similar interaction was also reported in Swiss 3T3 cells between insulin and the G-protein linked receptor for bombesin. Insulin induced a marked reduction in the dependence of DNA synthesis on MAP kinase activity in bombesin-stimulated cells (Seufferlein *et al.*, 1996). Bombesin caused PKC-dependent activation of mitogen-activated protein kinase kinase-1 (MEK-1) and p42^{mapk} which were blocked by PD098059, a MEK-1 inhibitor. In the presence of insulin, which had no effect alone, bombesin promoted DNA synthesis in a PKC-independent manner even in the presence of PD098059.

Results presented here are consistent with mediation of proliferative effects of CCK peptides including G-Gly *via* CCK₂ receptors and suggest that GH₃ cells, in contrast to AR42J cells, do not contain any novel G-Gly-responsive site. These findings further reinforce the role of CCK₂ receptors in control of cell proliferation and may suggest that there are similarities in the intracellular targets stimulated by insulin and CCK₂ receptors. Finally, the type and size of stimulus required to initiate mitogenesis in a particular cell clearly depends on the combination of other factors which may be present in the extracellular environment.

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Taurine modulates I_{Kr} but not I_{Ks} in guinea-pig ventricular cardiomyocytes

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1 Effects of taurine on the delayed rectifier K^+ current (I_K) in isolated guinea-pig ventricular cardiomyocytes were examined at different intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), using whole-cell voltage and current clamp techniques. Experiments were performed at 36°C.

2 Addition of taurine (10–20 mM) decreased the action potential duration (APD) at pCa 8, but increased the APD at pCa 6. Taurine (20 mM) enhanced I_K at 70 mV by $22.4 \pm 3.1\%$ ($n=6$, $P<0.01$) at pCa 8, whereas taurine inhibited the I_K by $27.1 \pm 2.7\%$ ($n=6$, $P<0.01$) at pCa 6. These responses behaved in a concentration-dependent manner.

3 The I_K is composed of the rapid and slow components (I_{Kr} and I_{Ks}). When $[Ca^{2+}]_i$ was pCa 6, taurine at 20 mM reduced the tail current of I_{Kr} at 70 mV by $16.5 \pm 2.7\%$ ($n=5$, $P<0.05$) and that of I_{Ks} at 70 mV by $27.1 \pm 2.8\%$ ($n=6$, $P<0.01$). In contrast, at pCa 8, the tail currents of I_{Kr} and I_{Ks} at 70 mV were enhanced by $13.4 \pm 3.2\%$ ($n=7$, $P<0.05$) and by $22.4 \pm 3.1\%$ ($n=7$, $P<0.01$), respectively. The voltages of half-maximum activation ($V_{1/2}$) for I_{Kr} and I_{Ks} were not modified by taurine.

4 Addition of E-4031 (5 μ M) to taurine had a complete blockade of the tail current of I_{Kr} , but not I_{Ks} . The remained tail current (I_{Ks}) in the presence of E-4031 (5 μ M) was not affected by taurine (20 mM), but was blocked by 293B (30 μ M).

5 These results indicate that taurine modulates I_{Kr} but not I_{Ks} , depending on $[Ca^{2+}]_i$, resulting in regulation of the APD.

Keywords: Taurine; E-4031; 293B; action potential duration; delayed rectifier K^+ current; I_{Kr} ; I_{Ks} ; patch clamp; guinea-pig ventricular myocytes

Abbreviations: ANOVA, analysis of variance; APD, action potential duration; APD₇₅, 75% repolarization of action potential duration; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; I_K , delayed rectifier K^+ current; I_{K1} , inwardly rectifying K^+ current; I_{Kr} , rapid component of delayed rectifier K^+ current; I_{Ks} , slow component of delayed rectifier K^+ current; I_{Ca} , L-type Ca^{2+} current; I_{Na} , fast Na^+ current; S, slope factor; TTX, tetrodotoxin; $V_{1/2}$, voltage of half-maximum activation; V_m , membrane potential

Introduction

The outward K^+ currents play an important role in the regulation of repolarization of the action potential in cardiac cells. The blockade of K^+ channels prolongs the action potential duration (APD) but the stimulation shortens APD. Of the K^+ currents, the delayed rectifier K^+ current (I_K) mainly affects APD, regulated strongly by the intracellular or extracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$ or $[Ca^{2+}]_o$) (Meech, 1974; Isenberg, 1975) Satoh (1995) has recently reported that, in embryonic chick cardiomyocytes, taurine inhibited I_K at high $[Ca^{2+}]_i$, whereas taurine enhanced I_K at low $[Ca^{2+}]_i$.

Taurine (2-aminoethanesulphonic acid) is a sulphur-containing amino acid, and is abundant in cardiac myocardium and other tissues. During ischaemia and cardiac failure, taurine content in the heart decreases, with the depletion correlated with the degree of mechanical dysfunction (Lombardini, 1980; Kramer *et al.*, 1981). This suggests that taurine may be essential to maintain cardiac function. Thus, the actions of taurine on cardiomyocytes have been investigated (Franconi *et al.*, 1982; Failli *et al.*, 1992). Taurine inhibited the TTX-sensitive Na^+ current (I_{Na}) (Satoh, 1998a; Satoh & Sperelakis, 1992). Taurine also inhibited the L-type Ca^{2+} current (I_{Ca}) at pCa 7, whereas taurine enhances I_{Ca} at pCa 8 (or 10) (Satoh & Horie, 1998; Satoh & Sperelakis, 1993). From these effective actions, taurine appears to play an

important role for regulating $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$. If so, taurine may possess and exhibit cardioprotective activity related to its ability to alter Ca^{2+} movement (Huxtable & Sebring, 1983; Satoh & Sperelakis, 1998).

The aim of the present experiments was to examine the modulations of I_K by taurine, using adult cardiomyocytes at different $[Ca^{2+}]_i$ levels (pCa 6 and 8). Recently, the I_K has shown to be composed of rapidly and slowly activated currents (I_{Kr} and I_{Ks}) (Sanguinetti & Jurkiewicz, 1990). So, the effects of taurine on I_{Kr} and I_{Ks} and the modulation of the APD were also examined.

Methods

Cell preparation

Cells were prepared from tissue taken from the ventricle muscle of guinea-pig hearts, using methods similar to those described previously (Satoh, 1998b; Satoh & Sperelakis, 1992, 1993). Under anaesthesia with sodium pentobarbital (30 mg kg⁻¹, i.p.), the chest was opened and the aorta was cannulated *in situ*. The heart was dissected out and perfused with normal Tyrode solution on the Langendorff apparatus. After a washout of blood, the heart was perfused with Ca^{2+} -free Tyrode solution, and spontaneous beating ceased. Then, the perfusate was switched to low- Ca^{2+} (30–60 μ M) Tyrode

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solution containing 0.4 mg ml^{-1} collagenase (Type I, Sigma Chemical, St. Louis, MO, U.S.A.) for about 20 min. The heart was washed out by high- K^+ and low- Cl^- solution (KB solution), and was dissected with scissors. The temperature of all solutions was maintained at 36°C .

Whole-cell voltage-clamp experiments

Whole-cell voltage-clamp recordings were performed using an Axopatch patch-clamp amplifier (Axon Instruments, Burlingame, CA, U.S.A.) and standard techniques. Patch pipettes from borosilicate glass capillaries were fabricated using a two-stage puller, and had a resistance of $5\text{--}7 \text{ M}\Omega$ for whole-cell experiments. The series resistance error was less than $3\text{--}7 \text{ mV}$, and no compensation was used. The liquid junction potential between the pipette solution and the external solution (less than 10 mV) was corrected for all membrane potential recording. Experiments were carried out at temperature of 36°C . The data were stored and analysed on an IBM-AT microcomputer, using the PCLAMP analysis program (Axon Instruments). Current traces were filtered using a cut-off frequency of 1 KHz for plotting. All values are given as means $\pm$ s.e.mean. The differences of the mean values were analysed by Student's *t*-test and analysis of variance (ANOVA) for paired data, and a *P* value less than 0.05 was considered significant.

Experimental solutions

The composition of the modified Tyrode solution was (in mM): NaCl 137, KCl 5.4, CaCl_2 1.8, MgCl_2 1.0, NaH_2PO_4 0.3, glucose 5.0, and HEPES 5.0. The pH was adjusted to 7.4 with NaOH. To avoid the interference of other currents, $1 \mu\text{M}$ nifedipine was added to the external Tyrode solution to block the L-type Ca^{2+} current. Taurine (Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved to the desired concentrations directly in the bath solution, and the solution was superfused. Also, E-4301 (Eizai Pharmaceutical Co., Tokyo, Japan), a blocker of I_{Kr} , and 293B (Hoechst Pharmaceutical Co., Germany), a blocker of I_{Ks} , were used. The pipette solution (intracellular) contained (in mM): K-aspartate 110, KCl 20, MgCl_2 , EGTA 10, Mg-ATP 5, creatine phosphate 5, and HEPES 5 (pH 7.2). The concentrations of free Ca^{2+} (pCa 8 and 6) in the internal solution were calculated on the basis of apparent stability constants for EGTA, Ca^{2+} -ATP, Ca^{2+} -creatine phosphate, Mg^{2+} -EGTA, Mg^{2+} -ATP, and Mg^{2+} -creatine phosphate, according to the calculation of Fabiato & Fabiato (1979) and the correction of Tsien & Rink (1980).

Results

Effects on the action potential

To examine the effect of taurine on the action potential configuration, current-clamp experiments were carried out. The single cell was stimulated at 1 Hz . As shown in Figure 1A,B, taurine (20 mM) markedly shortened the action potential duration (APD) at pCa 8, but prolonged it at pCa 6. The 75% repolarization of APD (APD_{75}) at pCa 8 was shortened by $7.5 \pm 1.8\%$ ($n=6$, $P>0.05$) and $19.7 \pm 2.3\%$ ($n=6$, $P<0.05$) in the presence of 10 and 20 mM taurine, respectively (Figure 1C). At pCa 6, taurine at 10 and 20 mM prolonged APD_{75} by $64.5 \pm 2.8\%$ ($n=6$, $P<0.001$) and $93.2 \pm 4.3\%$ ($n=6$, $P<0.001$), respectively. The resting potential was unaffected.

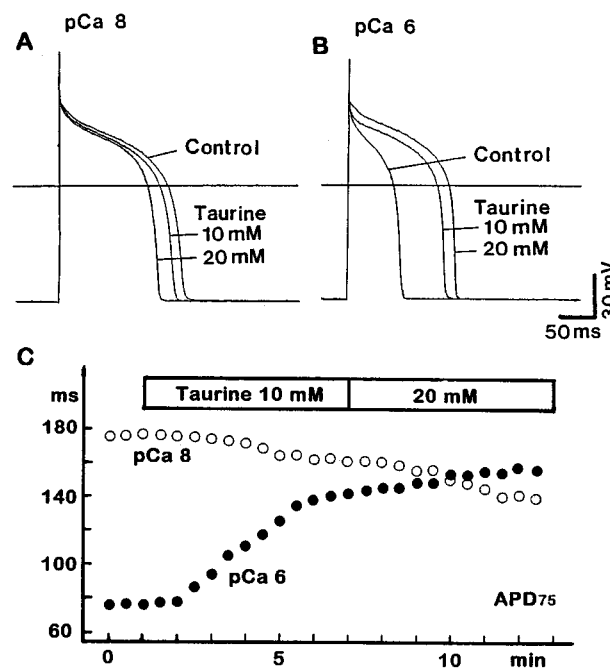


Figure 1 Effects of taurine on the action potentials in ventricular cardiomyocytes. (A) Action potentials at pCa 8 before and after taurine application. (B) Action potentials at pCa 6. Taurine (10 and 20 mM) was cumulatively added to the bath solution. (C) Time-dependent changes in APD_{75} at pCa 8 and pCa 6.

Taurine's action on I_K

The I_K current was measured by applying test pulses (2 s duration) to -30 – $+70 \text{ mV}$ from a holding potential of -40 mV . The I_K current at 90 mV was $16.7 \pm 0.5 \text{ pA pF}^{-1}$ at pCa 8 (Figure 2A). The average capacitance was $88.9 \pm 3.1 \text{ pF}$ ($n=27$). Cumulative administrations of taurine (10 and 20 mM) to the bath solution enhanced I_K at 70 mV by $13.4 \pm 3.2\%$ ($n=6$, $P<0.05$) and by $22.4 \pm 3.1\%$ ($n=6$, $P<0.01$), respectively. Current-voltage (I – V) relations in the absence and presence of taurine (10 and 20 mM) are given in Figure 2B.

On the other hand, the I_K at pCa 6 was inhibited by application of taurine in a concentration-dependent manner (Figure 3A,B). The I_K and its tail current were strongly activated as compared with those at pCa 8. Figure 3B shows the I – V relationship for I_K . The inhibition at 70 mV in the presence of 10 and 20 mM of taurine was $16.5 \pm 2.8\%$ ($n=6$, $P<0.05$) and $27.1 \pm 2.8\%$ ($n=6$, $P<0.01$), respectively. The responses to taurine were incompletely recovered by a 20-min washout. Low concentrations ($1\text{--}5 \text{ mM}$) of taurine had little or no effect on I_K .

Effects on activation curves for I_{Kr} and I_{Ks}

To examine the degree of activation of the I_K current, the amplitude of the outward tail current of I_K was plotted along the voltage axis. At pCa 8, 10 and 20 mM taurine enhanced the tail current at 70 mV by $36.2 \pm 2.1\%$ ($n=5$, $P<0.01$) and $72.3 \pm 3.8\%$ ($n=5$, $P<0.01$), respectively (Figure 4A). The curves in Figure 4C were fitted by an empirical equation; $p = 1 / \{1 + \exp[(V_m - V_{1/2})/S]\}$. $V_{1/2}$ is the potential of half-activation, and S is the slope factor. The slope factor at pCa 8 was 9.8 ± 2.1 ($n=5$) in the control and 9.9 ± 1.3 ($n=5$) in the taurine (10 and 20 mM)-treated cells. The $V_{1/2}$ value in five cells

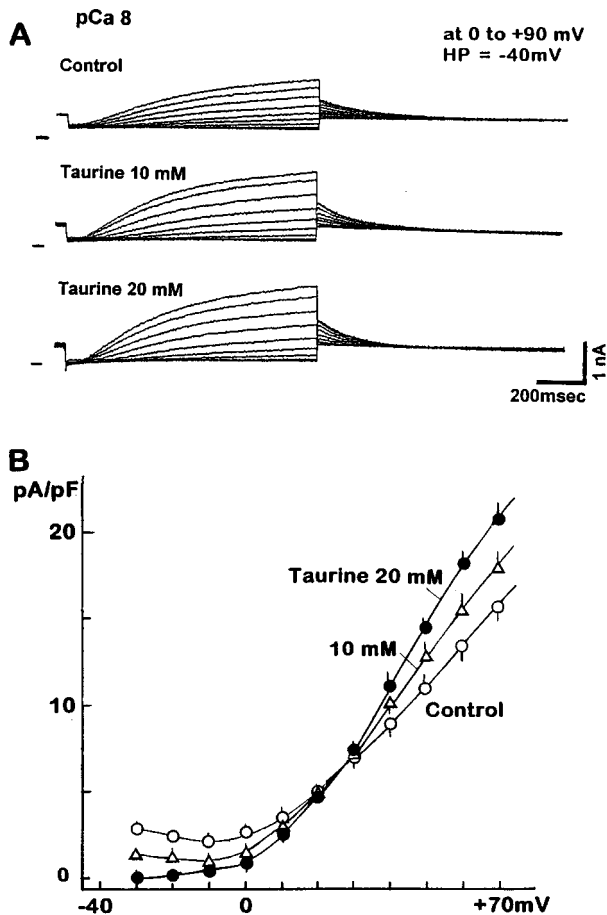


Figure 2 Enhancement of the delayed rectifier K^+ current (I_K) induced by taurine at pCa 8. (A) Current traces in the absence and presence of 10 and 20 mM taurine. A test pulse (for 2 s) was applied between 0–90 mV from a holding potential of -40 mV. The short line at the left of the current records represents the zero current level. (B) Current-voltage relationship for I_K ($n=6$).

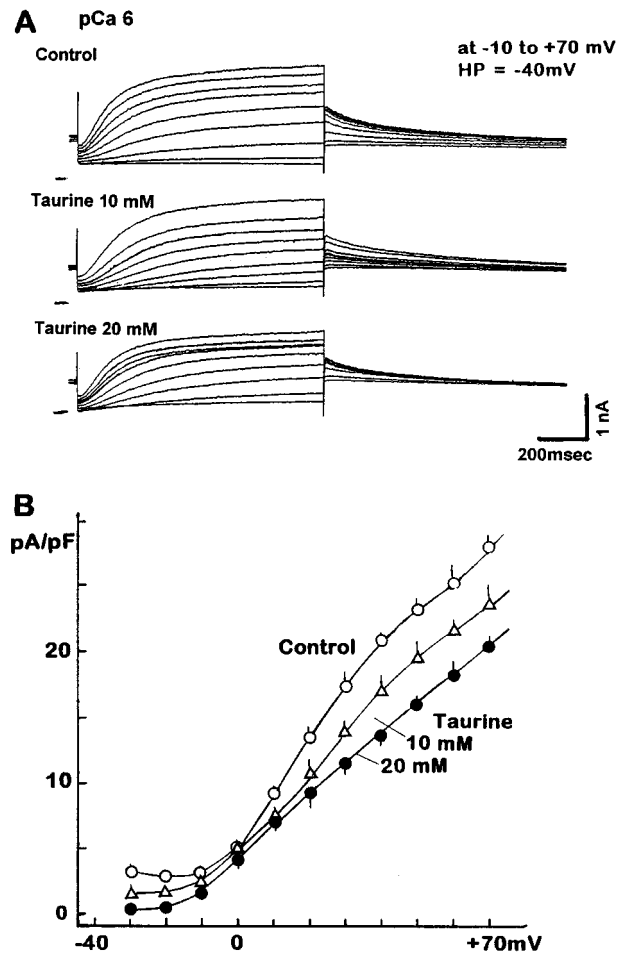


Figure 3 Inhibition of I_K induced by taurine at pCa 6. (A) Current traces in the absence and presence of taurine. Test pulses for 2 s were applied between -10 – $+70$ mV from a holding potential of -40 mV. The short line at the left of the current records represents the zero current level. (B) Current-voltage relationship for I_K in control and in 10 and 20 mM taurine ($n=6$).

was 42.3 ± 2.6 mV ($n=5$) in control and 44.8 ± 2.2 mV ($n=5$) at 20 mM taurine. The difference was not significant ($P < 0.05$).

At pCa 6, the tail current at 70 mV was inhibited by $12.4 \pm 2.0\%$ ($n=6$, $P < 0.05$) at 10 mM taurine and by $35.1 \pm 2.4\%$ ($n=6$, $P < 0.01$) at 20 mM taurine (Figure 4B). Increasing $[Ca^{2+}]_i$ (from pCa 8 to 6) shifted the normalized curve to a more negative potential in normal Tyrode solution; by approximately 10 mV at $V_{1/2}$ from the curves in Figure 4C,D. The slope factors were 10.2 ± 0.3 ($n=5$) in the control, and 10.8 ± 0.4 in the presence of taurine (10 and 20 mM). The $V_{1/2}$ value was 32.3 ± 2.2 mV ($n=5$) in the control, and 30.1 ± 2.8 mV ($n=5$) in the presence of 20 mM taurine; the difference was not significant ($P < 0.05$).

The I_K activated by the long test pulse is composed of rapidly and slowly activated currents (I_{Kr} and I_{Ks}). Effects of taurine on the tail current of I_{Kr} were examined by test pulse for 200 ms duration to separate the I_{Ks} component. Taurine had the stimulatory effects on the tail current of I_{Kr} at 50 mV at pCa 8 and the inhibitory effects at pCa 6 (Figure 5A,B). The tail currents are plotted in Figure 5C,D. Additional application of E-4031 ($5 \mu M$) blocked I_{Kr} completely. The $V_{1/2}$ values were -10.2 ± 0.8 mV ($n=5$) at pCa 8, and 4.3 ± 0.9 mV ($n=5$) at pCa 6. Taurine did not affect the $V_{1/2}$ values at both pCa levels (Figure 5E,F).

Effects of taurine on the tail currents of I_{Kr} activated by less 200 ms duration and on that of I_K (or I_{Ks}) by 1 or 2 s duration

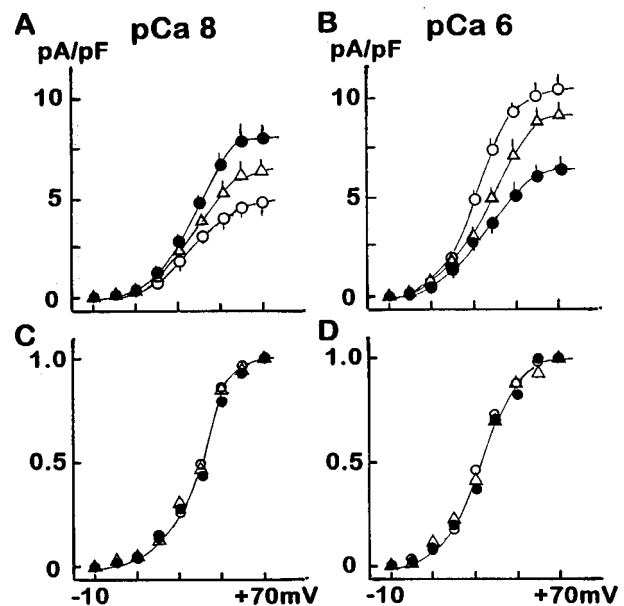


Figure 4 Effects of taurine on the activation process for I_K . Holding potential was -40 mV. (A,B) Outward tail currents in control and in 10–20 mM taurine at pCa 8 ($n=5$) and 6 ($n=6$). (C,D) Normalized activation curves for I_K at pCa 8 and 6, respectively. The curve was fitted by Boltzmann equation.

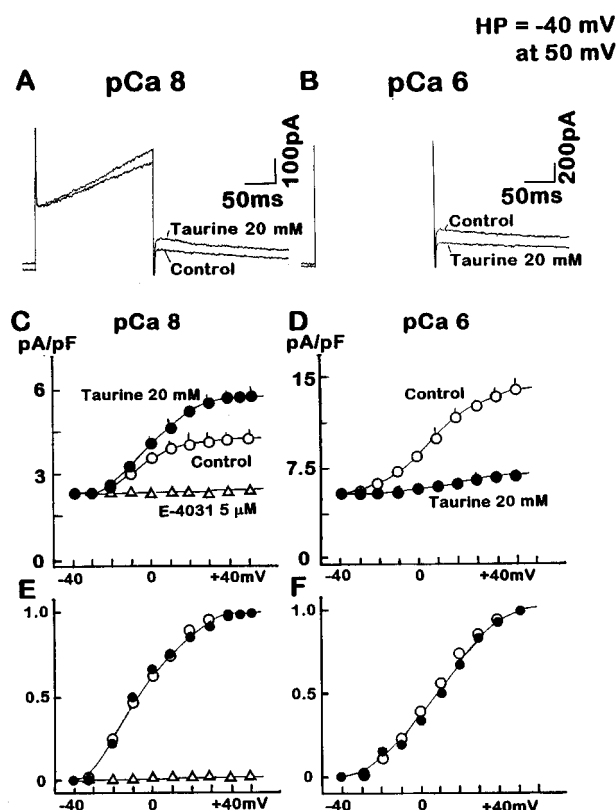


Figure 5 Modulation by taurine of the tail current and the activation curve for rapid component (I_{Kr}) of I_K . (A,B) Outward tail currents for 200 ms in control and 20 mM taurine at pCa 8 and 6. At pCa 6, the current traces during test pulses disappeared through saturation. (C,D) Peak of the tail currents ($n=5$) at pCa 8 and 6. (E,F) Normalized curves of the activation for I_{Kr} at pCa 8 and 6 respectively.

were examined. Taurine (20 mM) inhibited the tail current of I_{Kr} , as shown in Figure 6A. The inhibition was $35.7 \pm 2.8\%$ ($n=6$, $P<0.05$) at 10 mM and $56.0 \pm 2.3\%$ ($n=6$, $P<0.01$) at 20 mM taurine. Addition of E-4031 (5 μ M) blocked the I_{Kr} completely, but not I_K fully. The effects on I_{Kr} at pCa 8 and 6 are summarized in Figure 6B. The tail current was blocked by E-4031 (5 μ M). Taurine had little or no effect on the remained tail current, that is I_{Ks} , in the presence of E-4031 (5 μ M) at both pCa levels. However, addition of 293B (30 μ M) had almost blockade of I_{Ks} (Figure 6C).

Discussion

Taurine, an amino acid largely obtained from the diet, has been found to exert many important physiological functions. Its electrical and mechanical actions are independent of many known regulators, such as cyclic AMP and cyclic GMP levels, (Na,K)-ATPase activity and calmodulin-dependent protein kinase action, although taurine may inhibit protein kinase C activity (Segawa *et al.*, 1985; Lombardini, 1992). The experiments were designed to examine the effects of taurine on I_K (I_{Kr} and I_{Ks}) at low and high $[Ca^{2+}]_i$ in single isolated (adult) guinea-pig ventricular cardiomyocytes. The present experiments showed the following. (1) The APD_{75} in the presence of taurine decreased at pCa 8, whereas taurine increased it at pCa 6. (2) Taurine enhances I_K at pCa 8 and inhibited I_K at pCa 6. (3) Taurine stimulated the tail

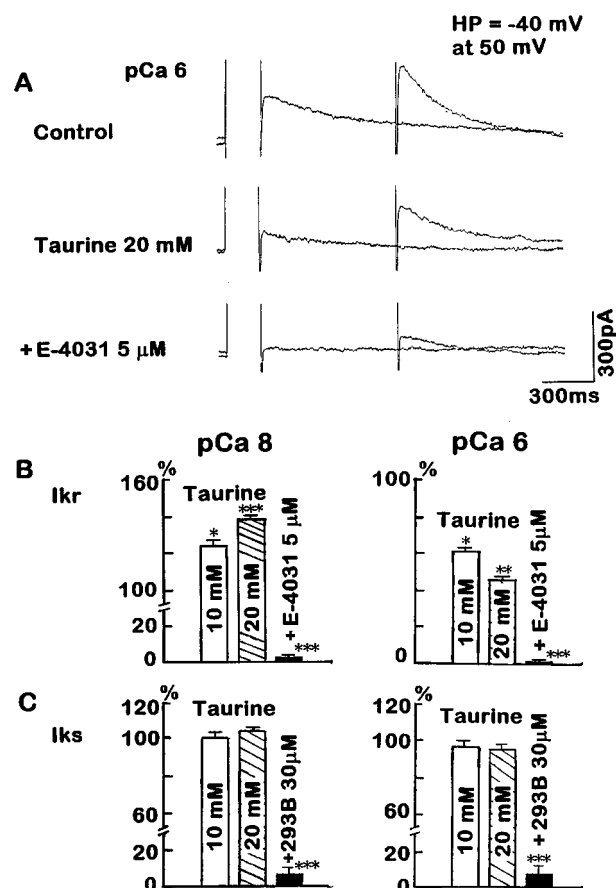


Figure 6 Effects of taurine on the tail currents of rapid and slow components of I_K (I_{Kr} and I_{Ks}). (A) Current traces of I_{Kr} and I_{Ks} at pCa 6. Test pulses were applied to 50 mV from -40 mV for 200 ms and 1 s. (B) Percentage changes in peak of I_{Kr} ($n=6$) at pCa 8 and 6 by taurine (10 and 20 mM) and E-4031 (5 μ M). Ordinate axis is represented control value as 100%. (C) Changes in I_{Ks} at pCa 8 and 6 by taurine and 293B (30 μ M) in the presence of 5 μ M E-4031 ($n=6$).

current of I_K at low pCa, but depressed it at high pCa. (4) The tail current of I_K was not affected by taurine. (5) The voltage of half-maximum activation of I_{Ks} was unaffected by taurine at both pCa 8 and 6. (6) The taurine-induced effects were irreversible even after a 30-min washout, consistent with our previous reports (Satoh & Sperelakis, 1992, 1993, 1998). The basis for this is unclear, but some possibilities exist: (a) the recovery from taurine may occur slowly and be not observed by 30 min, and (b) the recovery may be masked by time-dependent damage to the myocytes.

Action potential duration

At the plateau, the inward and outward currents are practically in balance, and a small change in one of the currents will greatly affect the course of the potential. From a theoretical point of view, APD is strongly regulated by the delayed rectifier (and time-dependent activated) K^+ current (I_K). Taurine stimulated I_K at pCa 8, whereas it depressed I_K at pCa 6. Since the I_K in cardiac cells is dependent on $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$ (Meech, 1974; Isenberg, 1975), the Ca^{2+} -activated I_K might be stimulated through the enhancement of I_{Ca} even when $[Ca^{2+}]_i$ is low. Since, in the present experiments, $[Ca^{2+}]_i$ was buffered at pCa 6 or 8 (EGTA 10 mM in the pipette), the actions on I_K induced by taurine would be independent of $[Ca^{2+}]_i$.

The APD may also be modulated by such ionic currents as I_{Ca} and the fast Na^+ current (I_{Na}) (Carmeliet & Saikawa, 1982). Taurine increased I_{Ca} at low pCa, but decreased I_{Ca} at high pCa. The inactivation of I_{Ca} is composed of both fast ($[Ca^{2+}]_i$ -dependent) and slow (voltage-dependent) exponentials (Kass & Sanguinetti, 1984; Satin & Cook, 1989). In embryonic chick ventricular myocytes, taurine affected only the slow time constant for I_{Ca} inactivation, but not the fast time constant (Satoh & Sperelakis, 1993). The increase in the time constant for inactivation would result in APD prolongation. In this study, thus, the Ca^{2+} channel was blocked by Ca^{2+} antagonist. On the other hand, taurine inhibits I_{Na} , independent of $[Ca^{2+}]_i$ level (Satoh, 1998a). The time constant for I_{Na} inactivation (with a single component) is not modified by taurine (Satoh & Sperelakis, 1992). In this study, the I_{Na} channel is not activated at -40 mV of holding potential. Therefore, these results suggest that taurine-induced APD modulation is mainly due to its actions on I_K . In addition, taurine inhibits the inwardly rectifying K^+ current (I_{K1}), which may also partly modulate the APD (Satoh, 1998b).

I_K at different pCa levels

In the present experiments, the different pCa levels were used to elucidate the effects of taurine on I_K current. The amplitude of I_K was greater at pCa 6 than at pCa 8, and its activation curve was shifted at $V_{1/2}$ by approximately 10 mV to the negative potential (see Figures 3B and 4B). The potentiation of I_K amplitude is consistent with previous reports (Meech, 1974; Isenberg, 1975).

Taurine inhibited I_K at pCa 6, but enhanced I_K at pCa 8, consistent with the previous report (Satoh, 1995). Recently, I_K is separated by two distinct components of rapidly and slowly activation (I_{Kr} and I_{Ks}) (Sanguinetti & Jurkiewicz, 1990). The I_{Kr} is selectively E-4031-sensitive and is activated fully within 200 ms. Taurine markedly affected I_{Kr} . In the presence of E-4031, however, taurine had little or no effect on the remained current (I_{Ks}), and addition of 293B (a selective I_{Ks} inhibitor) (Suessbrich *et al.*, 1996) blocked it completely. Therefore, these results indicate that the dual action of taurine at different pCa

levels is mostly due to its effects on I_{Kr} , but not I_{Ks} . The selective inhibition would somewhat play an important role for cardioprotective actions of taurine.

Cardioprotection

Our recent experiments showed that taurine possesses a potent cardioprotective action in embryonic chick cardiomyocytes and guinea-pig ventricular myocytes (Satoh & Sperelakis, 1992, 1998; Satoh, 1998a,b). Taurine enhanced I_{Ca} at low pCa, but inhibited I_{Ca} at high pCa. Sperelakis and colleagues (Sperelakis & Satoh, 1993; Sperelakis *et al.*, 1992, 1988) concluded that one of taurine's actions is to normalize Ca^{2+} movement through the sarcolemma, dampening the effects of variation in perfusate Ca^{2+} concentration. These results indicate that taurine acts on I_{Ca} in a manner to keep $[Ca^{2+}]_i$ levels relatively constant, and consequently plays an important role in maintaining cell viability and cardiac function.

The decline in $[Ca^{2+}]_i$ induced by taurine should lead to a reduction of cellular calcium overload during ischaemia. Taurine also promotes the sudden emergence of a dormant I_{CaT} , contributing to the generation of pacemaker activity during diastole (Satoh & Sperelakis, 1993). Since automaticity is depressed under calcium overload (Satoh & Hashimoto, 1988; Satoh *et al.*, 1989), the stimulation of I_{CaT} may also be one of the protective actions of taurine. Furthermore, taurine stimulates the hyperpolarization-activated inward current (that is a pacemaker current, I_f) (unpublished observation). In the present experiments, high pCa itself shortened APD, whereas low pCa itself prolonged APD. Extreme prolongation or shortening of APD may alter the refractory period and elicit arrhythmias (Satoh, 1993b). The antiarrhythmic actions of taurine are also produced by the inhibitions of such as the fast Na^+ , Ca^{2+} , and the ATP-sensitive channels. These findings demonstrate that the effects induced by high and low $[Ca^{2+}]_i$ are antagonized and many ionic channels were modulated by taurine application, indicative of a cardioprotective action of taurine. Although many of taurine's actions on heart still remain unclear, further studies should reveal possible therapeutic uses of taurine.

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Nitric oxide limits the eicosanoid-dependent bronchoconstriction and hypotension induced by endothelin-1 in the guinea-pig

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1 This study attempts to investigate if endogenous nitric oxide (NO) can modulate the eicosanoid-releasing properties of intravenously administered endothelin-1 (ET-1) in the pulmonary and circulatory systems in the guinea-pig.

2 The nitric oxide synthase blocker N^ω-nitro-L-arginine methyl ester (L-NAME; 300 μM; 30 min infusion) potentiated, in an L-arginine sensitive fashion, the release of thromboxane A₂ (TxA₂) stimulated by ET-1, the selective ET_B receptor agonist IRL 1620 (Suc-[Glu⁹,Ala^{11,15}]-ET-1(8-21)) or bradykinin (BK) (5, 50 and 50 nM, respectively, 3 min infusion) in guinea-pig isolated and perfused lungs.

3 In anaesthetized and ventilated guinea-pigs intravenous injection of ET-1 (0.1–1.0 nmol kg⁻¹), IRL 1620 (0.2–1.6 nmol kg⁻¹), BK (1.0–10.0 nmol kg⁻¹) or U 46619 (0.2–5.7 nmol kg⁻¹) each induced dose-dependent increases in pulmonary insufflation pressure (PIP). Pretreatment with L-NAME (5 mg kg⁻¹) did not change basal PIP, but increased, in L-arginine sensitive manner, the magnitude of the PIP increases (in both amplitude and duration) triggered by each of the peptides (at 0.25, 0.4 and 1.0 nmol kg⁻¹, respectively), without modifying bronchoconstriction caused by U 46619 (0.57 nmol kg⁻¹).

4 The increases in PIP induced by ET-1, IRL 1620 (0.25 and 0.4 nmol kg⁻¹, respectively) or U 46619 (0.57 nmol kg⁻¹) were accompanied by rapid and transient increases of mean arterial blood pressure (MAP). Pretreatment with L-NAME (5 mg kg⁻¹; i.v. raised basal MAP persistently and, under this condition, subsequent administration of ET-1 or IRL 1620, but not of U-46619, induced hypotensive responses which were prevented by pretreatment with the cyclo-oxygenase inhibitor indomethacin.

5 Thus, endogenous NO appears to modulate ET-1-induced bronchoconstriction and pressor effects in the guinea-pig by limiting the peptide's ability to induce, possibly *via* ET_B receptors, the release of TxA₂ in the lungs and of vasodilatory prostanoids in the systemic circulation. Furthermore, it would seem that these eicosanoid-dependent actions of ET-1 in the pulmonary system and on systemic arterial resistance in this species are physiologically dissociated.

Keywords: Pulmonary insufflation pressure; mean arterial blood pressure; ET_B receptors; eicosanoids; nitric oxide

Abbreviations: BK, Bradykinin; BQ-788, (N,cis-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-L-methoxy-carbonyltryptophanyl-D-norleucine); ET-1, Endothelin-1; INDO, Indomethacin; IRL-1620, (Suc-[Glu⁹,Ala^{11,15}]-endothelin-1(8-21)); L-NAME, N^ω-nitro-L-arginine methyl ester; MAP, Mean arterial pressure; NO, Nitric oxide; PBS, Phosphate-buffered saline; PG, Prostaglandin; PIP, Pulmonary insufflation pressure; TxA₂, Thromboxane A₂; TxB₂, Thromboxane B₂

Introduction

It is well established that eicosanoids contribute importantly to the pronounced bronchoconstrictor effects of ET-1 in the guinea-pig. Inhibition of eicosanoid production with the cyclo-oxygenase blocker indomethacin markedly attenuates the increase in pulmonary insufflation pressure (PIP) caused by intravenous (Payne & Whittle, 1988) and, to a lesser extent, aerosol administration of ET-1 (Lagente *et al.*, 1989; Pons *et al.*, 1991b). The fact that ET-1 effectively triggers release of TxA₂ and prostacyclin from guinea-pig perfused lungs (de Nucci *et al.*, 1988) further strengthens this view. Considering that TxA₂ receptor blockers effectively attenuate ET-1-induced contractions in guinea-pig isolated trachea, upper bronchi and parenchymal strips (Filep *et al.*, 1990), as well as increase in PIP *in vivo* (Noguchi *et al.*, 1993), TxA₂ seems to be the main eicosanoid mediator involved in the peptide's bronchoconstrictor effect.

The modulatory properties of eicosanoids are not limited to the pulmonary effects of exogenous ET-1. The pressor effects of ET-1 in the anaesthetized rat are also strongly limited by the concomitant release of vasodilatory prostaglandins (de Nucci *et al.*, 1988), as well as NO (Warner *et al.*, 1989). Interestingly, the NO-releasing properties of ET-1 in many vascular circuits *in vitro* or *in vivo* (Webb, 1991; Warner *et al.*, 1993; D'Orléans-Juste *et al.*, 1994; Gellai *et al.*, 1997), as well as its ability to trigger TxA₂ release from guinea-pig perfused lungs (D'Orléans-Juste *et al.*, 1994) are both related to stimulation of endothelin ET_B receptors. Furthermore, these receptors also mediate ET-1-induced bronchoconstriction in the guinea-pig, as this effect is insensitive to blockade by the selective endothelin ET_A receptor antagonist BQ-123 (Noguchi *et al.*, 1993). The ET_B receptors coupled to NO release are most likely expressed on the vascular endothelium, but the cellular sources of ET_B-receptor mediated release of TxA₂ in the guinea-pig lung remain to be fully identified and could include tracheal epithelial cells, lung parenchyma, resident inflamma-

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tory cells and/or alveolar macrophages (Filep *et al.*, 1990, 1991; Ninomiya *et al.*, 1992; Fleisch *et al.*, 1996).

Cross-talk between cyclo-oxygenase and NO synthase activities in various *in vitro* systems have been suggested (Stadler *et al.*, 1993; Swierkosz *et al.*, 1995; Bishop-Bailey *et al.*, 1997), but similar studies in more complex *in vivo* systems are limited. It has been proposed that disruptions in the homeostatic balance between NO, eicosanoids and endothelins in the pulmonary circulation, involving deregulation of the synthesis and/or expression of these factors, may underlie disabling respiratory diseases such as cystic fibrosis and primary pulmonary hypertension (Giaid *et al.*, 1993a, 1995; Saleh *et al.*, 1997; Park *et al.*, 1997).

To our knowledge, there has been no report in the literature on the possible functional cross-talk between endothelins, NO and eicosanoids *in vivo* and, especially, between NO and ET-1-induced bronchoconstriction. To this effect, we have assessed the influence of an NO synthase inhibitor, N^ω-nitro-L-arginine methyl ester (L-NAME; Rees *et al.*, 1990), on ET-1-induced release of vasodilator and vasoconstrictor eicosanoids, as well as eicosanoid-mediated bronchoconstriction in the anaesthetized and ventilated guinea-pig. Our results lead us to conclude that indeed there is a significant interplay between NO, endothelins and arachidonic acid metabolites *in vivo*, whereby NO very effectively limits the ability of intravenous ET-1 to cause ET_B receptor-mediated eicosanoid release and hence bronchoconstriction and hypotension in this species.

Methods

Measurements of eicosanoids from isolated perfused guinea-pig lungs

Duncan Hartley guinea-pigs (250–350 g) (Charles River, St-Constant, Canada) of either sex were killed by cervical dislocation. Following thoracotomy, the lung artery was cannulated for perfusion of the lung circulation with a heparinized Krebs' solution (100 units ml⁻¹). The lungs were then removed and immediately suspended in a heated chamber (37°C) and perfused (5 ml min⁻¹) with an oxygenated (95% O₂, 5% CO₂) Krebs' solution. The lungs were left to stabilize for 45 min before two successive 3 min infusions (conducted 45 min apart) of ET-1 (5 nM), the selective ET_B receptor agonist IRL 1620 (Takai *et al.*, 1992) (50 nM), BK (50 nM; a potent eicosanoid releaser in this system, Bakhle *et al.*, 1985) or the stable TxA₂ analogue U 46619 ((15S)-hydroxy-11 α ,9 α -(epoxymethano)prosta-5Z,13E-dienoic acid; Coleman *et al.*, 1981) (10 nM). In a second series of experiments, one of the agonists was administered in each lung in a first infusion following which the NO synthase inhibitor N^ω-nitro-L-arginine methyl ester (L-NAME; 300 μ M, infused intraarterially (i.a.)) was applied prior to (for 30 min) and during the second infusion of the same agonist. The concentrations of these agonists and inhibitors were chosen according to a previously reported study (Lewis *et al.*, 1998). In a third set of experiments, L-arginine (6 mM) was infused (i.a.) 15 min before and then throughout treatment of the lungs with L-NAME. All agents were infused at a rate of 50 μ l min⁻¹. The effluent from the lungs was collected (1-min samples) before, during and after infusion of the various agents. The samples were stored at -20°C until determination of their levels of stable hydrolytic metabolites of prostacyclin (6-Keto-prostaglandin F_{1 α} (6-Keto-PGF_{1 α})) and thromboxane A₂ (thromboxane B₂(TxB₂)) by radioimmunoassay (Salmon, 1978).

In vivo experiments

Guinea-pigs of the same breed and characteristics as before were anaesthetized with ketamine/xylazine (90/15 mg kg⁻¹, intramuscular). Polyethylene catheters (PE-50) were inserted into the left external jugular vein and the right carotid artery for drug administration and recording of both mean arterial blood pressure (MAP) and heart rate, respectively. A cannula (PE-240) was then inserted into the trachea following thoracotomy to facilitate respiration. A blood pressure analyser (Micro-Med, Louisville, KY, U.S.A.) was used to monitor MAP and heart rate. The data were recorded at fixed 5-s time intervals throughout the entire experiment by an automated computer data acquisition system (Blood Pressure Analyzer Communications Program; MicroMed, Inc.) linked to a Compaq Prolinea 4/33 computer.

Thirty minutes after surgery, spontaneous breathing was suppressed with succinylcholine (5 mg kg⁻¹, s.c.) and each animal was connected through the tracheal cannula to a rodent respirator (Model 141, Valley Scientific Co. apparatus, Medway, MA, U.S.A.) and ventilated (6 ml kg⁻¹, 60 strokes min⁻¹). PIP was monitored with a pressure transducer (Statham, model P23AC) connected to a side arm of the tracheal cannula, according to the method of Konzett & Rössler (1940), and coupled to a Grass polygraph (model 7D) for the recording of bronchoconstrictor responses. The pharmacological responses to the various agonists were monitored for at least 40 min following their administration. Each animal received a single dose of one of the above-mentioned agonists (ET-1, IRL 1620, BK or U 46619). In some experiments, L-NAME (5 mg kg⁻¹, i.v.) was administered 3 min prior to agonist injection. Alternatively, this L-NAME treatment was preceded by an injection of either L-arginine (100 mg kg⁻¹, 5 min beforehand and with L-NAME, i.v.), the selective ET_B receptor antagonist, BQ-788 (N,cis-2,6-dimethyl-piperidinocarbonyl-L-L-methylleucyl-D-L-methoxy-carbonyl-tryptophanyl-D-norleucine; Ishikawa *et al.*, 1994) (0.25 mg kg⁻¹, 2 min beforehand, i.v.), or indomethacin (10 mg kg⁻¹, 15 min beforehand, i.v.).

In vivo experiments lasted no longer than 90 min and maintenance doses of anaesthetics were not required. Vehicles (phosphate-buffered saline (PBS) or Trizma base) were systematically tested and were found not to alter either basal MAP or PIP or the responses of the guinea-pig to the agonists used in the present study.

Drugs

ET-1 and IRL 1620 were purchased from American Peptide Company Inc. (Sunnyvale, U.S.A.). BQ-788 was purchased from Peptides International (Louisville, U.S.A.). BK was synthesized in our laboratory. U 46619 (dissolved in methyl acetate and then freshly diluted in phosphate-buffered saline, pH 7.4) was purchased from Cayman Chemical Company (Ann Arbor, U.S.A.). L-NAME, L-arginine, heparin, PBS, 6-Keto-PGF_{1 α} , TxB₂, 6-Keto-PGF_{1 α} and TxB₂ antisera were obtained from Sigma (St. Louis, U.S.A.). Tritiated 6-Keto-PGF_{1 α} and TxB₂ were purchased from Amersham (Oakville, Canada). The TxB₂ antiserum has a 100% cross-reactivity with authentic TxB₂, less than 2% cross-reactivity with prostaglandin D₂ (PGD₂) and prostaglandin F_{1 α} (PGF_{1 α}) and less than 0.1% cross-reactivity with 6-Keto-PGF_{1 α} , prostaglandin E₁ (PGE₁) and prostaglandin E₂ (PGE₂). The 6-Keto-PGF_{1 α} antiserum has 100% cross-reactivity with authentic 6-Keto-PGF_{1 α} , 23% with PGE₁, 4% with PGE₂,

7% with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and less than 1% with TxB_2 . Both 6-Keto- $PGF_{1\alpha}$ and TxB_2 radioimmunoassays have a detection limit of 0.4 ng ml^{-1} . The eicosanoid antisera employed in the present study did not cross-react with any of the agonists or inhibitors used. All agents were dissolved in PBS, except for indomethacin which was dissolved in Trizma-base (0.2 M).

Data analysis

Results are shown as means $\pm$ s.e.mean for n experiments. Statistical comparisons between the release of eicosanoids from the perfused lungs triggered by the first (control) and second infusions (in presence of L-NAME, with or without L-arginine) of each agonist were made by paired Student's t -test. Since the pressor and bronchoconstrictive properties of ET-1 were very poorly reversible, guinea-pigs were not used as their own controls for the *in vivo* experiments with inhibitors or antagonists. Statistical comparisons between responses of guinea-pigs pretreated or not with L-NAME, L-arginine, indomethacin or BQ-788 *in vivo* were made using unpaired Student's t -test. Changes in MAP, increases in PIP or increased release of eicosanoids from the perfused lung when compared to basal at time 0, were analysed by ANOVA followed by Dunnett's test for multiple comparisons. Differences in which $P < 0.05$ were considered significant.

Ethics

The care of animals and all the research protocols conformed to the guiding principles for animal experimentation, as enunciated by the Canadian Council on Animal Care and approved by the Ethical Committee on Animal Research of the University of Sherbrooke.

Results

L-NAME selectively potentiates TxA_2 release induced by ET-1, IRL 1620 or BK from the guinea-pig perfused lung

A first control 3-min infusion of ET-1 (5 nM) or BK (50 nM) triggered a significant release of prostacyclin and TxA_2 from the guinea-pig perfused lung (Figures 1a,b and 2). In contrast, the selective ET_B receptor agonist IRL 1620 (50 nM) markedly increased only the release of TxA_2 (Figure 1c,d), confirming previous findings (D'Orléans-Juste *et al.*, 1994). The TxA_2 -mimetic U 46619 (50 nM) failed to alter eicosanoid release (TxA_2 release: from 0.72 ± 0.07 to peak of $0.74 \pm 0.09 \text{ ng ml}^{-1}$; prostacyclin release: from 0.65 ± 0.08 to peak of $0.73 \pm 0.05 \text{ ng ml}^{-1}$, $n = 3$).

The TxA_2 -releasing properties of ET-1 and IRL 1620 were quite reproducible, as a second control infusion with either

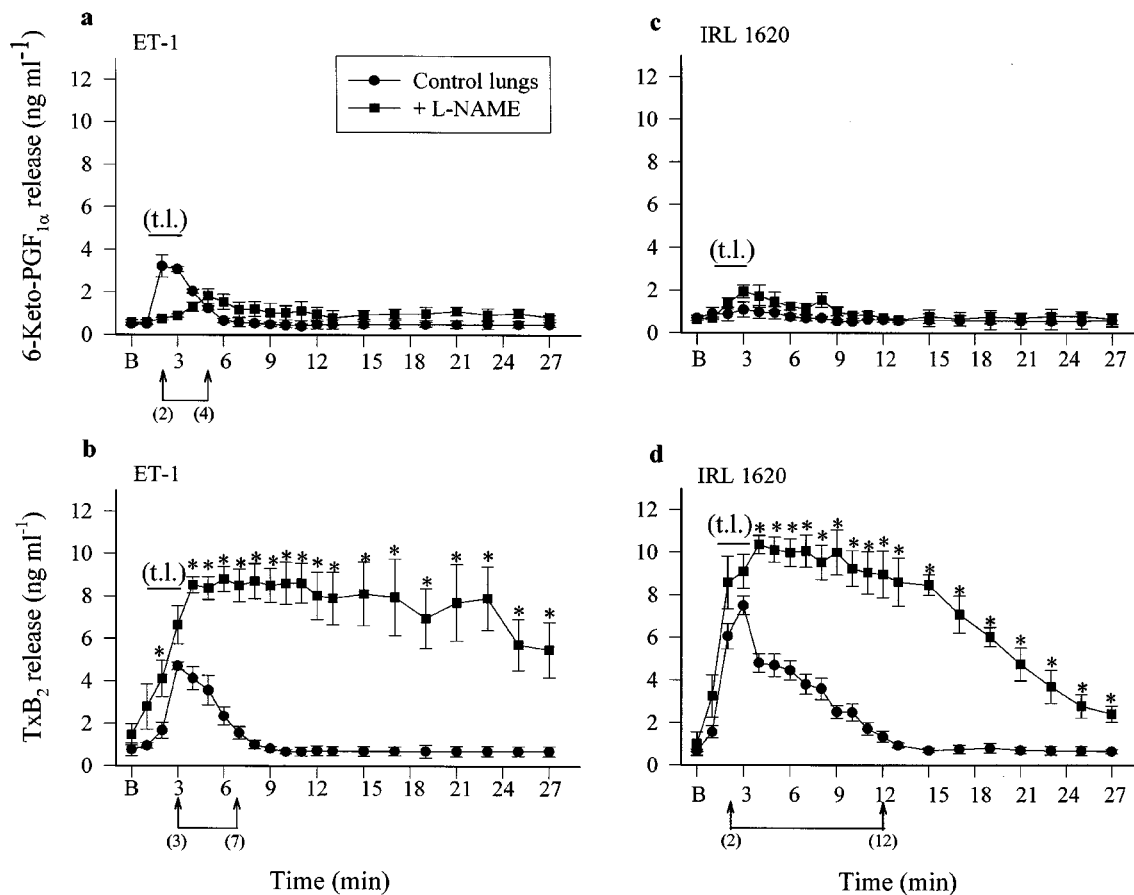


Figure 1 Profile of prostacyclin (measured as 6-keto- $PGF_{1\alpha}$) and thromboxane A_2 (measured as TxB_2) release from the isolated perfused guinea-pig lung induced by ET-1 (5 nM) (a, b) and IRL 1620 (50 nM) (c, d) in the absence or presence of L-NAME ($300 \text{ } \mu\text{M}$). The 6-keto- $PGF_{1\alpha}$ and TxB_2 release was monitored for 24 min after the interruption of a 3 min infusion of the agonist through the lung (t.l.) arterial vasculature. (b) Basal levels of eicosanoid were measured before the infusion of the agonist. Arrows represent the time points (min) at which a first infusion of ET-1 or IRL-1620 induce a significant release of eicosanoids ($P < 0.05$) when compared to basal. Each value represents the mean $\pm$ s.e.mean of 4–8 experiments. *, $P < 0.05$ when compared with the levels of eicosanoid from the lungs without added L-NAME at each time point.

agonist produced similar magnitudes of release as the first (peak releases: ET-1: first infusion 4.78 ± 0.43 ng ml⁻¹, second infusion 3.77 ± 0.34 ng ml⁻¹, $n=6$; IRL 1620: first infusion 6.84 ± 0.48 ng ml⁻¹, second infusion 7.24 ± 1.25 ng ml⁻¹, $n=4$). In contrast, TxA₂ release triggered by BK showed significant tachyphylaxis (peak releases: first infusion 2.44 ± 0.36 ng ml⁻¹, second infusion 1.2 ± 0.52 ng ml⁻¹, $n=7$, $P<0.05$). Conversely, release of prostacyclin was reproducible when triggered by BK (peak releases: first infusion 8.54 ± 1.99 ng ml⁻¹, second infusion 5.48 ± 1.26 ng ml⁻¹, $n=7$; $P>0.05$), but as previously reported (D'Orléans-Juste *et al.*, 1994) not by ET-1 (peak releases: first infusion 3.40 ± 0.61 ng ml⁻¹, second infusion 1.22 ± 0.55 ng ml⁻¹, $n=16$, $P<0.05$). Finally, two successive infusions of IRL-1620 did not induce a significant release of PGI₂ (peak releases: first infusion 0.8 ± 0.2 ; second infusion: 0.9 ± 0.1 ng ml⁻¹, $n=3$).

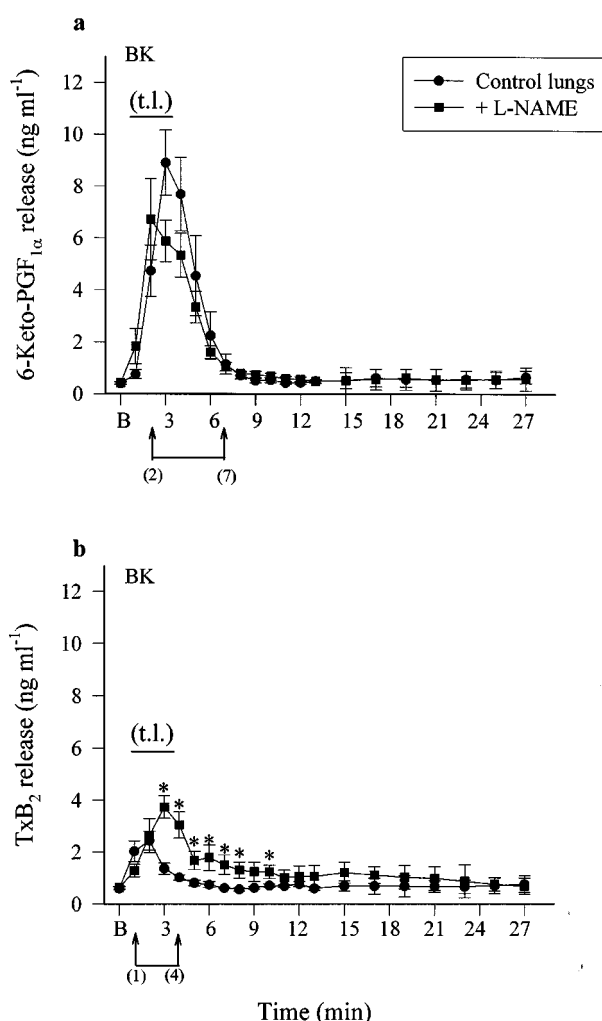


Figure 2 Profile of prostacyclin (measured as 6-keto-PGF_{1α}) (a) and thromboxane A₂ (measured as TxB₂) (b) release from the isolated perfused guinea-pig lung induced by BK (50 nM) in the absence or presence of L-NAME at 300 μM. The 6-keto-PGF_{1α} and TxB₂ release was monitored for 24 min after the interruption of a 3 min infusion of the agonist through the lung (t.l.) arterial vasculature. (b) Basal levels of eicosanoid were measured before the infusion of the agonist. Arrows represent the time points (min) at which a first infusion of bradykinin induces a significant release of eicosanoids when compared to basal ($P<0.05$). Each value represents the mean $\pm$ s.e.mean of eight experiments. *, $P<0.05$ when compared with the levels of eicosanoid from the lungs without added L-NAME at each time point.

When L-NAME (300 μM) was infused into the perfused lung for 30 min prior to a second infusion of ET-1, IRL 1620 or BK, the release of TxA₂ induced by each agonist was sharply potentiated (Figures 1b,d and 2b). It is noteworthy that the potentiating influence of L-NAME on TxA₂ release stimulated by ET-1 and IRL 1620 was more pronounced and longer-lasting than that triggered by BK. On the other hand, L-NAME pretreatment did not significantly alter the magnitude of prostacyclin released by IRL-1620 and BK whereas the inhibitor delayed by 2 min the ET-1-induced release of the same prostanoid, when compared to the amounts measured following the first infusion (Figures 1a,c and 2a). The ability of L-NAME to enhance TxA₂ release stimulated by ET-1, IRL 1620 or BK was almost fully prevented when the lungs were infused with L-arginine (6 mM) for 15 min before starting infusion with the NO-synthase blocker (Figure 3). Worthy of

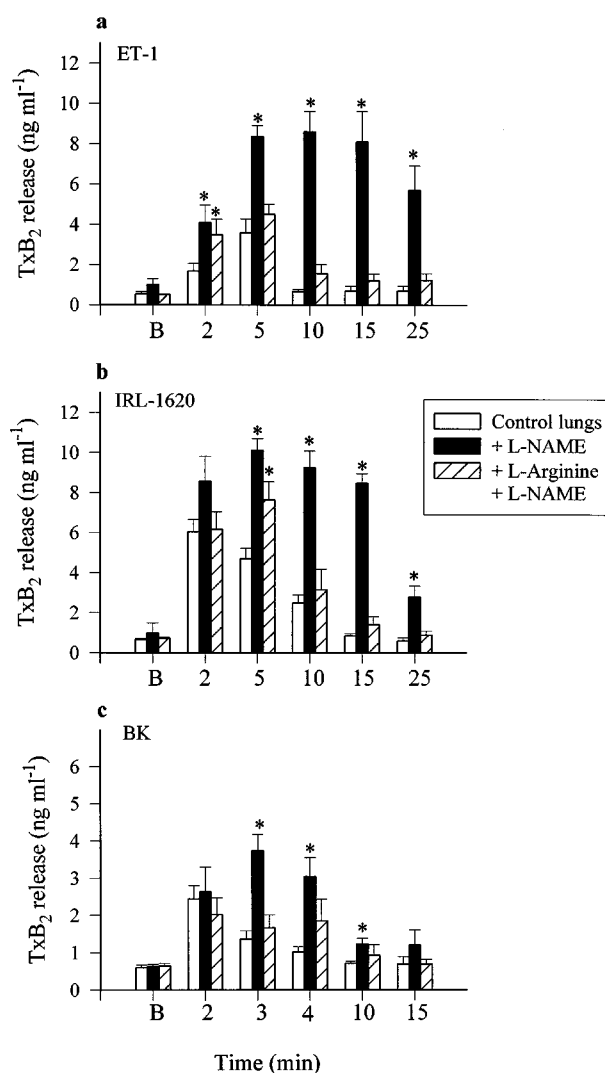


Figure 3 Profile of thromboxane A₂ release (measured as TxB₂) from the isolated perfused guinea-pig lung induced by ET-1 (5 nM) (a), IRL 1620 (50 nM) (b) or BK (50 nM) (c) in the absence or presence of 300 μM of L-NAME alone or with 6 mM of L-arginine. The TxB₂ release was illustrated at six time points (for ET-1 and IRL 1620: 0, 2, 5, 10, 15, 25 min; for BK: 0, 2, 3, 4, 10, 15 min) following the beginning of a 3 min infusion of the peptide through the lung arterial vasculature. (b) Basal levels of TxB₂ were measured before the infusion of the peptide. Each value represents the mean $\pm$ s.e.mean of at least five experiments. *, $P<0.05$ when compared with the levels of TxB₂ from the control value at each time point.

notice is the fact that L-NAME *per se* did not affect the basal release of 6-keto-PGF_{1 α} nor TxB₂ (Figures 1 and 2).

L-NAME in vivo modifies PIP and MAP responses to ET-1, IRL 1620 or BK, but not U 46619

Basal PIP and MAP in naïve anaesthetized and ventilated control guinea-pigs was averaged at 8 ± 0.3 mmHg ($n = 40$) and 57 ± 0.7 mmHg ($n = 31$), respectively. Intravenous injection of L-NAME (5 mg kg^{-1}) did not alter PIP (L-NAME-treated PIP 9 ± 0.5 mmHg, $n = 45$), but increased MAP rapidly and persistently, starting within 30 s of injection and reaching a sustained plateau within 2–3 min, with a peak Δ MAP of 31 ± 0.8 mmHg ($n = 45$), which remained unchanged throughout the full remainder of the experiment (Table 1). In untreated guinea-pigs, ET-1 ($0.25 \text{ nmol kg}^{-1}$, i.v.) triggered transient increases in PIP and MAP, whereas in L-NAME treated animals it induced far greater increases in PIP allied to a sustained hypotension (Figure 4 and Table 1). Although IRL 1620 (0.4 nmol kg^{-1} , i.v.) was as effective as ET-1 ($0.25 \text{ nmol kg}^{-1}$, i.v.) in increasing PIP in untreated control animals (Figure 5), the peak of its transient hypertensive effect was significantly smaller than that caused by the latter peptide (Table 1), confirming previous studies (Pons *et al.*, 1991a; Gratton *et al.*, 1995). Nevertheless, when injected into L-NAME-treated guinea-pigs, IRL 1620 caused, like ET-1, greater PIP responses and prolonged decreases in MAP.

Like ET-1 and IRL 1620, BK (1 nmol kg^{-1} , i.v.) increased PIP in control untreated guinea-pigs (Figure 5). However, unlike those peptides, it induced a clear decrease in MAP which was greatest at 2 min (Δ MAP of -18.6 ± 3.8 mmHg, $n = 4$) and reversed to baseline at 10 min. L-NAME treatment potentiated both BK-induced increases in PIP as well as the magnitude and duration of its hypotensive effect (Δ MAP of -46.5 ± 4.5 mmHg, $n = 4$). In sharp contrast, the TxA₂-mimetic U 46619 ($0.57 \text{ nmol kg}^{-1}$, i.v.) caused increases in both PIP and MAP in control animals which were unaffected by treatment with L-NAME (Figure 6 and Table 1). As shown in Figure 6, the failure of L-NAME to affect PIP responses to U 46619 was confirmed over a broad range of doses of this particular agonist, and is in clear contrast to the significant potentiation of bronchoconstriction triggered by submaximally effective doses of ET-1, IRL 1620 or BK. It is also noteworthy that doses higher than 1 and 1.6 nmol kg^{-1} of ET-1 or IRL 1620, respectively, were found to be lethal under these experimental conditions.

Influence of indomethacin, L-arginine and BQ-788 on the responses to of ET-1, IRL 1620 or BK in L-NAME-treated guinea-pigs

Pretreatment with indomethacin (10 mg kg^{-1}), L-arginine (100 mg kg^{-1}) or BQ-788 (0.25 mg kg^{-1}) did not affect basal

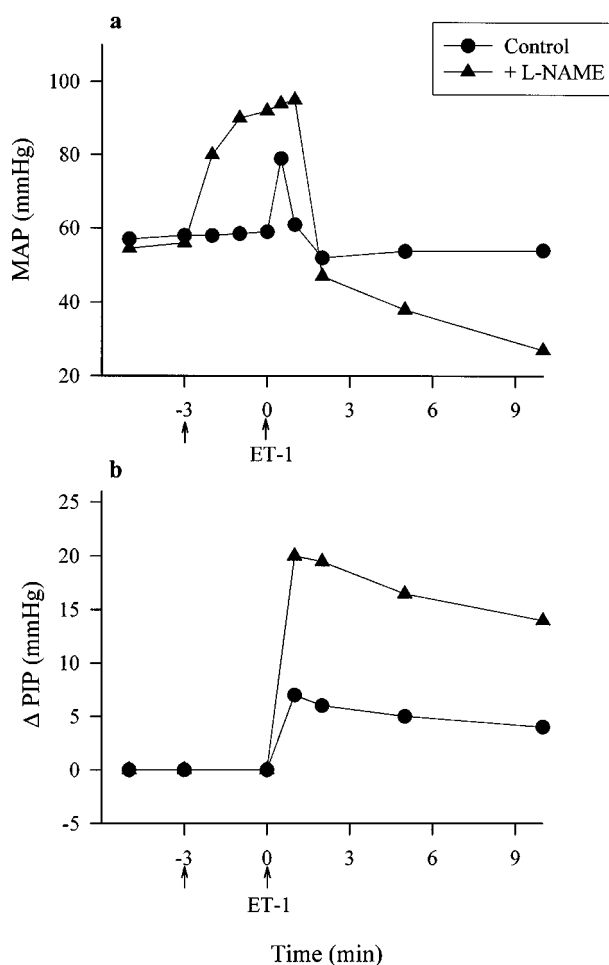


Figure 4 Original recordings showing the effects of an intravenous administration of ET-1 ($0.25 \text{ nmol kg}^{-1}$) alone or in the presence of L-NAME (5 mg kg^{-1} , injected 3 min before ET-1) on mean arterial pressure (MAP) and increase in pulmonary insufflation pressure (Δ PIP) in anaesthetized and ventilated guinea-pigs. The first arrow indicates administration of vehicle or L-NAME at time point -3 .

Table 1 Influence of L-NAME (5 mg kg^{-1}), indomethacin (10 mg kg^{-1}) or BQ-788 (0.25 mg kg^{-1}) on the mean arterial blood pressure (MAP) following an administration (at time point 0) of ET-1 ($0.25 \text{ nmol kg}^{-1}$), IRL 1620 (0.4 nmol kg^{-1}) or U 46619 ($0.57 \text{ nmol kg}^{-1}$) in the anaesthetized guinea-pig

Treatment	MAP (mmHg)						
	-3 min	0 min	0.5 min	1 min	2 min	5 min	10 min
L-NAME	62.7 ± 0.4	63.0 ± 0.7	$68.3 \pm 0.3^*$	$78.3 \pm 5.6^*$	$90.3 \pm 1.6^*$	$92.3 \pm 1.7^*$	$94.8 \pm 2.2^*$
ET-1	60.7 ± 1.2	63.3 ± 1.6	$85.7 \pm 3.7^*$	78.0 ± 5.9	70.0 ± 6.5	63.4 ± 4.9	59.5 ± 1.9
Indomethacin + ET-1	62.4 ± 4.4	65.4 ± 4.7	$75.6 \pm 4.3^*$	$78.2 \pm 3.7^*$	$78.3 \pm 3.2^*$	$79.0 \pm 4.1^*$	$74.9 \pm 5.5^*$
L-NAME + ET-1	62.6 ± 2.3	$88.2 \pm 5.0^\dagger$	95.5 ± 4.8	76.6 ± 9.0	$50.4 \pm 11.0^*$	$48.3 \pm 8.3^*$	$52.4 \pm 8.0^*$
Indomethacin + L-NAME + ET-1	66.2 ± 3.9	$92.5 \pm 1.8^\dagger$	$97.8 \pm 0.7^*$	$97.8 \pm 0.3^*$	$97.5 \pm 0.6^*$	$98.2 \pm 1.5^*$	$97.5 \pm 1.5^*$
BQ-788 + L-NAME + ET-1	60.1 ± 3.5	$85.5 \pm 2.7^\dagger$	$92.5 \pm 2.7^*$	$96.5 \pm 2.7^*$	$98.4 \pm 2.9^*$	$102.2 \pm 3.4^*$	$102.7 \pm 3.2^*$
IRL 1620	57.8 ± 2.1	56.2 ± 1.7	$66.3 \pm 1.9^*$	63.9 ± 4.3	57.6 ± 5.2	54.7 ± 5.6	55.9 ± 2.8
L-NAME + IRL 1620	59.5 ± 3.3	$93.5 \pm 2.5^\dagger$	106.1 ± 3.4	102.8 ± 1.7	84.5 ± 8.2	$59.2 \pm 11.6^*$	77.6 ± 6.7
Indomethacin + L-NAME + IRL 1620	59.6 ± 3.7	$94.2 \pm 3.6^\dagger$	100.8 ± 2.2	96.5 ± 1.4	92.5 ± 1.4	93.9 ± 3.3	95.8 ± 3.2
U 46619	56.0 ± 1.8	55.0 ± 1.4	$67.5 \pm 3.0^*$	$66.8 \pm 2.8^*$	$63.4 \pm 2.6^*$	60.0 ± 3.7	59.5 ± 3.0
L-NAME + U 46619	59.3 ± 3.1	$88.8 \pm 1.5^\dagger$	89.5 ± 0.7	$96.1 \pm 1.8^*$	$95.2 \pm 0.5^*$	$91.7 \pm 1.6^*$	90.4 ± 2.6

Each value represents the mean $\pm$ s.e. mean of at least five experiments. † , $P < 0.05$ when compared at time point -3 . * , $P < 0.05$ when compared to MAP at time point 0.

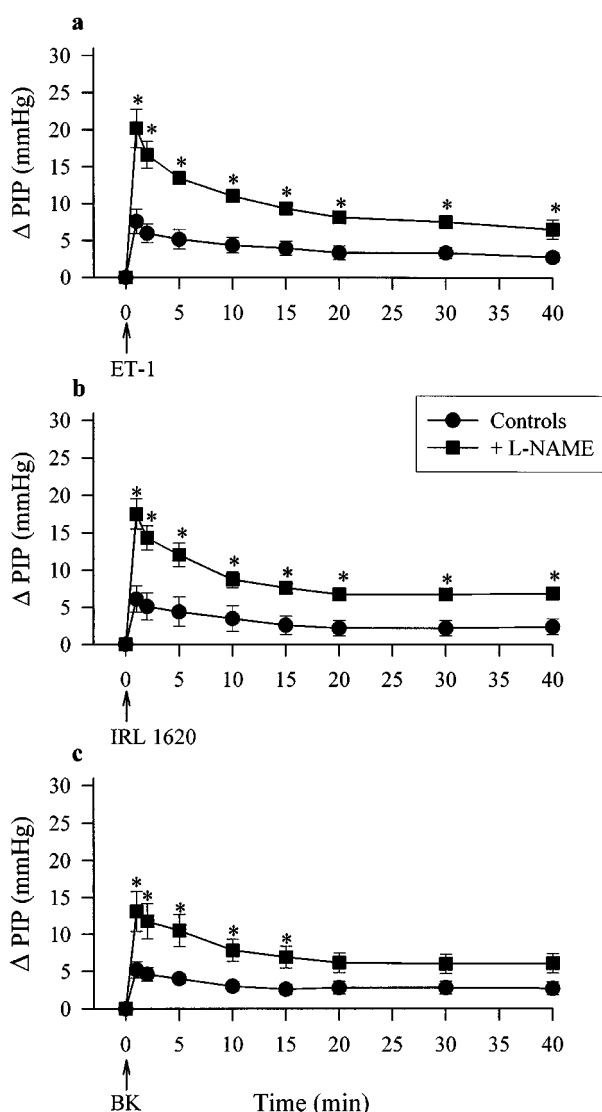


Figure 5 Effect of L-NAME on ET-1 ($0.25 \text{ nmol kg}^{-1}$) (a), IRL 1620 (0.4 nmol kg^{-1}) (b) or BK (1 nmol kg^{-1}) (c) evoked increase of pulmonary insufflation pressure (PIP) in the anaesthetized guinea-pig. Agonists were injected i.v. alone or 3 min after L-NAME administration (5 mg kg^{-1} , i.v.). The arrows indicate the time of administration of the peptides. Each value represents the mean $\pm$ s.e. mean of at least five experiments. *, $P < 0.05$ when compared with the variation of PIP induced without L-NAME at each time point.

PIP or MAP (results not shown). Indomethacin (INDO) fully suppressed the increases in PIP stimulated by ET-1 ($0.25 \text{ nmol kg}^{-1}$), IRL 1620 (0.4 nmol kg^{-1}) or BK (1 nmol kg^{-1}) in either untreated (ET-1: 7.6 ± 1.6 ; + INDO: 0, IRL-1620: 6.1 ± 1.7 ; + INDO: 0, BK: 5.2 ± 1.1 ; + INDO: 0 mmHg, $n = 5$, $P < 0.05$ when compared to control in absence of indomethacin) or L-NAME-treated guinea-pigs. (ET-1: 20.2 ± 2.6 ; + INDO: 0, IRL-1620: 17.5 ± 2.1 ; + INDO: 0, BK: 13.1 ± 2.7 ; + INDO: 0 mmHg, $n = 5$, $P < 0.01$ when compared to control in absence of indomethacin). Moreover, though the cyclo-oxygenase blocker did not change the magnitude of the peak ET-1-induced MAP increase in untreated guinea-pigs, it prolonged the peptide's hypertensive effect substantially (Table 1). Importantly, indomethacin did not modify the hypertensive response to L-NAME administration, yet the typical hypotensive response to subsequent ET-1 injection was abolished and replaced by a discrete additional increase in MAP. Almost identical alterations in MAP

responses were observed when IRL 1620 (Table 1) or BK (i.e. this peptide also induced a small but prolonged hypertensive effect) were injected instead of ET-1 in indomethacin and L-NAME pretreated animals.

An administration of L-arginine (100 mg kg^{-1}), 5 min prior to treatment with L-NAME co-administered with L-arginine (5 and 100 mg kg^{-1} , respectively) in the anaesthetized and ventilated guinea-pig, fully reversed the L-NAME-dependent potentiation of ET-1- ($0.25 \text{ nmol kg}^{-1}$), IRL 1620- (0.4 nmol kg^{-1}) or BK- (1 nmol kg^{-1}) induced increases in PIP (Figure 7), and significantly blunted the hypertensive response to L-NAME as well as the hypotension induced by subsequent ET-1 injection. The points chosen to study the above mentioned three effects, were set in the ED_{50} range, as interpolated from the dose-response curves for each agonist in presence of L-NAME, as illustrated in Figure 6.

On the other hand, pretreatment with the selective ET_B receptor antagonist BQ-788 (0.25 mg kg^{-1}), 2 min prior to L-NAME administration, markedly blunted the PIP responses to ET-1 (ET-1: 20.2 ± 2.6 ; + BQ-788: $1.5 \pm 0.1 \text{ mmHg}$, $n = 5$, $P < 0.01$) as well as its depressor effect on MAP and actually unmasked a pronounced and long-lasting hypertensive response to the peptide (Table 1).

Discussion

Our results demonstrate that endogenous NO potentially downregulates the ability of ET-1 to induce three ET_B receptor-mediated effects, namely the release of TxA_2 from the perfused lung, as well as eicosanoid-dependent bronchoconstriction and hypotension *in vivo* in the guinea-pig. Infusion with the NO-synthase blocker L-NAME selectively enhanced the TxA_2 release triggered by either ET-1 or the selective ET_B receptor agonist IRL 1620 in the guinea-pig perfused lung, without modifying their respective capacity to enhance prostacyclin output. In fact, IRL 1620 does not trigger prostacyclin release in the guinea-pig perfused lung (D'Orléans-Juste *et al.*, 1994). This modulatory influence of NO on ET-1 actions in the pulmonary system appears to be largely restricted to the production of TxA_2 . Furthermore, the finding that L-NAME also affected the eicosanoid-releasing effect of BK in a similar fashion indicates that the inhibitory influence of NO is not restricted to TxA_2 release stimulated *via* ET_B receptors.

As previously reported (D'Orléans-Juste *et al.*, 1994), the guinea-pig perfused lung displays a marked tachyphylaxis as far as ET-1-induced release of prostacyclin is concerned. In addition, L-NAME delayed the ET-1- induced release of the above-mentioned prostanoid. This state of event may not allow us to interpret accurately the potentiating properties of NO-synthase inhibition of the ET-1 induced release of prostacyclin. Similar phenomena occur with regards to the release of TxA_2 stimulated by two consecutive infusions of BK (tachyphylaxis and delayed response in L-NAME-treated lungs). It is however of interest that L-NAME still significantly potentiated the TxA_2 release induced by BK without enhancing the generation of prostacyclin by the same agonist in the perfused lungs. Furthermore, as discussed below, L-NAME also significantly potentiated the dose-dependent increases in PIP triggered by BK *in vivo*.

The bronchoconstrictor effects of intravenously injected ET-1 *in vivo* in the guinea-pig also seem to be mediated importantly, perhaps even exclusively, *via* an ET_B receptor-mediated eicosanoid mechanism. Firstly, they are mimicked by IRL 1620. Secondly, they are abolished following treatment

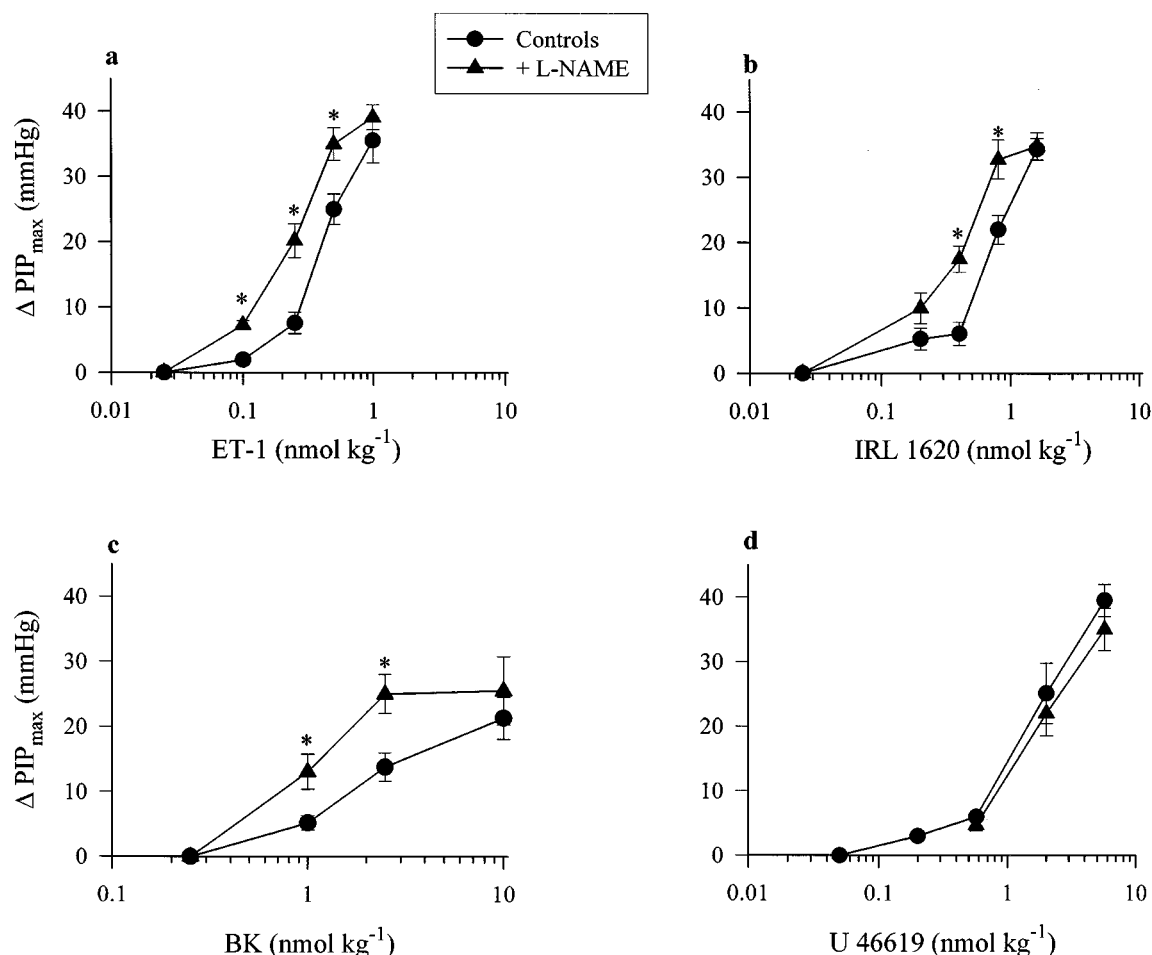


Figure 6 Dose-response curves of endothelin-1 (ET-1) (a), IRL 1620 (b), BK (c) and U 46619 (d) of evoked increase of pulmonary insufflation pressure (PIP) in the absence or presence of L-NAME (5 mg kg⁻¹) in the anaesthetized guinea-pig. Each value represents the mean $\pm$ s.e. mean of at least four experiments. *, $P < 0.05$ when compared with the variation of PIP without L-NAME at each dose.

with either a thromboxane A₂ blocker (L-670,596) (Noguchi *et al.*, 1993), the selective ET_B receptor antagonist BQ-788 (present study) or the cyclo-oxygenase inhibitor indomethacin (Payne & Whittle, 1988; this study). The findings that L-NAME potentiated and prolonged the increases in PIP induced by ET-1 and IRL-1620 (and BK) correlate remarkably well with the results obtained in the perfused lung experiments. The fact that in L-NAME-treated animals the PIP responses to all three peptides were again fully blocked by indomethacin, strongly suggests that selective inhibition by NO of the TxA₂ release stimulated by these agonists (as evidenced in the perfused lung experiments) also plays a major role in limiting their effects on airway resistance *in vivo*. This hypothesis is reinforced by the finding that L-NAME failed to potentiate PIP responses induced by the TxA₂-mimetic compound U 46619, which was also found to be devoid of eicosanoid-releasing properties in the perfused lung. Another noteworthy aspect is that L-NAME did not affect basal TxA₂ release in the perfused lung or PIP *in vitro*, indicating that NO truly modulates the action of eicosanoid-releasing substances in the pulmonary system.

In contrast to its well known sustained hypertensive effect in the rat (Yanagisawa *et al.*, 1988; de Nucci *et al.*, 1988), ET-1 induced only a transitory increase in MAP in the anaesthetized guinea-pig, confirming previous reports (Gratton *et al.*, 1995; Noguchi *et al.*, 1993; Pons *et al.*, 1991a). The current study now reveals that the transient nature of

this effect of ET-1 in the later species is due to ET_B receptor-mediated generation of vasodilator prostanoids which limit its pressor action, because indomethacin treatment prolonged markedly the duration of the elevation of MAP induced by the peptide. Moreover, treatment with L-NAME unveiled pronounced hypotensive actions of ET-1 and IRL 1620, as well as potentiated the already normally hypotensive responses triggered by BK. Since L-NAME, if anything, actually enhanced the pressor response to U 46619, the shift in the profile of activity of ET-1 on MAP afforded by the NO synthase blocker cannot be ascribed solely to the substantial pressor response it causes. In other words, the profile change in ET-1 action is not simply because L-NAME enhances the window for reduction of MAP induced by hypotensive agents. This view is further substantiated by the finding that indomethacin not only abolished the hypotension induced by ET-1 or IRL-1620 in L-NAME-treated animals, but unmasked as well a sustained hypertensive effect of ET-1 which was additive to the elevation of MAP induced by the NO synthase blocker. On the other hand, the findings that IRL 1620 also caused hypotension in L-NAME-treated guinea-pigs, and that MAP responses of these animals to ET-1 were changed by BQ-788 injection in a manner similar to that seen with indomethacin, are strong indications that ET_B receptors are critically involved in mediating the prostanoid-dependent hypotensive effects of both agonists. Our results also suggest that this

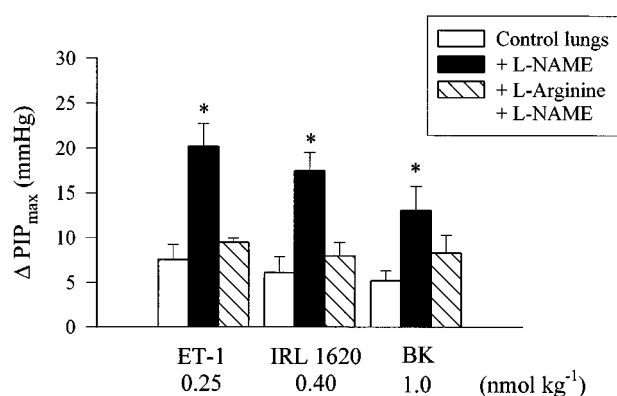


Figure 7 Maximal increase of pulmonary insufflation pressure evoked by an i.v. injection of ET-1 (0.25 nmol kg⁻¹), IRL 1620 (0.4 nmol kg⁻¹) or BK (1 nmol kg⁻¹) in absence or presence of L-NAME alone (5 mg kg⁻¹) or co-administered with L-arginine (100 mg kg⁻¹) in the anaesthetized guinea-pig. Each value represents the mean $\pm$ s.e. mean of at least five experiments. *, $P < 0.05$ when compared with the variation of PIP without L-NAME.

particular pathway is substantially limited by endogenous NO.

Although L-NAME enhanced eicosanoid-dependent ET_B receptor-mediated effects of intravenously injected ET-1 in both the pulmonary and cardiovascular systems, the particular eicosanoids involved in increasing PIP and reducing MAP in this condition are suggested to be distinct. As already discussed, the main eicosanoid mobilized by ET-1 in the lung is likely to be TxA₂, but this potent vasoconstrictor (Hamberg *et al.*, 1975) elevates MAP in the guinea-pig, as evidenced by our results with the TxA₂-mimetic U 46619 and those of Cui *et al.* (1997). Thus, although we have not demonstrated which is the hypotensive prostanoid(s) released *via* ET_B receptors in response to intravenous ET-1 in this species, the two most likely candidates would be prostacyclin and prostaglandin E₂. Either of these vasodilators could act to limit the peptide's pressor activity in the guinea-pig, as previously illustrated in the rat by de Nucci *et al.* (1988). However, prostacyclin release is not coupled to ET_B receptors (present study) and ET-1 is a poor stimulant of prostaglandin E₂ production in the guinea-pig perfused lungs (Botting & Vane, 1990). Based on the above mentioned premises we suggest that the generation of prostacyclin/prostaglandin E₂ and TxA₂ triggered by systemically administered ET-1 in this species occurs at different anatomical sites in the circulation, but NO effectively controls this action in both cases.

Conceivably, the hypotensive effect of ET-1 in L-NAME-treated animals could be ascribed to a sudden reduction in cardiac output, as the peptide can induce an indomethacin- and TxA₂ blocker-sensitive decrease in cardiac output in the guinea-pig heart and lung preparation (Del Basso & Argiolas, 1995). Furthermore, another TxA₂-mimetic, U 44069, reduces cardiac output in thoracotomized guinea-pigs (Dube *et al.*, 1995). However, this action was always associated with an increase in MAP, a result similar to that we obtained using U 46619 in the present study. These observations allow us to dissociate the hypotensive effect of ET-1 from a possible reduction in cardiac output by TxA₂ generated by the peptide.

The alterations caused by L-NAME of ET-1 induced eicosanoid release *in vitro* and PIP and MAP responses *in*

vivo appear to be related to specific inhibition of NO production, as they were all significantly attenuated by concomitant treatment with L-arginine. However, as L-NAME is a non-selective blocker of all NO synthase isoforms (Moore & Handy, 1997), it is not yet possible to pinpoint precisely which of these enzymes are responsible for the NO that normally limits ET_B receptor-mediated eicosanoid release, or which are the cells that express them. In the systemic circulation, eNOS in endothelial cells are an almost certain possibility, but iNOS in pulmonary macrophages, as well as eNOS in other blood borne cells and in airway epithelial cells may also participate importantly. Indeed, removal of the airway epithelium potentiates contractions induced by ET-1 in isolated guinea-pig trachea (Filep *et al.*, 1993).

Interestingly, the NO synthase blocker N^G-monomethyl-L-arginine increases prostaglandin E₂ and TxA₂ production in rat Kupffer cells, resident macrophages of the liver (Stadler *et al.*, 1993). Furthermore, a cross-talk between prostaglandin H synthase-2 (also known as cyclo-oxygenase-2) and NO has only recently been suggested by Gunther *et al.* (1997), in which NO radical may interfere with the activity of the cyclo-oxygenase pathway by nitrotyrosine formation. Also, Swierkosz *et al.* (1995) reported that large amounts of NO inhibit cyclo-oxygenase-2 expression and activity in murine macrophages incubated with bacterial endotoxin. All three of these studies were performed in conditions of upregulated iNOS and/or cyclo-oxygenase-2 activities. Since the experimental conditions adopted in the present study do not favour such an upregulation, we believe that we were dealing with the constitutive eNOS and cyclo-oxygenase-1 enzymes. Based on the later premise, we suggest that in the guinea-pig, NO would interfere with the metabolic pathway of the arachidonic acid cascade, thus acting as a potent modulator of the generation of vasodilatory and bronchoconstrictive eicosanoids stimulated by endothelins or kinins *in vivo*.

In a pathophysiological perspective, there are an increasing number of reports suggesting that variations in NO production, cyclo-oxygenase activity and ET-1 expression may be useful indicators of pulmonary dysfunction, such as in cystic fibrosis and primary pulmonary hypertension (Giaid *et al.*, 1993a,b; 1995; Kuitert *et al.*, 1996; Park *et al.*, 1997). Such considerations warrant further investigation of the possible cross-talk between these three vaso- and broncho-modulatory factors on a functional basis, as attempted in the present study.

In summary, our study demonstrates that endogenous NO limits ET-1-induced bronchoconstriction and pressor effects in the guinea-pig by downregulating the peptide's ability to induce ET_B receptor-mediated release of TxA₂ in the lungs and of vasodilatory prostanoids in the systemic circulation. Furthermore, it would seem that these eicosanoid-dependent actions of ET-1 in the pulmonary system and on systemic arterial resistance in this species are physiologically dissociated.

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Transcriptional down-regulation of the rabbit pulmonary artery endothelin B receptor during phenotypic modulation

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1 We confirmed that endothelium-independent contraction of the rabbit pulmonary artery (RPA) is mediated through both an endothelin A (ET_AR) and endothelin B (ET_{B2}R) receptor.

2 The response of endothelium-denuded RPA rings to endothelin-1 (ET-1, pD₂ = 7.84 ± 0.03) was only partially inhibited by BQ123 (10 μM), an ET_AR antagonist.

3 Pretreatment with 1 nM sarafotoxin S6c (S6c), an ET_BR agonist, desensitized the ET_{B2}R and significantly attenuated the response to ET-3 (pD₂ = 7.40 ± 0.02 before, < 6.50 after S6c).

4 Pretreatment with S6c had little effect on the response to ET-1, but BQ123 (10 μM) caused a parallel shift to the right of the residual ET_AR-mediated response to ET-1 (pD₂ = 7.84 ± 0.03 before S6c, 7.93 ± 0.03 after S6c, 6.81 ± 0.05 after BQ123).

5 Binding of radiolabelled ET-1 to early passage cultures of RPA vascular smooth muscle cells (VSMC) displayed two patterns of competitive displacement characteristic of the ET_AR (BQ123 pIC₅₀ = 8.73 ± 0.05) or ET_{B2}R (S6c pIC₅₀ = 10.15).

6 Competitive displacement experiments using membranes from late passage VSMC confirmed only the presence of the ET_AR (ET-1 pIC₅₀ = 9.3, BQ123 pIC₅₀ = 8.0, S6c pIC₅₀ < 6.0).

7 The ET_AR was functionally active and coupled to rises in intracellular calcium which exhibited prolonged homologous desensitization.

8 Using a reverse transcriptase polymerase chain reaction for the rabbit ET_{B2}R, we demonstrated the absence of mRNA expression in phenotypically modified VSMC.

9 We conclude that the ET_{B2}R expressed by VSMC which mediates contraction of RPA is rapidly down-regulated at the transcriptional level during phenotypic modulation *in vitro*.

Keywords: endothelin receptor; pulmonary artery; vascular smooth muscle; phenotypic modulation; intracellular calcium; mRNA

Abbreviations: ET, endothelin; ETR, endothelin receptor; FBS, foetal bovine serum; GRGESP, Gly-Arg-Gly-Glu-Ser-Pro hexapeptide; RPA, rabbit pulmonary artery; S6c, sarafotoxin 6c; VSMC, vascular smooth muscle cells

Introduction

Three distinct endothelin receptors (ETR) have been sequenced and characterized (Bax & Saxena, 1994). The ET_AR has greater affinity for endothelin-1 (ET-1) than ET-3, whereas the ET_BR has similar affinity for all ET isoforms and sarafotoxin S6c (S6c). A receptor with highest affinity for ET-3, the ET_CR, has been identified in *Xenopus laevis* (Karne *et al.*, 1993) and a response with similar ligand specificity has been described in rabbit saphenous vein (Douglas *et al.*, 1995). On the basis of functional assays and ligand selectivity, subtypes of ET_BRs have been described. The ET_{B1}R on endothelial cells mediates endothelium-dependent relaxation of vascular smooth muscle cells (VSMC) while the ET_{B2}R on VSMC mediates contraction (Warner *et al.*, 1993). Rabbit pulmonary artery (RPA) preparations were among the first described examples of ET_{B2}R-mediated vasoconstriction (Fukuroda *et al.*, 1994; La Douceur *et al.*, 1993; Panek *et al.*, 1992; Warner *et al.*, 1993) but others have also been identified (Lodge & Halaka, 1993; Moreland *et al.*, 1992; Shetty *et al.*, 1993; Sudjarwo *et al.*, 1994; Webb *et al.*, 1993). Whilst BQ788

antagonizes effects attributable to both B receptor subtypes (Ishikawa *et al.*, 1994) the antagonists PD142893 and IRL 1038 have been used to discriminate between them (Sudjarwo *et al.*, 1994; Warner *et al.*, 1993).

The structural basis for the pharmacological differences between ET_BR subtypes is uncertain. All of the sequenced ETRs are members of the G protein-coupled receptor superfamily and low stringency screening has failed to identify multiple genes. The interpretation of standard pharmacological assays is complicated by 'cross-talk' between different receptors in preparations where both the ET_AR and ET_{B2}R are expressed (Clozel & Gray, 1995). Post-translational modifications or alternative mRNA splicing could account for different ligand binding characteristics. However, the only reported splice variant of the ET_BR does not affect the coding region (Cheng *et al.*, 1993) and the binding characteristics of alternatively spliced ET_AR products have not yet been reported (Miyamoto *et al.*, 1996). The inferred protein sequence of a putative human ET_{B2}R was not significantly different from that of the human endothelial ET_{B1}R (Webb *et al.*, 1995). However, a recent study using the endothelin B receptor gene knockout mouse suggests that both ET_{B1}R- and ET_{B2}R-mediated contractions may be due to ET_B receptors derived from the same gene (Mizuguchi *et al.*, 1997).

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Many studies of second messenger systems have been performed using cell lines, including those stably transfected with cloned receptors. Although facilitating biochemical characterization of the responses, they need not have a bearing on the behaviour of normal cells, many of which express more than one receptor (including RPA VSMC). While the events in contractile cells which are coupled to signalling through the ET_AR are relatively well described, particularly calcium transients (Pollock *et al.*, 1995), those activated through the ET_BR have not been well characterized. In attempting to identify second messenger systems coupled to ETRs on normal VSMC from the RPA and the nature of 'cross-talk' between them, we have confirmed that both the ET_B2R and ET_AR mediate RPA contraction. We have shown that the ET_AR expressed in both early and late passage cells is functional and that ligand binding stimulates a rise in intracellular calcium. However, during phenotypic modulation of VSMC early in culture (Campbell *et al.*, 1988), expression of ET_B2R mRNA is rapidly down-regulated, leading to the loss of ET_B2R expression at the cell membrane. We have correlated the down-regulation of the ET_B2R with that of smooth muscle high molecular weight caldesmon, a marker of the contractile phenotype (Birukov *et al.*, 1993; Kashiwada *et al.*, 1997).

Methods

Animals

Main pulmonary arteries were obtained from heparinized (500 U, i.v. male and female New Zealand White rabbits (<1.5 kg) after Nembutal anaesthesia (120 mg pentobarbitone Na, Boehringer Ingelheim, Sydney) according to a protocol approved by the Garvan/St. Vincent's Hospital Animal Experimentation and Ethics Committee.

Organ bath experiments

Endothelium was removed by gentle rubbing of the luminal surface with a wooden swab stick. Rings (2 mm) were suspended at 37°C in 10 ml jacketed organ baths containing freshly prepared Krebs-bicarbonate solution (in mM) NaCl 118, KCl 4.7, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.18, glucose 5, NaHCO₃ 25, CaCl₂·2H₂O 2.54 continuously gassed with 5% CO₂ and 95% O₂. The basal tension was 2.00±0.05 g and isometric tension was measured using Grass FT03 force transducers (Quincy, MA, U.S.A.) coupled to a MacLab data acquisition system (Analog Digital Instruments, Sydney, Australia). In cumulative concentration-response experiments, drugs were administered sequentially after the tension had stabilized (≥2 min). In some experiments rings were pre-treated with 1 nM S6c, an ET_BR-selective agonist, for 30 min prior to the concentration-response protocols.

Tissue culture

VSMC from RPA were obtained from vessels which were washed several times in Hanks balanced salt solution (GIBCO, Life Technologies, Melbourne) and incubated in 1000 u ml⁻¹ collagenase type II (Sigma Chemical Co, Sydney) for 30 min at 37°C to remove endothelium. Strips of vessel wall media were peeled off using watchmakers' forceps, diced and incubated in a solution containing collagenase and 60 u ml⁻¹ porcine pancreatic elastase (Calbiochem-Novabiochem, Sydney) for 2–3 h at 37°C with periodic pipetting. Either the tissue explants or dispersed cells were seeded in

Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin (100 u ml⁻¹), streptomycin (100 µg ml⁻¹), fresh L-glutamine (4 mM, all from GIBCO) and 10% foetal bovine serum (FBS, PA Biologicals, Sydney). Cultured cells expressed smooth muscle actin but not Factor VIII:RAG, an endothelial cell marker, using an indirect immunofluorescence assay.

Expression of high molecular weight caldesmon

At subculture between passages 2–4, a small portion of cells from the main culture batch were seeded into 8-well chamber slides (Nunc, Medos, Sydney). The cells were rinsed twice with phosphate-buffered saline (PBS), and fixed for 10 min in 3% formaldehyde/0.1% glutaraldehyde in PBS. After three washes in PBS, the cells were permeabilized with 0.5% Triton X-100 (Rohm & Haas) in PBS for 30 min. After a further three washes with PBS, mouse monoclonal anti-high molecular weight caldesmon (clone hCD, Sigma Australia) was applied to the cells for 30 min at room temperature in 0.1% bovine serum albumin (BSA, Sigma Australia) in PBS. Antibody binding was detected using FITC-rabbit anti-mouse antibody (Silenus Laboratories, Hawthorn, Australia) for 30 min at room temperature in 0.1% BSA/PBS. Slides were mounted in glycerol containing 1 mg ml⁻¹ paraphenylenediamine (BDH Sydney) and fluorescence visualized using a laser confocal microscope (Molecular Dynamics, Melbourne, Australia).

Whole cell binding assays

Passage 2 cells were seeded into 24-well plates (Corning, Sydney) at 4 × 10⁴ cells well⁻¹ and cultured to confluence (6–8 × 10⁴ cells well⁻¹). The cells were washed twice with binding buffer (phosphate-buffered saline containing CaCl₂ 1 mM, MgCl₂ 0.5 mM and 0.1% BSA) at 30°C (Serradeil-Le Gal *et al.*, 1991). Wells were incubated at 22°C with 1 ml binding buffer containing 10 pM [¹²⁵I]-ET-1 (Amersham, Sydney) ± unlabelled ET-1, BQ123, S6c or an unrelated control hexapeptide of sequence GRGESP (all American Peptide Co, Sunnyvale, CA, U.S.A.). Non-cell-associated (free) counts were determined in the aspirated supernatant which was pooled with two 1 ml washes of binding buffer. Cell associated (bound) counts were determined following solubilization of the cells with two 0.5 ml aliquots of NaOH (0.5 M). Tubes were counted for 1 min in a Packard Cobra Auto Gamma counter (Meriden, CT, U.S.A.).

Membrane preparation and binding assays

Passage 6–8 cells in 150-cm² flasks were rinsed with ice-cold phosphate-buffered saline and scraped from the flask. After two further washes the cell pellet was lysed with HEPES (5 mM) lysis buffer (5 × 10⁸ cells 20 ml⁻¹) according to published protocols (Lagny-Pourmir *et al.*, 1989; Servin *et al.*, 1987). The lysate was pelleted at 24,500 × g for 15 min at 4°C in a Heraeus Sepatech Supafuge (Osterode, Germany) with a HFA20.16 fixed angle rotor. The pellets were resuspended at a cell equivalent concentration of 2 × 10⁷ ml⁻¹ in RBM buffer (Servin *et al.*, 1987) containing (in mM) Tris HCL (pH 7.5) 50, NaCl 100, MgCl₂ 5, EDTA 1; bacitracin 100 µg ml⁻¹, leupeptin 10 µg ml⁻¹ (both Calbiochem, Sydney) and phenylmethylsulphonyl fluoride (100 µM) (Sigma). The crude receptor preparations were then gently syringed with a 23 gauge needle and stored at –70°C in 50 µl aliquots for up to 1 week. Aliquots were thawed and sonicated on ice using a Branson Sonifier 250 (6 × 10 s cycles at 50% power setting). Protein concentration was determined using a bicinchoninic

acid assay kit (Pierce, Lab Supply, Sydney). After the optimum protein concentration had been defined, competitive receptor binding assays were performed according to published protocols (Servin *et al.*, 1987). In a final volume made up to 200 μ l with KRB buffer (see below), membrane protein was added to 15 μ l [^{125} I]-ET-1 (final concentration 30 pM) and 20 μ l of 10 fold dilutions (range 10^{-6} – 10^{-12} M final concentration) of unlabelled competitors ET-1, ET-3, BQ123 and sarafotoxin 6c or control hexapeptide GRGESP (American Peptide Co.). Ligands were diluted in KRB buffer (in mM) (NaCl 68, KCl 2.4, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.6, KH_2PO_4 0.6, NaHCO_3 2.5, glucose 2.8, HEPES 10, CaCl_2 1, 0.2% BSA, 1 mg ml $^{-1}$ bacitracin (Boehringer Mannheim, Sydney), pH 7.4) in Eppendorf microfuge tubes. The tubes were incubated for 2 h in a 37°C waterbath, after which each incubation mix was underlaid with 500 μ l FBS using a 1 ml tuberculin syringe fitted with a 25G needle. The tubes were spun for 10 min at $13,000 \times g$ at 4°C to separate receptor-bound radioactivity in the pellet from unbound radioactivity in the supernatant. The supernatants were aspirated and kept for counting the unbound radioactivity, and the tips of the tubes containing the pellets were cut off for counting the bound radioactivity. Results were expressed as percentage bound/control as a function of ligand concentration.

Intracellular calcium assays

At least 3 days before intracellular calcium assays, passage 2–3 cells (2×10^5 /well) were plated onto glass coverslips and serum-deprived for 24 h in DMEM containing 4% Monomed (Commonwealth Serum Laboratories, Melbourne). They were incubated for 30 min with Fura-2 acetoxymethyl ester (5 μ M) (Fura-2 AM; stock solution 1 mM in dimethyl sulphoxide; Molecular Probes, BioScientific, Sydney) in 4% Monomed and washed twice in perfusion solution (in mM) NaCl 145, KCl 5, HEPES 10, MgCl_2 1.2, CaCl_2 1 and 0.1% bovine serum albumin; pH 7.45). The loaded cells were mounted on the stage of a Nikon Diaphot microscope, modified for microfluorescence studies as previously described (Gibb *et al.*, 1994) and superfused with perfusion solution at 37°C using a peristaltic pump (Ismatech, Extech Equipment, Melbourne). The ratio of fluorescence emission at 510 nm by Fura-2 at excitation wavelengths of 340 nm and 380 nm were used to measure $[\text{Ca}^{2+}]_i$ as previously described (Gryniewicz *et al.*, 1985):

$$[\text{Ca}^{2+}]_i = K \frac{(R - R_{\min})}{(R_{\max} - R)} \cdot \frac{(S_{f2})}{(S_{b2})}$$

where $K = 224$ nM (Fura-2 at 37°C); $R_{\min}$ = ratio value in Ca^{2+} free conditions; $R_{\max}$ = ratio value at a maximal Ca^{2+} concentration; $S_{f2} = 380$ nm reading in low Ca^{2+} conditions (corrected for background) and $S_{b2} = 380$ nm reading in high Ca^{2+} conditions (corrected for background). $R_{\min}$ and S_{f2} were determined at the end of an experiment by perfusing the cells with Ca^{2+} -free perfusion solution containing EGTA (5 mM, Calbiochem) and a divalent cation ionophore, ionomycin (2 μ M, Calbiochem). $R_{\max}$ and S_{b2} were then determined by the addition of CaCl_2 (5 mM) in ionophore-containing perfusion solution.

Ligands and inhibitors

Stock solutions of human ET-1, human ET-3, BQ123, S6c (all American Peptide Co.), adenosine 5'-triphosphate (ATP, disodium salt) and uridine 5'-triphosphate (UTP, monosodium

salt, both from Sigma) were stored at -20°C . For organ bath experiments, drugs were freshly diluted in Krebs bicarbonate solution and for intracellular calcium experiments in perfusion solution.

Data analysis

Organ bath results from at least four experiments are presented as means $\pm$ s.e.mean. Graphs were obtained by fitting the results to a four parameter logistic function (SigmaPlot, Jandel Corporation, San Rafael, CA, U.S.A.). The IC_{50} from binding experiments was calculated using the Inplot program (Graphpad Software, San Diego, CA, U.S.A.).

cDNA preparation

RNA from RPA (four rabbit donors) and cultured VSMC derived from the same RPA (primary through to passage 4 cells) was prepared according to standard procedures (Chomczynski & Sacchi, 1987). RNA pellets were resuspended in 50 μ l diethylpyrocarbonate (Sigma)-treated water. cDNA was prepared from 2 μ g RNA in a 50 μ l reaction volume containing dNTPs (0.25 mM each, Boehringer Mannheim), 200 ng oligo(dT), 4 U AMV reverse transcriptase and 2 U RNasin (all Promega, Sydney).

Reverse Transcriptase-Polymerase chain reaction (RT-PCR)

The rabbit ET_BR was cloned from a rabbit lung cDNA library screened with a PCR product derived from RPA VSMC using human primers (N. Yang *et al.*, in preparation). Specific primers based on the rabbit DNA sequence (forward 5'-ACTGGCCATTTGGAGCTGAGAT and reverse 5'-TTGCATTCCACTCTTCTTTCTTAA) were used to amplify a 430 base pair product, which spans an intron, from cDNA prepared from each RPA and cultured VSMC cells at each passage derived from the same RPA. Rabbit GAPDH was used as a control (forward 5'-GATGCTGGTGCCGAGTACGTGG and reverse 5'-TCAGCAACGCATCCTGCAC-CAC) for the integrity of the cDNA preparation. Reactions (50 μ l) were performed using 5 μ l cDNA, dNTPs (0.25 mM), Mg^{2+} for GAPDH (1.5 mM), or Mg^{2+} for ET_BR (2.5 mM and 1–2 U Taq polymerase (Boehringer Mannheim). The cycle conditions for ET_BR were: 95°C, 3 min; 57°C, 30 s, 75°C, 30 s, for 1 cycle; 95°C, 1 min; 57°C, 30 s; 75°C, 30 s for 28 cycles; 95°C, 1 min; 57°C, 30 s; 75°C, 7 min for 1 cycle final extension. The cycle conditions for GAPDH were 95°C, 1 min; 62°C, 30 s; 72°C, 30 s for 1 cycle; 95°C, 30 s; 62°C, 30 s; 72°C, 30 s for 32 cycles; 95°C, 30 s; 62°C, 30 s; 72°C, 5 min for 1 cycle final extension. RT-PCR products were run on 1.8% agarose gels (Amresco, Astral Scientific, Sydney) in Tris-borate-EDTA buffer and stained with ethidium bromide for visualization under UV light. Gels were photographed using a Polaroid MP-4 camera and type 665 film.

Results

ET-mediated contraction of rabbit pulmonary artery

Cumulative concentration-response experiments using ET-1 and ET-3, the latter being relatively selective for the ET_BR , confirmed that they both stimulated endothelium-independent contraction via ET_AR or ET_BR (ET-1 $\text{pD}_2 = 7.84 \pm 0.03$, $n = 8$; ET-3 $\text{pD}_2 = 7.4 \pm 0.02$, $n = 9$, Figure 1A). Pretreatment of

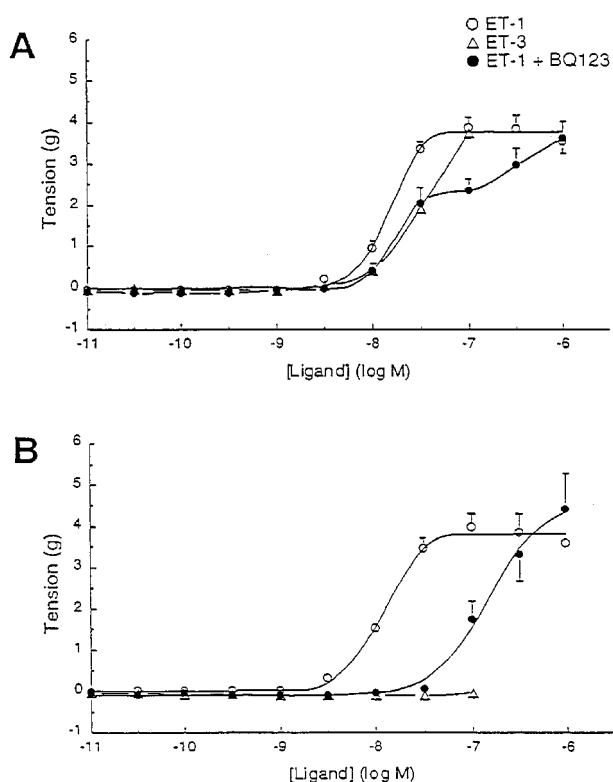


Figure 1 Contraction of endothelium-denuded RPA rings with increasing concentrations of (A) ET-1 ($n=8$), ET-3 ($n=9$) and ET-1 + 10 μM BQ123 ($n=8$), and (B) ET-1 ($n=4$), ET-3 ($n=3$) and ET-1 + 10 μM BQ123 ($n=4$) after pretreatment with S6c.

endothelium-denuded rings with the ET_B R-selective agonist S6c (1 nM) abolished the contractile response to ET-3 ($\text{pD}_2 < 6.50$, $n=3$, Figure 1B). However, pretreatment with S6c had no effect on the response subsequently induced by ET-1 ($\text{pD}_2 = 7.93 \pm 0.03$ after S6c, $n=4$, Figure 1B). The dose-response curve to ET-1 was bi-sigmoidal in the presence of the ET_A R-specific antagonist BQ123 (Ihara *et al.*, 1992; Figure 1A, $n=8$), suggesting that the contractile response to ET-1 was mediated *via* activation of both ET_A R and ET_B R. A parallel shift to the right of the response to ET-1 in the presence of BQ123 followed desensitization with S6c ($\text{pD}_2 = 6.81 \pm 0.05$, $n=4$, Figure 1B), indicating a pure ET_A R-mediated response.

Expression of high molecular weight caldesmon

Due to the small numbers of cells available at early passages, RPA VSMC were not examined immunohistochemically until passage 2 (Figure 2). At this stage, approximately 30% of cells stained positively for high molecular weight caldesmon, the staining being associated with cytoplasmic filaments running the length of the cells (Figure 2A). Thus, by passage 2, most of these cells had down-regulated their expression of high molecular weight caldesmon. In passage 3 and 4 cells (Figure 2B and C, respectively), caldesmon staining was localized around the nuclei, with little cytoplasmic staining present.

Down-regulation of ET_B R expression in cultured vascular smooth muscle cells

Small scale binding studies were used to identify the receptors expressed on low numbers of early passage adherent cells. Binding of radio-iodinated ET-1 increased over 1.5–2 h and

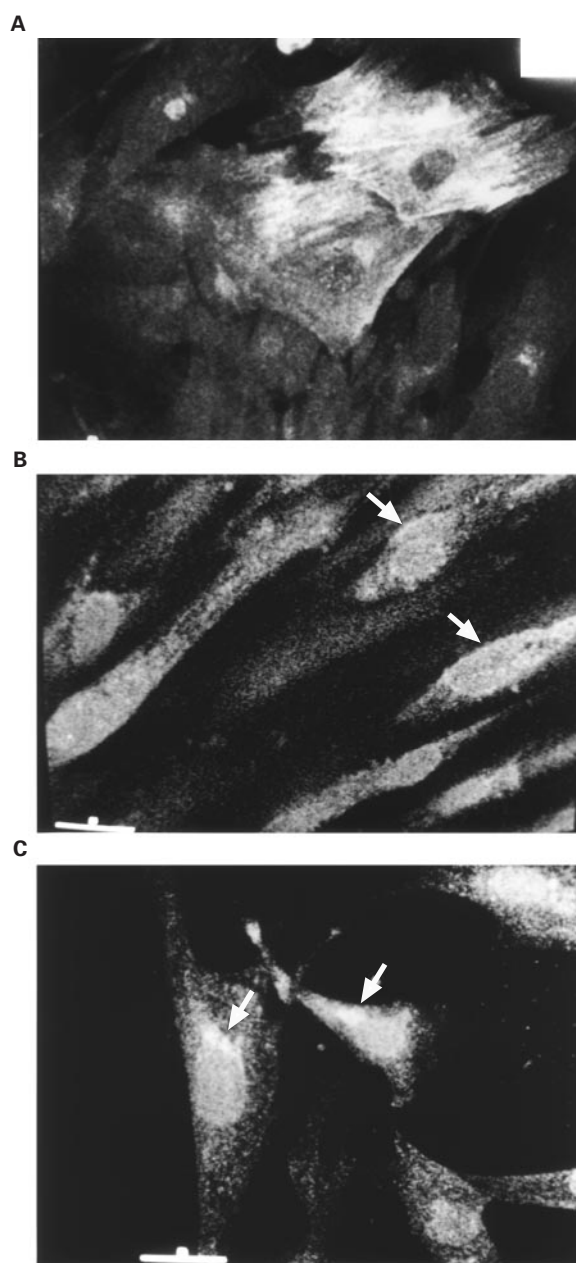


Figure 2 Expression of high molecular weight caldesmon. (A) Caldesmon immunofluorescence is associated with cytoplasmic filaments. Only 30% of cells exhibit positive staining at passage 2. Caldesmon expression is only perinuclear at passages 3 and 4 (arrows, B and C, respectively). Note that the perinuclear staining in the passage 4 cells extends slightly into the extended pseudopodia (arrows). Scale bar = 10 μm .

was specifically inhibited by unlabelled ET-1 (results not shown). Competition binding studies were performed using ET-1 and the ET_A R-selective ligands, BQ123 and S6c respectively. Representative experiments are shown in Figure 3 and the results of six experiments are summarized in Table 1. Individual cultures from single rabbits displayed two patterns of binding which were typical of either the ET_A R or ET_B R (Figure 3). Figure 3A illustrates competitive displacement by ET-1 ($\text{pIC}_{50} = 10.2$) and BQ123 ($\text{pIC}_{50} = 8.7$) but not S6c ($\text{pIC}_{50} < 6.0$) while Figure 3B illustrates competitive displacement by ET-1 ($\text{pIC}_{50} = 10.4$) and S6c ($\text{pIC}_{50} = 8.6$) but not BQ123 ($\text{pIC}_{50} < 6.0$). In experiments demonstrating ET_A R binding ($n=4$, preparations 2 and 4–6,

Table 1) the mean pIC_{50} for BQ123 was 8.73 ± 0.05 , while in experiments demonstrating ET_B R binding ($n=2$, preparations 1 and 3, Table 1) the mean pIC_{50} for S6c was 10.15 ($n=2$). The mean pIC_{50} s for ET-1 were 9.95 ± 0.25 and 10.25 respectively.

ET_AR expression in late passage vascular smooth muscle cells

Given the small number of cells obtained from a single rabbit, we were unable to follow the pattern of binding sequentially in cultured cells derived from the same animal. However, there were sufficient cells from late passages (6–8) to obtain

satisfactory membrane preparations for competitive binding studies ($n=3$). These cells were clearly 'synthetic' as judged by their morphological characteristics (transition from spindle-shaped to polygonal cells, cellular hypertrophy) and decreased expression of high molecular weight caldesmon isoforms by immunofluorescence. These binding studies demonstrated concentration-dependent inhibition of radiolabelled ET-1 binding by unlabelled ET-1 ($pIC_{50}=9.3$) and BQ123 ($pIC_{50}=8.0$) but not S6c (Figure 4). This was consistent with the binding characteristics of an ET_A R. Thus phenotypic modulation of RPA SMC is associated with down-regulation of ET_B R ligand binding.

ET_AR-mediated $[Ca^{2+}]_i$ responses in cultured vascular smooth muscle cells

In passage 2 and 3 cultured cells ET-1 (200 nM) induced a typical biphasic rise in $[Ca^{2+}]_i$ (Figure 5A). The resting $[Ca^{2+}]_i$ in RPA VSMC was 60–100 nM with a peak response up to ~500 nM. Oscillations with a periodicity of ~3 min were occasionally observed, particularly in cultures approaching confluence (data not shown). In keeping with the binding studies which suggested that the ET_A R was the predominant receptor expressed by cultured RPA VSMC, the ET_B R-selective agonists S6c and ET-3 failed to induce a significant rise in $[Ca^{2+}]_i$ (Figure 5B). Whereas the ET-1 response demonstrated homologous desensitization, the responses to P_{2Y} purinoceptor agonists (ATP and UTP) were unaffected by pre-exposure to ET-1 (Figure 5A and B, respectively). We have shown previously that the P_{2Y2} receptor on RPA does not demonstrate homologous desensitization (Qasabian *et al.*, 1997). The $[Ca^{2+}]_i$ response to 200 nM ET-1 was completely abolished by pretreatment and co-perfusion with 1 μ M BQ123 (Figure 6). This confirms that the ET-1 induced rise in $[Ca^{2+}]_i$ is mediated through the ET_A R. There was no significant difference in the responses of early versus late passage cells (data not shown).

Mechanism of ET_BR down-regulation during phenotypic modulation

A reverse transcriptase-PCR assay was developed to examine mRNA expression on small numbers of cells using primers designed from the sequence of the cloned RPA ET_B R (N. Yang *et al.*, in preparation). In preliminary experiments we

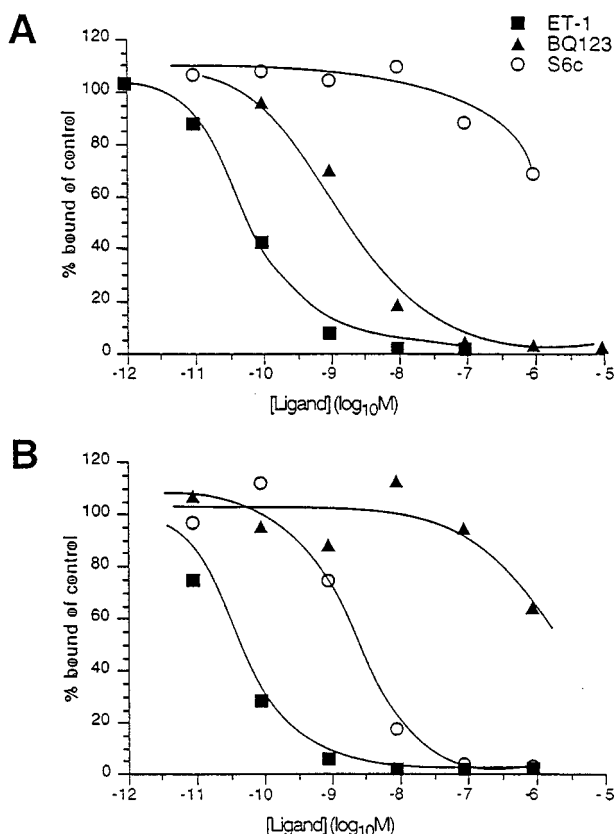


Figure 3 Inhibition of $[^{125}I]$ -ET-1 binding to whole, early passage VSMC in the presence of increasing concentrations of unlabelled ET-1, BQ123, or S6c. Representative experiments are illustrated which demonstrate binding typical of the ET_A R (A) and ET_B R (B).

Table 1 Competitive displacement of $[^{125}I]$ -ET-1 (10 pM) by unlabelled ligands in six early passage whole cell binding experiments

Expt #	ET-1 (pIC_{50})	BQ123 (pIC_{50})	S6c (pIC_{50})
1	10.4	<6.0	8.6
3	10.1	<6.0	11.7
mean	10.25	<6.0	10.15
2	10.2	8.7	<6.0
4	9.6	8.7	<6.0
5	10.0	8.8	<6.0
6	10.0	8.7	<6.0
mean	9.95 ± 0.25	8.73 ± 0.05	<6.0

Experiments 1 and 3 show evidence for ET_B R in early passage RPA VSMC; experiments 2 and 4–6 show evidence for ET_A R only.

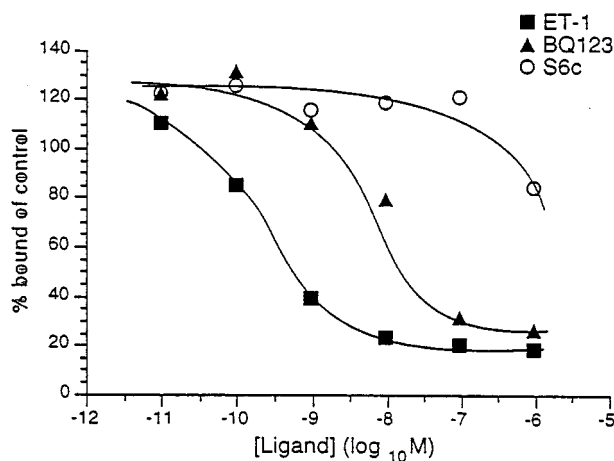


Figure 4 Inhibition of $[^{125}I]$ -ET-1 (30 pM) binding to late passage (P6) VSMC membranes ($n=3$) by increasing concentrations of unlabelled ET-1, and BQ123 but not S6c.

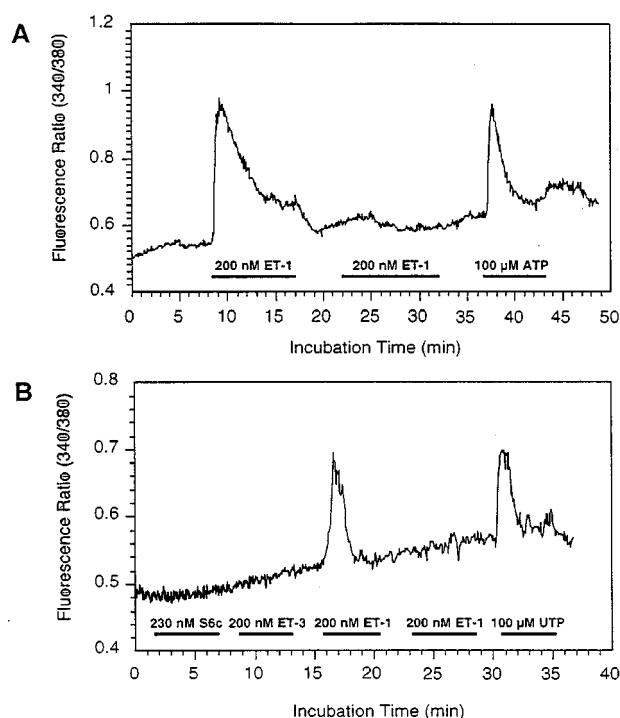


Figure 5 Homologous desensitization of the intracellular calcium response in passages 2 and 3 RPA VSMC to ET-1 (A,B) and no response to the ET_B R-selective agonists, S6c and ET-3 (B). Responses to the P_2 purinoceptor agonists ATP and UTP were unaffected by previous exposure to 200 nM ET-1.

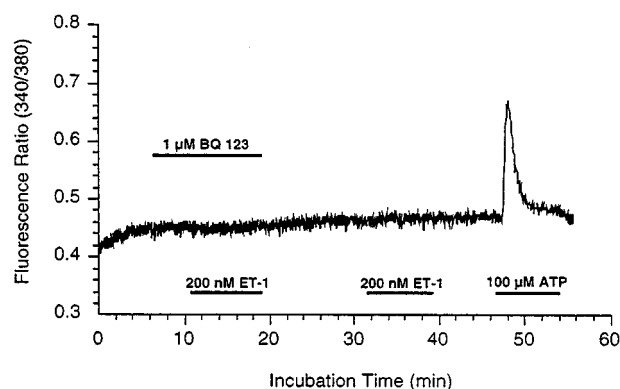


Figure 6 Abolition of the intracellular calcium response in passage 2 and 3 RPA VSMC to ET-1 (200 nM) following preincubation and co-perfusion with the ET_A R-selective antagonist, BQ123 (1 μM).

demonstrated that there was no significant contamination with genomic DNA and that the exponential phase of the reaction extended to 30–35 cycles. We examined ET_B R expression in RPA from four rabbits and series of cultured VSMC derived from these. ET_B R mRNA was present in RNA prepared from RPA, and rabbit lung (used as positive control) but was not detected in cultured VSMC (primary culture through to 4th passage) derived from the RPA (Figure 7). GAPDH PCR confirmed the integrity of the cDNA in these samples. Down-regulation of the ET_B R during phenotypic modulation thus appears to be at the level of mRNA transcription.

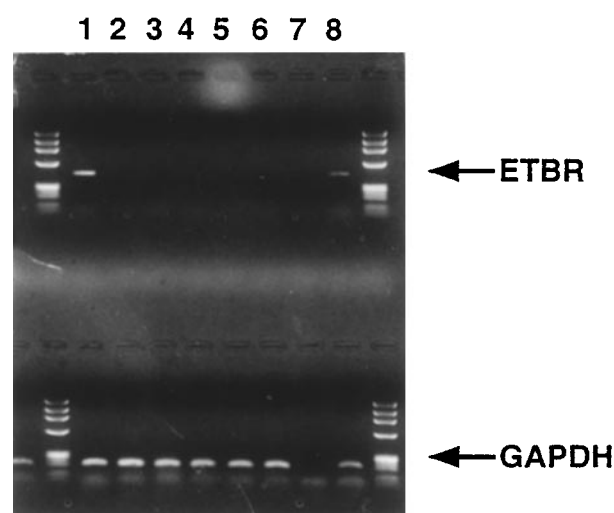


Figure 7 Reverse transcription-polymerase chain reactions for rabbit ET_B R (upper panel) and GAPDH (lower panel) using RPA ($n=4$) and derived VSMC. Lane 1: RPA; lane 2: primary cultured VSMC; lanes 3–6: passages 1–4 of VSMC; lane 7: negative control; lane 8: positive control.

Discussion

In this paper we describe several series of experiments which provide evidence for the rapid transcriptional down-regulation of the ET_B R and its functional consequences upon initiation of *in vitro* culture of RPA VSMC. By several overlapping lines of evidence we show that: (i) both ET_A R and ET_B R are present and functional in intact RPA (organ bath studies, RT-PCR); (ii) ET_B R is present in a minority of early passage (passage 2) RPA VSMC cultures (ligand binding) and (iii) ET_B R is absent in late passage (passage 6, synthetic) cultured RPA VSMC (ligand binding, RT-PCR). This represents a spectrum of ET_B R expression, ranging from its functional presence in VSMC of the contractile phenotype, to its absence in synthetic or proliferative VSMC. Given that significant differences between similar rabbit vascular preparations have been reported in different laboratories (Douglas *et al.*, 1995; Gray *et al.*, 1994), it was important for us to confirm the characteristics of ET_A R- and ET_B R-mediated contraction of the RPA. The maximum tension generated by ET-1, a non-selective agonist, was similar whether or not the ET_B R had been desensitized by prior exposure to S6c (Figure 1). Blockade and/or desensitization of both receptors was necessary to block the action of ET-1 in the RPA. Similar functional responses to ET-1-induced contraction have been reported in rat trachea which is also known to express both ET_A R and ET_B R (Clozel & Gray, 1995; Henry, 1993). Activation of either receptor in the RPA produces a similar contractile response, which is compatible with a model of receptor 'cross-talk' (Clozel & Gray, 1995).

In attempting to define the second messenger system(s) coupled to the ET_B R, we found that the ET_A R mediated increases in $[Ca^{2+}]_i$ in cultured cells at passage 2 and 3. Whereas ET_B R ligand binding patterns were observed in 2/6 membrane preparations from passage 2 cultured cells, neither of the ET_B R-selective agonists S6c nor ET-3 elevated $[Ca^{2+}]_i$ in Fura-2 loaded cells from early or late passage indicating that the ET_B R was rapidly down-regulated. Previous binding studies on RPA membrane preparations have estimated the proportions of ET_B R and ET_A R to be 60:40 (La Douceur *et*

et al., 1993) or 77:23 (Fukuroda *et al.*, 1994). Since both the ET_AR and ET_BR are biochemically and functionally demonstrable on the RPA, this implied that in cultured VSMC either the ET_BR was not coupled to [Ca²⁺]_i responses or that it was down-regulated *in vitro*. Therefore we systematically examined its expression *in vitro* using small scale binding assays and microfluorescence techniques in order to study individual animals. Although we did demonstrate the predominant expression of the ET_BR in binding experiments in some cultures of early passage RPA VSMC, the majority demonstrated competition binding typical of an ET_AR. These differences arose despite careful control of assay conditions. We never saw a mixed pattern of ET receptor responses and we inferred that there must have been a relatively rapid down-regulation of ET_BR expression during early culture. In keeping with this, membrane preparations from later passage cells whose phenotype had clearly become synthetic only expressed ET_AR. Although data from the RT-PCR indicated that ET_BR transcription was rapidly down-regulated after initiation of *in vitro* culture, we attributed the presence of the ET_BR in some of the early passage binding studies to the persistence or slow turnover of the ET_BR at the cell surface in the absence of transcription.

Two previous studies have addressed changes in ET receptor expression in cultured VSMC. Contractile rabbit aortic VSMC express a high affinity ET_AR which is down-regulated in parallel with phenotypic modulation, but differences in the expression of receptor subtype as found in this study were not identified (Serradeil-Le Gal *et al.*, 1991). Cultured rat aortic VSMC express predominantly the ET_AR during passages 10–15, but later passage cells (>30) expressed both the ET_AR and ET_BR (Eguchi *et al.*, 1994). However, phenotypic modulation of VSMC occurs within days, rather than weeks of culture (Campbell *et al.*, 1988). Therefore, these observations on late passage cells are unlikely to be relevant to the process of phenotypic modulation.

Because the ET_BR is down-regulated rapidly in culture, we have not been able to study the second messenger system(s) to which it is coupled. However, we have shown that the ET_AR is coupled to intracellular calcium transients (Pollock *et al.*, 1995). The rise in [Ca²⁺]_i seen in response to ET-1 would be sufficient to activate intracellular calcium-dependent protein kinases and calmodulin. The ET_AR characteristically demonstrated rapid and prolonged homologous desensitization, the mechanism of which probably involves receptor phosphorylation (Cyr *et al.*, 1993). In this study, the mechanism of ET_BR down-regulation *in vitro* occurs at the transcriptional level, and in parallel with VSMC phenotypic modulation, suggesting the existence of a local factor maintaining expression of ET_BR *in*

vivo. This phenomenon is distinct from the well-described process of agonist-mediated down-regulation of G protein linked receptors modelled by other groups using β -adrenergic receptors (Karoer *et al.*, 1996), and resembles the phenotype-related alteration of atrial natriuretic peptide receptor expression in rat aortic SMC (Suga *et al.*, 1992). In this study we made no attempts to re-induce the contractile phenotype in these cells through manipulation of culture conditions in order to maintain expression of the ET_BR.

Phenotypic modulation of VSMC *in vitro* from the 'contractile' to the 'synthetic' phenotype is thought to reflect the changes seen in certain pathophysiological states, such as atherosclerosis and vascular remodelling. However, experimental evidence concerning the role of ETR subtypes in these conditions is scant and conflicting. Winkles *et al.* (1993) found that ET_BR mRNA was down-regulated in 6/7 human aortic atherosclerotic plaque specimens compared to normal aortas, however the authors acknowledged that the cell types involved were not known. In contrast, Dagassan *et al.* (1996) found that ET_BR were up-regulated in human atherosclerotic coronary arteries, but again, no cell type was implicated in this up-regulation. Bacon *et al.* (1995) found that ET_BR was expressed only in areas of neovascularization in human atherosclerotic coronary arteries, co-localizing with von Willebrand factor staining, suggesting that ET_BR expression was found only on endothelial cells. The increase in pulmonary vascular resistance in patients with pulmonary hypertension (Wagenvoort & Mooi, 1989) and in animal models of the disease is associated with structural changes in the resistance vessels. Phenotypic modulation of the VSMC and their migration into the intima contribute to luminal narrowing in pulmonary hypertension and local production of ET-1 may play a role in this process (Giaid *et al.*, 1993; Stewart *et al.*, 1991). Given the potential therapeutic applications of selective ET receptor antagonists in such conditions, it is important to recognize that the target receptor may be different from that expressed in normal cells. The identification of factors which regulate ET_BR expression in this system should facilitate further studies on the interaction between the ETRs.

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Role of iNOS in the vasodilator responses induced by L-arginine in the middle cerebral artery from normotensive and hypertensive rats

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1 The substrate of nitric oxide synthase (NOS), L-arginine (L-Arg, 0.01 μ M–1 mM), induced endothelium-independent relaxations in segments of middle cerebral arteries (MCAs) from normotensive Wistar-Kyoto (WKY) and hypertensive rats (SHR) precontracted with prostaglandin $F_{2\alpha}$ (PGF_{2 α}). These relaxations were higher in SHR than WKY arteries.

2 L-N^G-nitroarginine methyl ester (L-NAME) and 2-amine-5,6-dihydro-6-methyl-4H-1,3-tiazine (AMT), unspecific and inducible NOS (iNOS) inhibitors, respectively, reduced those relaxations, specially in SHR.

3 Four- and seven-hours incubation with dexamethasone reduced the relaxations in MCAs from WKY and SHR, respectively.

4 Polymyxin B and calphostin C, protein kinase C (PKC) inhibitors, reduced the L-Arg-induced relaxation.

5 Lipopolysaccharide (LPS, 7 h incubation) unaltered and inhibited these relaxations in WKY and SHR segments, respectively. LPS antagonized the effect polymyxin B in WKY and potentiated L-Arg-induced relaxations in SHR in the presence of polymyxin B.

6 The contraction induced by PGF_{2 α} was greater in SHR than WKY arteries. This contraction was potentiated by dexamethasone and polymyxin B although the effect of polymyxin B was higher in SHR segments. LPS reduced that contraction and antagonized dexamethasone- and polymyxin B-induced potentiation, these effects being greater in arteries from SHR.

7 These results suggest that in MCAs: (1) the induction of iNOS participates in the L-Arg relaxation and modulates the contraction to PGF_{2 α} ; (2) that induction is partially mediated by a PKC-dependent mechanism; and (3) the involvement of iNOS in such responses is greater in the hypertensive strain.

Keywords: Rat middle cerebral artery; inducible NO synthase; L-arginine; lipopolysaccharide; dexamethasone; polymyxin B; protein kinase C

Abbreviations: AMT, 2-amine-5,6-dihydro-6-methyl-4H-1,3-tiazine; BK, bradykinin; iNOS, inducible nitric oxide synthase; KHS, Krebs-Henseleit solution; L-Arg, L-arginine; LPS, lipopolysaccharide, L-NAME, L-N^G-nitroarginine methyl ester; MCA, middle cerebral artery; NO, nitric oxide; NOS, nitric oxide synthase; PGF_{2 α} , Prostaglandin $F_{2\alpha}$; PKC, Protein Kinase C; SHR, spontaneously hypertensive rats; WKY, Wistar Kyoto rats

Introduction

The generation of nitric oxide (NO) from L-arginine (L-Arg) by NO synthase (NOS) plays an essential role in the regulation of cerebrovascular tone under both physiological and pathological conditions (Faraci, 1993; Iadecola *et al.*, 1994). Three distinct genes encoding NOS isoforms have been cloned (Moncada & Higgs, 1993; Faraci & Brian, 1994; Marín & Rodríguez-Martínez, 1997). In cerebral arteries, the NOS constitutive (eNOS), present in endothelium and perivascular nerves (Katusic *et al.*, 1993; Chen & Lee, 1995), produce NO in basal conditions and in response to several physiological stimuli (Faraci, 1993). In contrast, the inducible form of NOS (iNOS) produces greater quantities of NO for long periods in response to bacterial lipopolysaccharide (LPS) and cytokines in smooth muscle, macrophages, neutrophils and endothelium (Faraci & Brian, 1994; Marín & Rodríguez-Martínez, 1997).

Because of the role of NO in the physiological control of blood pressure, alterations in NO synthesis may be an important factor in the pathogenesis of hypertension (Mon-

cada *et al.*, 1991; Welch & Loscalzo, 1994; Marín & Rodríguez-Martínez, 1997). Thus, an impairment of endothelium-dependent relaxations in some vessels, including the cerebral ones, from spontaneously hypertensive rats (SHR) has been reported (see Marín & Rodríguez-Martínez, 1997). This suggests that endothelial function could be affected in hypertension. Moreover, it has been reported that L-Arg induces inhibition of vasoconstriction and relaxation of precontracted vessels by an endothelium-independent mechanism mediated by NO formation (Schini & Vanhoutte, 1991; Moritoki *et al.*, 1992; Jovanovic *et al.*, 1994; Laing *et al.*, 1995; Alonso *et al.*, 1998). The NOS isoform responsible for this NO production has been suggested to be of inducible type (Moritoki *et al.*, 1992; Jovanovic *et al.*, 1994; Laing *et al.*, 1995; Alonso *et al.*, 1998). The involvement of the iNOS in the physiological control of blood pressure is still unclear. Some authors have described an increase of both basal iNOS expression and activity in SHR (Junquero *et al.*, 1992; Wu *et al.*, 1996; Chou *et al.*, 1998) probably to compensate the blood pressure increase. However, other investigators have suggested that although iNOS expression is similar in vascular smooth muscle of SHR and Wistar-Kyoto (WKY) rats, there are differences in the regulation of transcription of iNOS mRNA

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(Singh *et al.*, 1996). It has been also proposed that changes in vascular tone regulation observed in hypertension, may be due to a deficiency of NOS substrate, as L-Arg supplied to hypertensive patients reduces the high blood pressure (Marín & Rodríguez-Martínez, 1997). It is also possible that these changes are due to an alteration of iNOS that modifies its ability to synthesize NO from L-Arg in arteries subjected to hypertension, a mechanism still unexplored in cerebral vessels.

The aim of the present study was to further study the capacity of L-Arg to induce relaxations in segments of the middle cerebral artery (MCA) from WKY and SHR and the mechanisms involved in such responses, specially the iNOS involvement.

Methods

Male WKY and SHR 6-month-old rats were obtained from colonies maintained at the Animal Quarter of the Facultad de Medicina of the Universidad Autonoma of Madrid. Rats were anaesthetized with diethyl ether (Panreac, Barcelona, Spain) and systolic arterial pressure was registered by cannulating the carotid artery, and connecting the cannula to a pressure transducer (Letica, Barcelona, Spain) and this in turn to a recorder (Letica Polygraph 2006). The average systolic pressure of the animals (mean $\pm$ s.e.mean in mm of Hg) was 169.7 ± 6.9 and 111.7 ± 20.8 for SHR and WKY rats, respectively ($P < 0.05$). The rats were sacrificed by bleeding and the brain removed and immersed in cold Krebs-Henseleit solution (KHS) bubbled with a 95% O₂–5% CO₂ mixture. Then, the right and left MCAs were carefully dissected, divided in segments of 2 mm in length and transferred to the organ bath of a dual isometric myograph (JP-Trading, Denmark). Two tungsten wires (40 μ m diameter) were introduced through the lumen of the segments and mounted according to the method described by Mulvany & Halpern (1976).

After a 30 min equilibration period in oxygenated KHS at 37°C and pH = 7.4, segments were stretched to their optimal lumen diameter for active tension development. This was determined based on the internal circumference-wall tension ratio of the segments by setting their internal circumference, L_o , to 90% of which the vessels would have had if they were exposed to a passive tension equivalent to that produced by a transmural pressure of 100 mm Hg (Mulvany & Halpern, 1977). The effective lumen diameter was calculated as $L_o \pi^{-1}$.

Afterwards, segments were washed three times and left to equilibrate for 30 min; then, segments contractility was tested by an initial exposure to a high K⁺ solution (120 mM K⁺-KHS).

At the end of the experiment, segments were washed and when basal tone was reached, KHS was replaced by 120 mM K⁺-KHS; once the contraction was stable, 0.1 mM papaverine was added.

The presence of endothelium was determined by the ability of 1 μ M bradykinin (BK) to produce relaxation in segments precontracted with 10 μ M prostaglandin F_{2 α} (PGF_{2 α}). In some experiments, endothelium was removed by the technique described by Osol *et al.* (1989). A human hair was washed in ethanol and rinsed in KHS before being inserted into the lumen of the segment mounted under tension in the myograph.

Experimental design

Once determined the presence of endothelium, rings were contracted with 10 μ M PGF_{2 α} and after a stable plateau was reached, a cumulative concentration-response curve to L-Arg

(0.01 μ M–1 mM) was performed in the presence or absence of different drugs. Steady-state response at each concentration was reached at 3 min, and this was the response measured. Only one concentration-response curve to L-Arg was achieved with each segment because of the reduction of responses in successive concentration-response curves. Control and treated rings obtained from the same animal were studied in parallel.

To determine the role of prostanoids or superoxide anions on the response induced by L-Arg, segments were incubated for 30 min with indomethacin, a cyclo-oxygenase inhibitor, or with the scavenger of these anions, superoxide dismutase (SOD) before the concentration-response curve to L-Arg was performed.

To assess the involvement of NO on the response to L-Arg, some segments were incubated for 30 min with the unspecific NOS inhibitor L-N^G-nitroarginine methyl ester (L-NAME) or with the selective inhibitor of iNOS, 2-amine-5,6-dihydro-6-methyl-4H-1,3-tiazine (AMT), before the concentration-response curve to L-Arg was performed.

The participation of iNOS on this response was also checked by incubation of segments with dexamethasone, a compound able to inhibit NOS induction or with LPS, a stimulator of iNOS. The involvement of PKC was checked by incubation with polymyxin B or calphostin C, inhibitors of this enzyme. To test the hypothesis that small contaminant endotoxin concentrations in KHS can contribute to the relaxation induced by L-Arg, experiments were performed in the presence of gentamycin plus ampicillin. The ability of LPS to reverse the effect of dexamethasone or polymyxin B was also tested in experiments in which it was added associated with dexamethasone or polymyxin B. Dexamethasone, LPS, polymyxin B and gentamycin plus ampicillin were added to the bath since the time the brain was removed from the animal and maintained throughout the experiment. The period of incubation of the arteries with these drugs, before the concentration-response curve to L-Arg, was of 4 or 7 h. Calphostin C was added to the bath 1 h before the curve to L-Arg; higher incubation times were not used because of the marked inhibition of contractile responses.

Data analysis and statistics

Vasoconstrictor responses to PGF_{2 α} were expressed as active wall tension (calculated as the increase in vessel wall force above resting level divided by twice the vessel segment length), as a percentage of the maximum contraction (determined by the difference between the tone generated by 120 mM K⁺ and that produced by 0.1 mM papaverine) or as a percentage of the corresponding parallel control response. Vasodilator responses to L-Arg were expressed as a percentage of the previous tone induced by PGF_{2 α} . The concentrations of L-Arg producing 50% of maximum response (EC₅₀ values) were calculated from individual concentration-response curves according to the method of Fleming *et al.* (1972). The concentration-ratio (EC₅₀ value in the presence of antagonist/EC₅₀ in the absence of antagonist) was also determined.

Results are expressed as mean $\pm$ s.e.mean of the number of arterial segments indicated in each case. Statistically significant differences were calculated by means of Student's *t*-test for paired or unpaired experiments, with Bonferroni corrections for multiple comparisons, or by two way analysis of variance (ANOVA) to compare groups; a probability value of less than 5% was considered significant. At least four rats were used in each set of reactivity experiments.

Drugs and solutions

KHS contained (mM): NaCl 115, NaHCO₃ 25, KCl 4.7, MgSO₄·7H₂O 1.2, CaCl₂ 2.5, KH₂PO₄ 1.2, glucose 11.1 and Na₂EDTA 0.01. The high K⁺ solution was identical to KHS except that NaCl was replaced by KCl in an equimolar basis. Drugs used were: PGF_{2α} tris salt, L-Arg hydrochloride, D-Arg hydrochloride, calphostin C, dexamethasone 21-acetate, BK acetate, indomethacin, L-NAME dihydrochloride, SOD, papaverine hydrochloride, LPS (*Escherichia coli*, serotype 055:B5), polymyxin B sulphate and ampicillin sodium salt (Sigma Chemical, Co., St. Louis, MO, U.S.A.), gentamycin sulphate (Biological Industries, Kibbutz Beit-Haemek, Israel) and AMT hydrochloride (Research Biochemicals International, Natick, MA, U.S.A.). Drug solutions were made in bidistilled water, except PGF_{2α} which was dissolved in absolute ethanol, calphostin C in dimethylsulphoxide and indomethacin in water containing sodium carbonate 1.5 mM; these solvents did not modify basal tone or the vasoconstrictor responses. Stock solutions were kept at -20°C, and appropriate dilutions were made on the day of the experiment.

Results

Data of effective lumen diameter, maximum response, contraction induced by the first administration of 10 μM PGF_{2α} and relaxation induced by 1 μM BK in segments of MCAs from WKY and SHR are shown in Table 1. The effective lumen diameter, maximum response and vasoconstriction induced by PGF_{2α} were higher in segments from normotensive than hypertensive strain, whereas the relaxation elicited by BK was similar in both strains. The increase in contractions to PGF_{2α} in segments from WKY was also observed when the responses were compared in active wall tension values (mN mm⁻¹, Table 1). Successive administration of PGF_{2α} decreased contractile responses in a similar degree in normotensive and hypertensive strain (Figure 1A). Thus, after 7 h incubation, the responses to PGF_{2α} were of 73.6 ± 5.0% and 74.3 ± 4.0% of the initial value, in the normotensive and hypertensive strain ($P > 0.05$), respectively.

Effect of L-arginine

L-Arg (0.01 μM–1 mM) induced concentration-dependent relaxations in MCAs from both WKY and SHR precontracted with 10 μM PGF_{2α} (Figure 1B). The EC₅₀ values were not different in arteries from both strains [WKY: 2.8 (2.3–3.4) μM; SHR: 3.7 (2.9–4.8) μM, in parentheses 95% confidence interval], but the maximum relaxation evoked by L-Arg was

greater in segments from SHR than from WKY (WKY: 89.5 ± 1.7%; SHR: 101.9 ± 3.9% of previous tone, $P < 0.05$). The relaxation obtained in both strains was similar to that observed in the absence of endothelium (EC₅₀ values: WKY, 3.0 (2.5–3.6) μM, $n = 10$; SHR, 4.1 (3.1–5.4) μM, $n = 8$). The effect of L-Arg was stereospecific since the inactive enantiomer D-Arg did not cause any effect (data not shown).

Effect of indomethacin and SOD

Basal tone, PGF_{2α}-induced contraction and L-Arg-caused relaxation were not modified in the presence of 10 μM indomethacin or SOD (60 u ml⁻¹) in segments from both strains (data not shown).

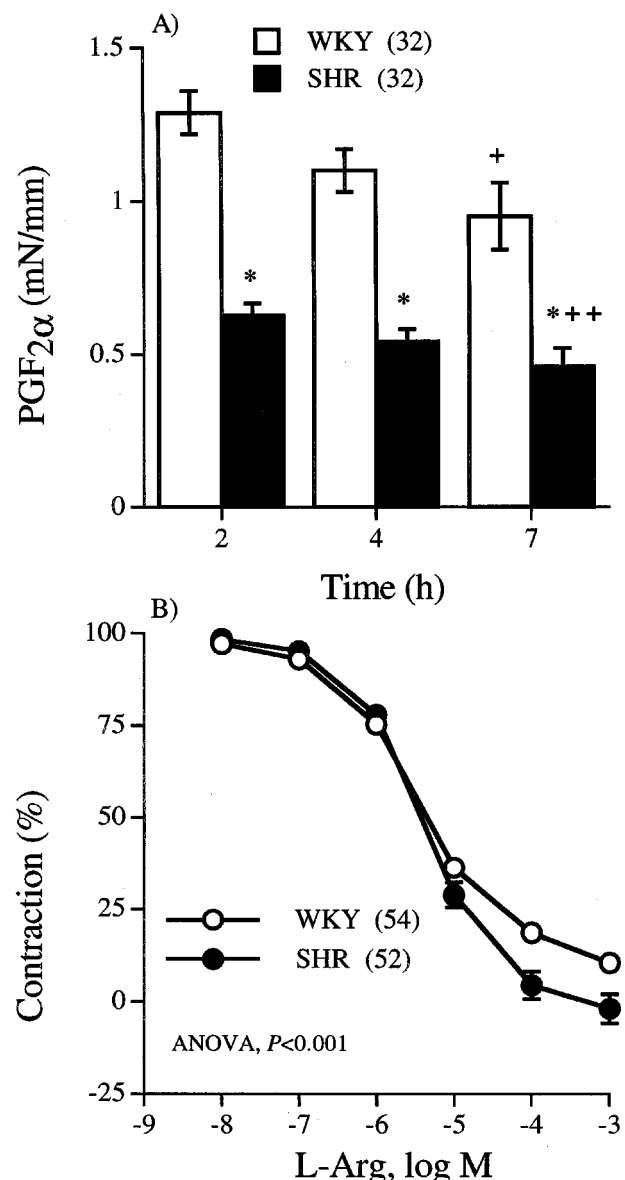


Table 1 Data of effective lumen diameter, maximum response and responses to PGF_{2α} and BK in segments of MCA from WKY and SHR

Parameter	WKY (n = 54)	SHR (n = 52)
Effective lumen diameter (μm)	215.0 ± 3.2	183.2 ± 3.1*
Maximum response (mN mm ⁻¹)	2.26 ± 0.06	1.56 ± 0.05*
10 μM PGF _{2α} (mN mm ⁻¹)	1.29 ± 0.07	0.62 ± 0.04*
(% Maximum response)	57.3 ± 2.7	36.7 ± 1.9*
1 μM BK (% previous tone)	88.0 ± 1.9	90.6 ± 3.6

Results (mean ± s.e.mean). * $P < 0.01$.

Figure 1 (A) Vasoconstrictor responses (mN mm⁻¹) induced by successive administration of 10 μM PGF_{2α} at different incubation times. (B) Concentration-response curves to L-Arg in middle cerebral arteries from WKY and SHR previously contracted with 10 μM PGF_{2α}. Results (mean ± s.e.mean) are expressed as active wall tension (mN mm⁻¹) or as a percentage of the contraction induced by PGF_{2α}. Number of arterial segments is indicated in parentheses. * $P < 0.05$ vs WKY. ⁺ $P < 0.05$ and ⁺⁺⁺ $P < 0.01$ vs response at 2 h (paired Student's *t*-test).

Effect of NOS inhibitors

To determine the role of NO on the L-Arg-induced relaxation, segments from MCA were incubated with 0.1 mM L-NAME or 5 and 10 nM AMT. In the presence of 0.1 mM L-NAME, the concentration-dependent curve to L-Arg was displaced to the right in segments from both WKY and SHR, this displacement being greater in the hypertensive strain (concentration-ratio WKY: 3.1 ± 0.9 ; SHR: 18.5 ± 7.0 , $P < 0.05$) (Figure 2). The L-Arg-induced relaxation was reduced by 10 nM AMT in MCAs from both WKY and SHR in a similar extent (concentration-ratio WKY: 2.6 ± 0.4 ; SHR: 1.9 ± 0.2 , $P > 0.05$). However, 5 nM AMT shifted to the right the curve to L-Arg only in segments from hypertensive animals (Figure 2).

L-NAME induced a basal tone increase of $33.2 \pm 8.4\%$ and $24.9 \pm 6.8\%$ of the maximum response, in segments from WKY and SHR, respectively. In this situation the tone reached by $10 \mu\text{M}$ $\text{PGF}_{2\alpha}$ was significantly potentiated (WKY: control, 65.6 ± 6.7 , L-NAME, $90.7 \pm 5.0\%$, $P < 0.05$; SHR: control, 39.6 ± 2.0 , L-NAME, $62.7 \pm 6.0\%$, $P < 0.05$). AMT induced slight contractile responses, which were similar in segments from normotensive and hypertensive animals (5 nM: 9.0 ± 2.6 and $5.4 \pm 1.5\%$, $P > 0.05$, and 10 nM: 14.7 ± 2.8 ($n = 10$) and $8.8 \pm 3.6\%$ ($n = 8$), $P > 0.05$, of the maximum response in segments from WKY and SHR, respectively). In addition, the contraction to $10 \mu\text{M}$ $\text{PGF}_{2\alpha}$ was significantly increased in the presence of both 5 nM AMT (WKY: control, 60.6 ± 3.5 , AMT, $78.6 \pm 7.1\%$, $P < 0.05$; SHR: control, 33.9 ± 4.8 , AMT, $50.1 \pm 4.5\%$, $P < 0.05$) and 10 nM AMT (WKY: control,

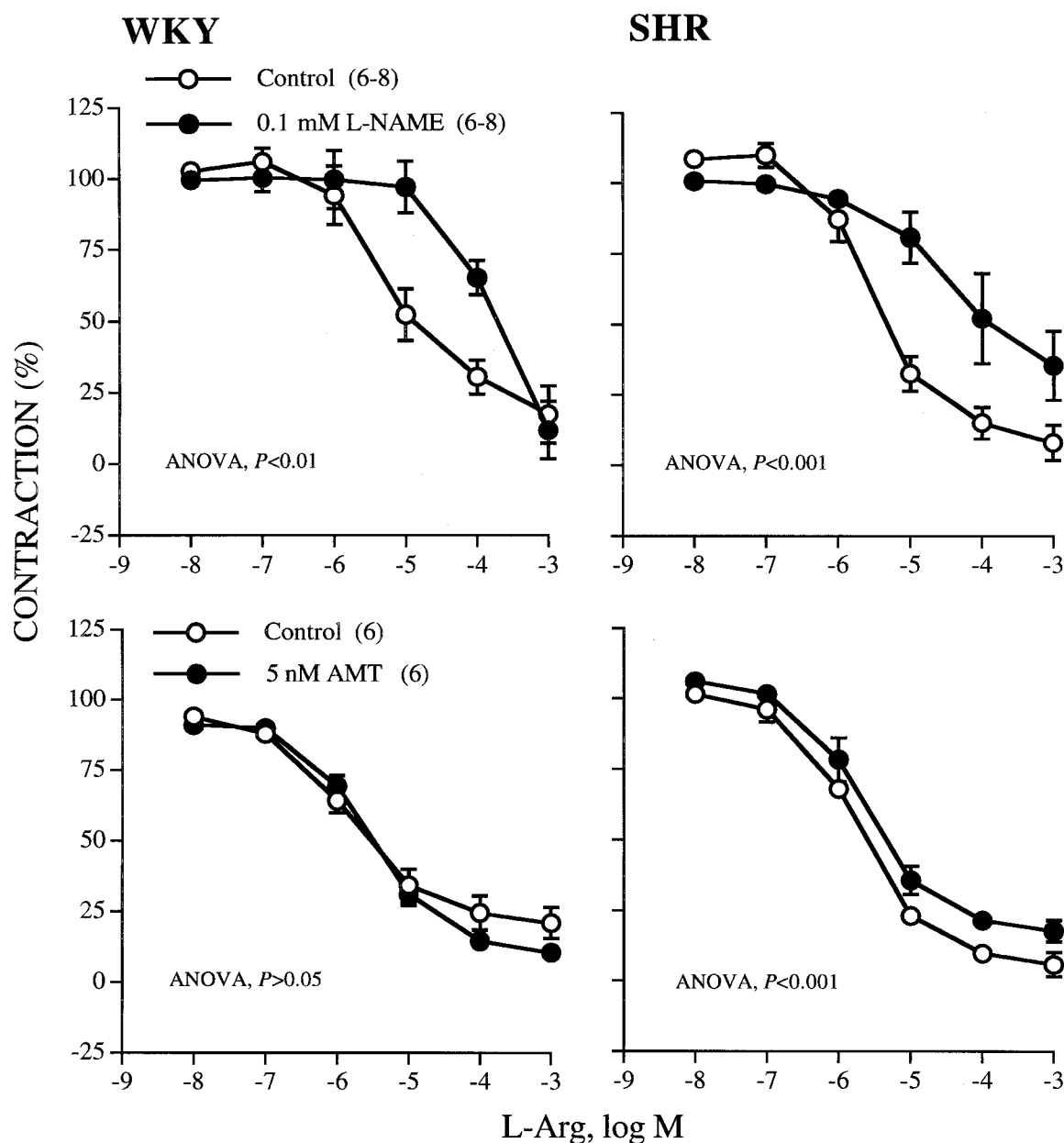


Figure 2 Effect of L-NAME and AMT on the concentration-response curves to L-Arg in middle cerebral arteries from WKY and SHR previously contracted with $10 \mu\text{M}$ $\text{PGF}_{2\alpha}$. Results (mean $\pm$ s.e.mean) are expressed as a percentage of the contraction induced by $\text{PGF}_{2\alpha}$ in each case (WKY: 1.29 ± 0.14 , 1.95 ± 0.08 and $1.69 \pm 0.26 \text{ mN mm}^{-1}$; SHR: 0.66 ± 0.05 , 1.05 ± 0.80 and $0.84 \pm 0.11 \text{ mN mm}^{-1}$, in control, L-NAME or AMT treated segments, respectively). Number of arterial segments is indicated in parentheses.

55.2 ± 6.2 , AMT, $74.5 \pm 8.4\%$, $P < 0.05$; SHR: control, 37.4 ± 4.3 , AMT, $52.1 \pm 3.2\%$, $P < 0.05$).

Effect of dexamethasone and LPS

L-Arg-induced relaxation was reduced after 4 or 7 h incubation of segments from WKY rats with dexamethasone, whereas the reduction was only observed after a 7 h incubation in segments from SHR (Figures 3 and 4). The relaxation induced by L-Arg was not modified by LPS in segments from WKY whereas it was slightly inhibited in those from SHR (Figure 4). The presence of both drugs reduced the inhibitory effect of dexamethasone on L-Arg-induced relaxation, only in segments from WKY rats (Figure 4).

Incubation of segments for 4 or 7 h with $1 \mu\text{M}$ dexamethasone did not modify the maximum response (results not shown), whereas the contraction caused by $\text{PGF}_{2\alpha}$ was similarly potentiated in both WKY and SHR (Figure 5). The presence of $10 \mu\text{g ml}^{-1}$ LPS since the time the brain was removed from the animal and for 7 h did not modify the maximum response in MCAs from WKY and SHR (results not shown). However, in these conditions, the contraction induced by $\text{PGF}_{2\alpha}$ was reduced in segments from normotensive and hypertensive animals, this reduction being greater in SHR (Figure 5). In the presence of LPS plus dexamethasone for 7 h the contraction to $\text{PGF}_{2\alpha}$ was unmodified in the normotensive strain and reduced in the hypertensive one (Figure 5).

Effect of PKC inhibitors

The incubation for 7 h with $10 \mu\text{g ml}^{-1}$ polymyxin B since the time the brain was removed from the animal, induced a significant increase of basal tone, which was greater in segments from SHR than WKY (percentage of maximum response: WKY, 12.8 ± 3.1 ; SHR, 31.4 ± 7.7 , $P < 0.05$). In these conditions, the contraction to $10 \mu\text{M}$ $\text{PGF}_{2\alpha}$ was potentiated in

segments from both strains, this potentiation being greater in SHR (Figure 5). In addition, the relaxation induced by L-Arg in segments from both types of animals was significantly reduced (Figure 6). Calphostin C ($0.5 \mu\text{M}$) produced a reduction of the L-Arg relaxation (ANOVA, WKY: $P < 0.05$, $n = 6$; SHR: $P < 0.005$, $n = 7$), whereas the contraction to $\text{PGF}_{2\alpha}$ was not modified (results not shown).

LPS abolished the increase in basal tone induced by polymyxin B. In addition, the contraction caused by $\text{PGF}_{2\alpha}$ in the presence of LPS plus polymyxin B was lower than that obtained in the presence of polymyxin B alone and similar or lower than the control in WKY and SHR, respectively (Figure 5). The inhibition L-Arg relaxation induced by polymyxin B in segments from both types of rats was abolished by LPS, but in the case of segments from SHR the relaxation obtained was higher than that found in control situation (Figure 6).

As polymyxin B is an antibiotic, in addition of PKC inhibitor, experiments were achieved with other antibiotics to analyse the specificity of the inhibitory effect of polymyxin B. Incubation of segments with gentamycin plus ampicillin for 7 h, both at $10 \mu\text{g ml}^{-1}$, did modify neither basal tone, the maximum response nor the contraction induced by $10 \mu\text{M}$ $\text{PGF}_{2\alpha}$. In addition, the concentration-response curve to L-Arg was not affected by these antibiotics in segments from both WKY and SHR (data not shown).

Discussion

This study investigates some functional consequences that hypertension produces in the rat MCA. The major and new finding of the present study is that the vasodilator responses induced by L-Arg are augmented in SHR-cerebral segments compared with WKY ones, whereas the vasoconstrictor responses induced by $\text{PGF}_{2\alpha}$ are reduced. A different functional expression and/or participation of iNOS through

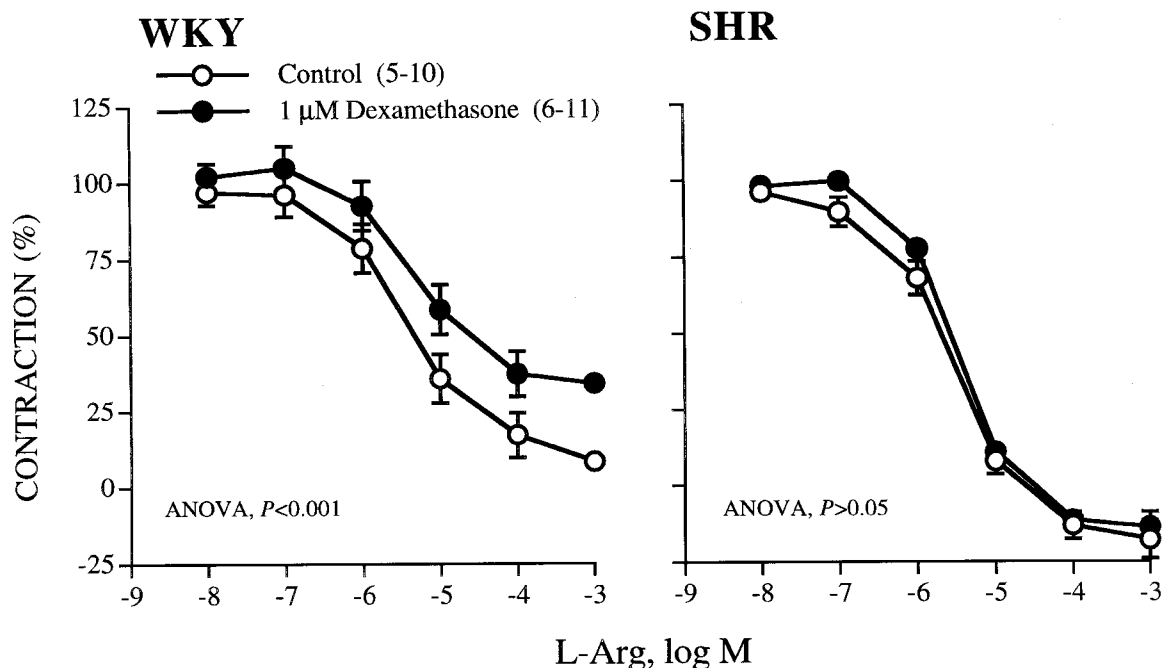


Figure 3 Effect of preincubation for 4 h with dexamethasone on the concentration-response curves to L-Arg in middle cerebral arteries from WKY and SHR previously contracted with $10 \mu\text{M}$ $\text{PGF}_{2\alpha}$. Results (mean $\pm$ s.e.mean) are expressed as a percentage of the contraction induced by $\text{PGF}_{2\alpha}$ in each case (WKY: 0.92 ± 0.09 and $1.21 \pm 0.07 \text{ mN mm}^{-1}$, SHR: 0.40 ± 0.06 and $0.66 \pm 0.06 \text{ mN mm}^{-1}$, in control and dexamethasone treated segments, respectively). Number of arterial segments is indicated in parentheses.

a PKC-dependent mechanism seems to be involved in these effects.

Vasodilator response to L-arginine

Previous studies have shown that the substrate of NO synthesis, L-Arg, has a weak effect in the vascular tone regulation of cerebral arteries and arterioles (Faraci, 1993; Kitazono *et al.*, 1996), and mild dilating effect on pial circulation under physiological conditions (Haberl *et al.*, 1991; Morikowa *et al.*, 1992; Rosenblum *et al.*, 1990; Riedel *et al.*, 1995). Recently, we have found that L-Arg induces an inhibitory effect of

vasoconstrictor responses induced by $\text{PGF}_{2\alpha}$ and K^+ in the rat middle cerebral artery through a mechanism that involves the iNOS activation (Alonso *et al.*, 1998).

In the present study, L-Arg induced an endothelium-independent relaxation that was more pronounced in SHR than in WKY, whereas EC_{50} values were not altered, suggesting that the sensitivity of both arteries to L-Arg is similar. Other investigators have also found that hypertension increases the vasodilator responses to L-Arg. Thus, Riedel *et al.* (1995) reported that the increase of diameter induced by L-Arg in pial arterioles was greater in the hypertensive than in normotensive rats. In peripheral arteries, hypertension may

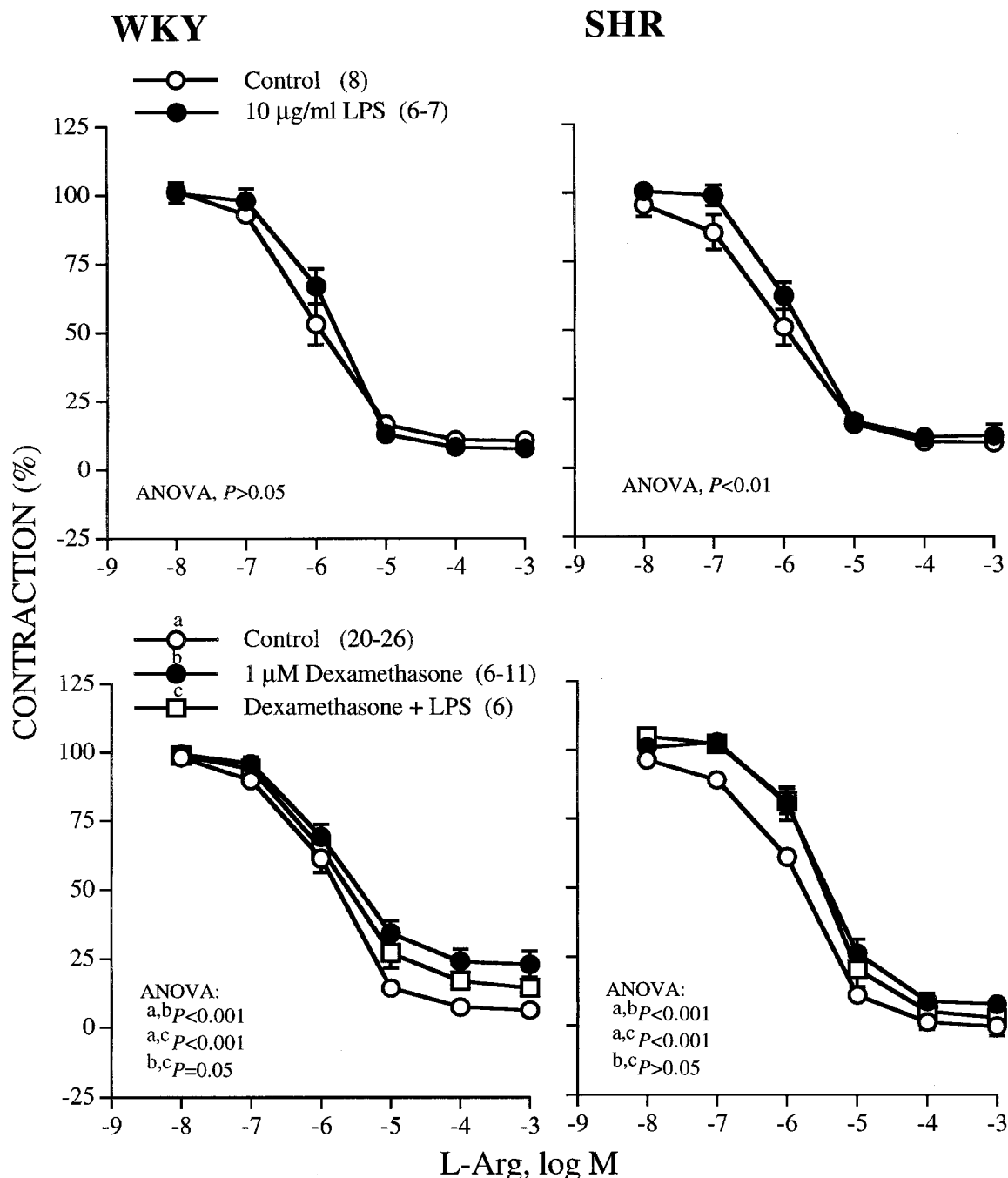


Figure 4 Effect of incubation for 7 h with LPS, dexamethasone, and dexamethasone plus LPS on the concentration-response curves to L-Arg in middle cerebral arteries from WKY and SHR previously contracted with 10 µM $\text{PGF}_{2\alpha}$. Results (mean $\pm$ s.e.mean) are expressed as a percentage of the contraction induced by $\text{PGF}_{2\alpha}$ in each case (WKY: 0.95 ± 0.11 , 0.75 ± 0.13 , 1.15 ± 0.14 and 1.04 ± 0.13 mN mm^{-1} ; SHR: 0.46 ± 0.07 , 0.26 ± 0.08 , 0.67 ± 0.12 and 0.36 ± 0.08 mN mm^{-1} , in control, LPS, dexamethasone and LPS plus dexamethasone treated segments, respectively). Number of arterial segments is indicated in parentheses.

modify the responses to L-Arg, e.g., Puci *et al.* (1995) and Wu *et al.* (1996) observed that L-Arg caused relaxation in aortic rings from hypertensive but not from normotensive rats. In contrast, the endothelium-dependent relaxation elicited by BK was not modified by hypertension. Other investigators, using the cranial window method, observed that the dilatation of the basilar artery in response to acetylcholine and BK is impaired in SHR and SHR stroke-prone (SHRSP) (Faraci, 1993; Kitazono *et al.*, 1996). Generally, hypertension impairs the endothelium-dependent vasodilator responses to different agonists in isolated vascular preparations (Lüscher, 1992),

although unchanged, an even, improved responses have been also reported (Marín & Rodríguez-Martínez, 1997).

In some vessels, the generation of prostanoids and/or free radicals has been described as a possible cause of L-Arg-induced dilatation (Brian *et al.*, 1995; Riedel *et al.*, 1995). However, in our study, their participation is ruled out as indomethacin and SOD failed to modify the responses to L-Arg in both strains.

The vasodilator responses induced by L-Arg were inhibited by L-NAME in segments from both WKY and SHR. The effects of L-Arg were enantiomerically specific, as D-Arg has no effect, and L-NAME is a competitive inhibitor of NOS. These results indicate that the synthesis of NO is a necessary step for the vasodilatation induced by L-Arg in both strains. Similar results with unspecific inhibitors of NOS have been described (Morikawa *et al.*, 1992; Moritoki *et al.*, 1992; Riedel *et al.*, 1995; Puci *et al.*, 1995). The specific inhibitor of iNOS, AMT (Nakane *et al.*, 1995; Tracey *et al.*, 1995) inhibited the L-Arg relaxation in both strains, indicating the participation of this isoform in such responses. However, the fact that the effect of 5 nM AMT on L-Arg relaxation was observed only in MCAs from SHR, and that the inhibitory effect of L-NAME was higher in SHR, suggests that the participation of iNOS in the vasodilator responses induced by L-Arg is higher in the hypertensive than in the normotensive strain. Wu *et al.* (1996) and Chou *et al.* (1998) found that the degree of both iNOS expression and activity, in basal conditions or after LPS stimulation, was greater in aorta homogenates from SHR than from WKY. These differences in the expression of iNOS could also explain the higher relaxation to L-Arg found in the hypertensive strain.

Previous studies in peripheral arteries from normotensive rats demonstrated that L-Arg relaxed only precontracted

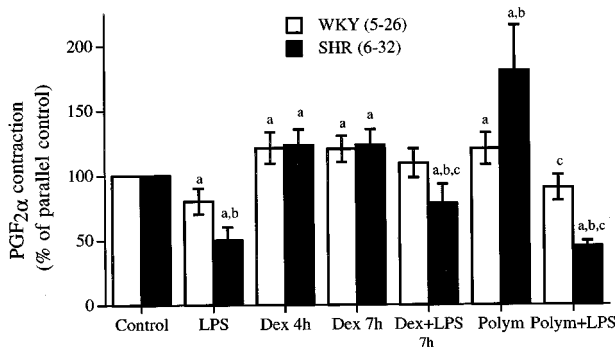


Figure 5 Vasoconstrictor responses induced by $10 \mu\text{M}$ $\text{PGF}_{2\alpha}$ in middle cerebral arteries from WKY and SHR treated for 4 or 7 h with $1 \mu\text{M}$ dexamethasone (Dex) and for 7 h with $10 \mu\text{g ml}^{-1}$ LPS, Dex plus LPS, $10 \mu\text{g ml}^{-1}$ polymyxin B (Polym) and polymyxin B plus LPS. Results (mean $\pm$ s.e.mean) are expressed as a percentage of the contraction induced by $\text{PGF}_{2\alpha}$ in a parallel control. Number of arterial segments is indicated in parentheses. ^a $P < 0.05$ vs control; ^b $P < 0.05$ vs WKY; ^c $P < 0.05$ vs Dex or Polym (unpaired Student's *t*-test with Bonferroni correction).

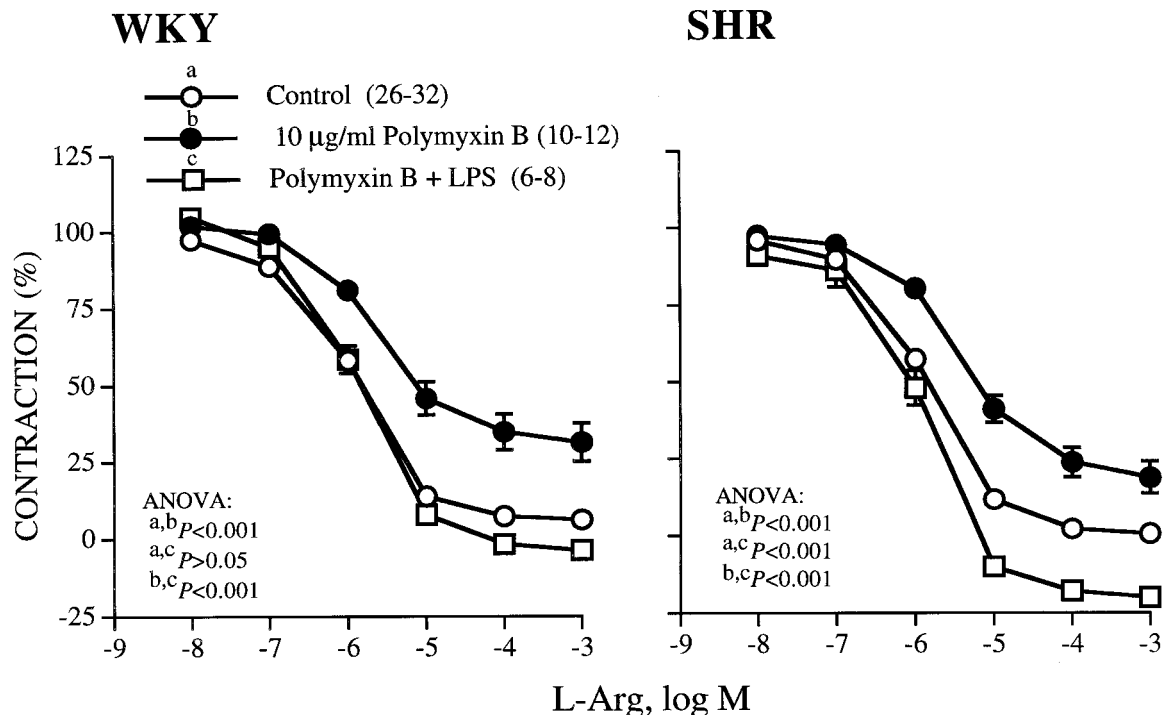


Figure 6 Effect of $10 \mu\text{g ml}^{-1}$ polymyxin B, and polymyxin B plus $10 \mu\text{g ml}^{-1}$ LPS on the concentration-response curves to L-Arg in middle cerebral arteries from WKY and SHR rats previously contracted with $10 \mu\text{M}$ $\text{PGF}_{2\alpha}$. Results (mean $\pm$ s.e.mean) are expressed as percentage of the contraction induced by $\text{PGF}_{2\alpha}$ in each case (WKY: 0.90 ± 0.10 , 1.19 ± 0.08 and $0.66 \pm 0.10 \text{ mN mm}^{-1}$; SHR: 0.42 ± 0.06 , 0.83 ± 0.18 and $0.21 \pm 0.02 \text{ mN mm}^{-1}$, in control polymyxin B and LPS plus polymyxin B treated segments, respectively). Number of arterial segments is in parentheses.

arterial vessels subjected to prolonged incubation in physiological buffers or after LPS treatments (Rees *et al.*, 1990; Schini & Vanhoutte, 1991; Moritoki *et al.*, 1992; Martínez *et al.*, 1996). In our study, however, L-Arg induced an early and marked relaxation of segments from normotensive and hypertensive strain in the absence of iNOS activators. *Ex vivo* induction of NO synthases by low levels of endotoxin present in the incubation medium was thought to be involved in this enhanced responsiveness to L-Arg (Rees *et al.*, 1990). It is unlikely that this mechanism was involved in the relaxant responses to L-Arg in MCAs, since these responses were not modified in the presence of the antibiotics ampicillin plus gentamycin. This finding is compatible with the notion that the important vasodilator responses observed in the MCA without previous exposure to endotoxin could be due, at least in part, to an *in vivo* induction of iNOS. The antibiotic polymyxin B induced a basal tone increase, potentiated the contraction induced by $\text{PGF}_{2\alpha}$ and reduced the vasodilator effect induced by L-Arg on the MCA from both strains. These effects, that were antagonized by LPS, could not be attributed to the presence of bacterial contaminants in the medium by the lack of effect of ampicillin plus gentamycin. The ability of polymyxin B to inhibit PKC has been extensively described and is currently used as PKC inhibitor (Kuo *et al.*, 1984; Yoon *et al.*, 1994). In vascular smooth muscle, PKC is involved in the contractile responses to different agonists (Salaices *et al.*, 1990; Singer *et al.*, 1996) and in the iNOS expression (McKenna *et al.*, 1994; Paul *et al.*, 1997). The results obtained in the present study with polymyxin B suggest the involvement of PKC in the activation of iNOS in the MCA from hypertensive and normotensive rats. The fact that calphostin C induced a similar reduction in L-Arg relaxation to that caused by polymyxin B supports this assumption.

Dexamethasone, an inhibitor of NOS induction (Radomski *et al.*, 1990), reduced, after a 4 h incubation, the relaxation induced by L-Arg in WKY, whereas in segments from SHR it was necessary to increase the incubation time up to 7 h to obtain such a reduction. The results obtained with dexamethasone suggest that a continuous protein synthesis is necessary for induction of L-Arg pathway, as previously indicated (Fleming *et al.*, 1993) and further confirm the participation of the iNOS in the vasodilator responses induced by L-Arg.

It has been described that LPS decreases vascular resistance, produces vascular hyporeactivity to different vasoconstrictors and potentiates the inhibitory effect of L-Arg by overproduction of NO mediated by activation of iNOS (Ueno & Lee, 1993; Berrazueta *et al.*, 1994; Brian *et al.*, 1995; Villamor *et al.*, 1995; Alonso *et al.*, 1998). In our study, LPS inhibited the vasoconstrictor responses induced by $\text{PGF}_{2\alpha}$ and the effect of dexamethasone and polymyxin B on basal tone, the contraction induced by $\text{PGF}_{2\alpha}$ and on the L-Arg-induced vasodilatation. These results confirm that the effects of LPS are mediated by activation of iNOS. Unexpectedly, LPS inhibited and unaltered the vasodilator response to L-Arg in SHR and WKY, respectively. The cause of this effect is unclear but it is possible that in normal conditions the iNOS was maximally induced in the presence of L-Arg in these arteries, and an ulterior induction by LPS was impossible. However, when the iNOS expression is inhibited by polymyxin B, the presence of LPS potentiated the L-Arg vasodilatation in SHR.

Contractile responses induced by $\text{PGF}_{2\alpha}$

The contractions elicited by $\text{PGF}_{2\alpha}$ were markedly attenuated in segments from SHR compared with those from WKY. Thus, we obtained not only a reduction in the active force

generated (the contraction in mN mm^{-1} was approximately a half of that obtained in MCA from WKY rats), but also in the percentage of maximum responses of the arteries. These changes could be independent, at least in part, of receptor-signal transduction mechanisms, since the maximum responses were also reduced. The reduction of the contractions induced by K^+ , a non specific stimulus, suggests a decreased vasoconstrictor ability of the smooth muscle cells in the cerebral vessels of the hypertensive strain. In some peripheral vessels an increase of vasoconstrictor responses in hypertension has been reported (Vila *et al.*, 1993; Tabernero *et al.*, 1996; Dickhout & Lee, 1997). However, a similar reduction of vasoconstrictor responses has been obtained in cerebral arteries of SHR and SHRSP compared with WKY (Winquist & Bohr, 1983; Mulvany, 1986; Yokota *et al.*, 1994; Arribas *et al.*, 1996). Alterations of vascular structure associated with vascular remodelling and vascular smooth muscle cell geometric disorganization observed in rat basilar arteries (Arribas *et al.*, 1996), could explain, in part, the impairment of the contraction.

The vasoconstrictor responses to $\text{PGF}_{2\alpha}$ after 7 h of incubation were about a 70% of those obtained in the first administration, in segments from SHR and WKY. This reduction could be due to an induction of iNOS because it was potentiated by LPS. Similar results have been obtained in the porcine basilar artery (Ueno & Lee, 1993), the rat aorta (Rees *et al.*, 1990) and the human mammary artery (Berrazueta *et al.*, 1994) in normotension. The participation of iNOS in this time-dependent reduction of $\text{PGF}_{2\alpha}$ contraction was also supported by the fact that in the presence of dexamethasone or polymyxin B, such a reduction was transformed in an increase of contraction that was antagonized by LPS. An inhibition by dexamethasone and polymyxin B of the time-dependent loss of agonist-induced tone has been reported (Rees *et al.*, 1990; Berrazueta *et al.*, 1994). The presence of gentamycin plus ampicillin did not alter the contractile response reduction which indicates that this reduction is not due to the presence of bacterial contaminant in the medium. Therefore, the effect of polymyxin B is independent of its antibiotic activity and probably related to its capacity to inhibit PKC (Kuo *et al.*, 1984; Yoon *et al.*, 1994), and therefore to the inhibition of iNOS expression. The fact that polymyxin B induced an increase in basal tone that was antagonized by LPS, suggests a continuous production of NO mediated by iNOS activation in basal condition. The increase in basal tone induced by L-NAME and AMT also supports this assumption.

Both the reduction and the potentiation of $\text{PGF}_{2\alpha}$ -induced vasoconstrictor responses caused by incubation for 7 h with LPS and polymyxin B, respectively, were greater in the MCA from SHR than from WKY. In addition, LPS antagonized the increase of $\text{PGF}_{2\alpha}$ response elicited by polymyxin B and dexamethasone in a greater extent in segments from SHR. These results agree with those obtained by Wu *et al.* (1996) and Ghou *et al.* (1998) in aortic homogenates from SHR and WKY in which a higher expression of iNOS in the hypertensive strain was observed. In addition, Junquero *et al.* (1992) using cultured aortic smooth muscle cells from 20 week old age-matched SHR and WKY, reported that cells obtained from the hypertensive strain produce three times more NO than those from WKY rats in response to interleukin-1 β . These authors suggested that the quantitative differences in NO production between these strains may reflect differences in the induction process by cytokines or a differential modulation of the L-Arg pathway. However, Singh *et al.* (1996) did not find significant differences in either the level of iNOS mRNA expression or the amount of NO generated in vascular smooth muscle cells from

prehypertensive SHR and WKY after stimulation with interleukin-1 β , whereas the cyclic GMP concentration was smaller in SHR cells than in WKY cells. The results obtained in the present study suggest that an increase in the NO production in the MCA vascular wall from SHR could be involved in its hyporesponsiveness to PGF_{2 α} . Similar conclusion was obtained by Michielsen *et al.* (1995) in aortic rings from portal hypertensive rats. One alternative is a greater sensitivity of the MCA from SHR than WKY to NO produced through iNOS activation.

In conclusion, the results obtained in the present study suggest that the induction of iNOS, mediated by a PKC-

dependent mechanism, participates in both the L-Arg-induced relaxation and the hyporeactivity to PGF_{2 α} in MCA from WKY and SHR rats, the participation of iNOS in such responses being greater in the hypertensive strain.

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NMDA receptor characterization and subunit expression in rat cultured mesencephalic neurones

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1 NMDA-induced changes in free intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) were determined in individual cultured rat mesencephalic neurones by the fura-2 method. mRNA expression encoding NMDA receptor subunits (NR1, NR2A–D) was examined by RT–PCR.

2 NMDA (1–100 μM , plus 10 μM glycine) induced a concentration-dependent increase in $[\text{Ca}^{2+}]_i$ ($\text{EC}_{50} = 5.7 \mu\text{M}$). The effect of NMDA was virtually insensitive to tetrodotoxin (0.3 μM) and nitrendipine (1 μM), but dependent on extracellular Ca^{2+} . 5,7-Dichlorokynurenic acid (10 μM), a specific antagonist at the glycine binding site on the NMDA receptor, abolished the NMDA response.

3 Memantine, an open-channel blocker, and ifenprodil, a preferential non-competitive NR1/NR2B receptor antagonist diminished the NMDA effect with an IC_{50} value of 0.17 and 1 μM , respectively. Ethanol at 50 and 100 mM caused about 25 and 45% inhibition, respectively.

4 Agarose gel analysis of the PCR products followed by ethidium bromide fluorescence or CSPD chemiluminescence detection revealed an almost exclusive expression of the NR1 splice variants lacking exon (E) 5 and E22. The 3' splice form without both E21 and E22 exceeded that containing E21 by approximately 4 fold. The relative amounts of NR2A, NR2B, NR2C corresponded to approximately 1:2:1. NR2D mRNA was also detectable.

5 In conclusion, mesencephalic neurones bear ethanol-sensitive NMDA receptors which might be involved in the development of ethanol dependence and withdrawal. The high affinity of NMDA to this receptor, its sensitivity to ifenprodil and memantine may suggest that the mesencephalic NMDA receptor comprises the NR1 splice variant lacking E5, NR2B, and NR2C, respectively.

Keywords: Cultured mesencephalic neurones; NMDA receptors; intracellular Ca^{2+} ; ethanol; ifenprodil; memantine; fura-2 method; RT–PCR; NR1 splice variants; NR2 subunits

Abbreviations: AP5, DL-2-amino-5-phosphonopentanoic acid; DIV, days *in vitro*; DMSO, dimethyl sulphoxide; E, exon; EC_{50} , 50% effective concentration; IC_{50} , 50% inhibitory concentration; NMDA, *N*-methyl-D-aspartate; NR1, NMDA receptor subunit 1, NR2A–D, NMDA receptor subunits 2A–D; RT–PCR, reverse transcriptase-polymerase chain reaction; SDS, sodium dodecyl sulphate; SSC, standard saline-citrate; TTX, tetrodotoxin acetate; TH, tyrosine hydroxylase; VTA, ventral tegmental area

Introduction

The *N*-methyl-D-aspartate (NMDA) receptor is essentially involved in a number of important central processes such as neuronal development, long-term-potential, and neuronal death (excitotoxicity) (Nakanishi, 1992). Moreover, this receptor mediates some of the acute and chronic CNS effects of ethanol (Harris *et al.*, 1992; Hoffman, 1995; Grant & Lovinger, 1995). At concentrations associated with moderate to severe intoxications [10–100 mM, corresponding to 0.046–0.46% (w/v) in blood] ethanol acutely inhibits NMDA receptor-mediated responses in cultured neurones (Lovinger *et al.*, 1989), brain slices (Göthert & Fink, 1989; Woodward & Gonzales, 1990; Poelchen *et al.*, 1997; Darstein *et al.*, 1998; Nieber *et al.*, 1998), and transfected expression systems (Koltchine *et al.*, 1993; Mirshahi & Woodward, 1995).

The NMDA receptor is a ligand-gated ion channel highly permeable to Ca^{2+} . In addition to glutamate, glycine is required as a co-agonist (Kleckner & Dingledine, 1988; Kemp & Leeson, 1993). Several NMDA receptor subunits (NR1, NR2A–D) have been cloned (Monyer *et al.*, 1992; Laurie & Seeburg, 1994; Zukin & Bennett, 1995). The NR1 subunit with

eight known splice variants is expressed throughout the brain, while the NR2A–D subunits display regional differences. *Xenopus* oocyte expression studies have shown that only the NR1 subunit is required to generate functional NMDA receptors and that co-expression with various NR2 subunits results in the assembly of ion channels with conductances that resemble native NMDA receptors. Natively expressed receptors presumably comprise two NR1 subunits (Behe *et al.*, 1995) and at least one member of the NR2 family (Schoepfer *et al.*, 1992). Heteromers containing different NR2 subunits display distinct pharmacological and physiological characteristics (Monyer *et al.*, 1992). For example, ifenprodil blocks the NR1/NR2B heteromer with considerably higher potency than the NR1/NR2A heteromer (Williams, 1993; Lovinger, 1995), while ethanol preferentially affects both NR1/NR2A and NR1/NR2B receptors (Masood *et al.*, 1994; Mirshahi & Woodward, 1995).

There is considerable evidence that the ventral tegmental area (VTA)-nucleus accumbens system is implicated in the stimulant and reinforcing properties of ethanol and other drugs of abuse (Harris *et al.*, 1992). Like morphine and nicotine, acutely administered ethanol has been shown to increase the firing rate of dopaminergic principal neurones of the VTA by a mechanism which is not completely understood.

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Ethanol might directly act on the dopamine neurones or inhibit GABAergic interneurons. In the present study, NMDA receptor function and mRNA subunit expression were studied on cultured rat ventral mesencephalic neurones by means of single-cell microfluorimetry (fura-2 method) and reverse transcriptase-polymerase chain reaction (RT-PCR). According to electrophysiological data, burst firing in dopamine neurones is induced by activation of somatodendritic NMDA receptors (Johnson *et al.*, 1992). Preliminary accounts of the present results were given previously (Scheibler *et al.*, 1998).

Methods

Mesencephalic cultures

For a detailed description of tissue preparation and cell culture conditions see Shimoda *et al.* (1992) and Kriegstein & Unsicker (1994), respectively. In brief, 1 mm³ of ventral mesencephalic tissue was dissected out from Wistar rat fetuses of gestational day 14 and pooled in ice-cold Hanks buffered salt solution. The tissue was dissociated by a consecutive treatment with trypsin (0.25%) and DNase (≈ 2000 u), followed by careful trituration. Subsequent to centrifugation, the supernatant was discarded and the pellet was dispersed in a 1:1 mixture of Dulbecco's modified Eagle's Medium and Nutrient F12 supplemented with 20% foetal calf serum, 2 mM L-glutamine, 50 $\mu\text{g ml}^{-1}$ gentamycin, and 36 mM D-glucose. The cell suspension was seeded in the centre of circular glass coverslips (30 mm in diameter) coated with poly-L-lysine at a density of 250,000 cells cm⁻². Medium was replaced every 3 days.

[Ca²⁺]_i measurement

Usually, 7–10 day-old cultures (DIV 7–11) were loaded with fura-2 acetoxymethyl ester (5 μM) for 30 min at 37°C, then rinsed with HEPES buffer (composition in mM: NaCl 133, KCl 4.8, CaCl₂ 1.3, KH₂PO₄ 1.2, HEPES 10; D-glucose 10; pH 7.4 adjusted with NaOH) and left to equilibrate for approximately 30 min. Subsequently, the preparation was placed in a closed superfusion chamber, mounted onto the stage of an inverted microscope (Diaphot 200, Nikon) and superfused with HEPES-buffer at a flow rate of 0.6 ml min⁻¹ for 15 min. During the washout period, the preparation was focused in normal light using a 1000 fold magnification (Fluor 100 $\times$ oil immersion objective, Nikon). Fura-2 fluorescence excited alternately at 340 and 380 nm by the light of a 75-W xenon bulb was recorded at 510/20 nm with a photomultiplier. The fluorescence field chosen by means of an adjustable rectangular diaphragm was slightly larger than the bipolar neurone in focus. Monochromator settings, chopper frequency, and complete data acquisition were computer-controlled (PTI, Wedel, Germany). The cells were stimulated by the application of NMDA for 1 min (usually in the presence of 10 μM glycine), and in some experiments by electrical stimulation (30 pulses/3 Hz; voltage drop: 37.5 V cm⁻¹; current strength: 58 mA; pulse width: 0.5 ms); the latter was mainly done to check the contribution of voltage-dependent sodium channels to the NMDA response. Up to three stimulations, separated by 15 min-intervals, were applied. During intervals the cells were protected from the excitation light to prevent bleaching and phototoxic action. Substances, such as NMDA receptor antagonists and ethanol were administered 10 min before and during the second stimulation

period. Cells were exposed to excitation lights over a period of 3 min, starting 1 min before stimulation. Calibration was performed according to Grynkiewicz *et al.* (1985) at the end of the experiment; Ca²⁺-saturated fura-2 signals (R_{max}) were measured in the presence of 10 μM ionomycin, and Ca²⁺-free (R_{min}) signals with buffer containing no Ca²⁺ but 25 mM EGTA.

Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was prepared using UltraspecTM-RNA (Biotech Laboratories, Inc). First-strand cDNA was synthesized by reverse transcription of 1 μg total RNA with 200 u SuperscriptTM II reverse transcriptase (Gibco-BRL) in first strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂) additionally containing 10 mM dithiothreitol, 500 μM of each dNTP, and 50 ng μl^{-1} oligo (dT)_{12–18} primer (Pharmacia). Subsequent to a 10-min pre-incubation at 70°C followed by chilling on ice for denaturation, the samples were kept for 60 min at 45°C. The reaction was stopped by heating for 10 min at 65°C.

To identify mRNAs coding for the different isoforms of the NR1 receptor subunit, primer pairs flanking the regions of alternative splicing were designed. Splice variants with and without exon (E) 5 were detected using the primer pair E5 sense (5'-TCAGCGACGACCACGAGGGACG-3') and E5 antisense (5'-TTGTAGATGCCCACTTGACCA-3') binding to position 495 and 1162 (position 1 represents the first nucleotide of the initiation codon of the full length isoform), respectively. PCR was initiated by a 3-min denaturation period (94°C) followed by 35 cycles of denaturation (94°C for 30 s), annealing (64°C for 30 s), and extension (72°C for 1 min), and terminated by a final extension at 72°C for 10 min. Predicted product lengths were 667 bp (+E5) and 604 bp (–E5). Alternative splicing at E21 and E22 was analysed with the primer pair E21/E22 sense (5'-AAGCTGCACGCTT-TATCTG-3'; binding position 2230) and E21/E22 antisense (5'-CTGACCGAGGGATCTGAGAG-3'; binding position 3150), giving rise to the following PCR products: +E21/+E22, 940 bp; –E21/+E22, 830 bp; +E21/–E22, 583 bp; –E21/–E22, 473 bp. PCR consisted of initial denaturation (94°C for 2 min), 30 cycles of denaturation (94°C for 30 s), annealing (58°C for 1 min), and extension (72°C for 1.5 min plus additional 2 s cycle1), and a final extension at 72°C for 10 min. The amplified PCR products allowed an estimation of the ratio of the respective NR1 splice variants.

The expression pattern of the NR2 subunits was investigated with primers common to NR2A, 2B, and 2C cDNA fragments (sense: 5'-GGGGTTCTGCATCGA-CATCC-3'; antisense: 5'-GACAGCAAAGAAGGCCCA-CAC-3') yielding a 547 bp amplified product from each NR2A–2C (Audinat *et al.*, 1994). Aliquots of the product were digested by restriction enzymes *BpmI*, *BfaI*, and *ScaI* (New England Biolabs), which selectively cut the NR2A, 2B and 2C PCR products, respectively. Cleavage by *BpmI*, *BfaI*, and *ScaI* yielded 226 and 321, 392 and 155, and 361 and 186 bp fragments, respectively. The primers used for the amplification of the NR2D cDNA fragment were 5'-CGATGGCGTCTG-GAATGG-3' (sense; position 1541) and 5'-CTGGCAA-GAAAGATGACCGC-3' (antisense; position 1986) yielding a 465 bp product (Audinat *et al.*, 1994). All PCR reactions were performed in a total volume of 25 μl PCR buffer containing 1 u AmpliTaq[®]DNA polymerase (Perkin Elmer), 1 μl cDNA, 200 μM dNTPs, 200 nM of each primer, and 1 mM MgCl₂ on a PTC-200 thermal cycler (MJ Research). Amplification

products were visualized by ethidium bromide staining or Southern hybridization followed by CSPD detection subsequent to agarose gel electrophoresis (1.5–2%); semi-quantitation of ethidium bromide fluorescence and chemiluminescence intensities were performed using a Diana II Digital Image Analyzer (raytest). Southern hybridization of E21/E22 PCR products was carried out with a digoxigenin-labelled oligonucleotide (5'-GCC ACC AGC ATG AAG ACC CCT GCC ATG TTC TCA AAA GTG A-3') after vacuum blotting of the amplification products onto positively charged nylon membranes (Qiagen). Hybridization was performed at 55°C in 5 × standard saline-citrate (SSC), 1% blocking reagent (Boehringer), 0.1% lauroylsarcosine, 0.02% SDS for at least 4 h. Stringent washing was carried out for 2 × 10 min at 55°C in 2 × SSC, 0.1% sodium dodecyl sulphate (SDS), and 2 × 15 min at 55°C in 0.1 × SSC, 0.1% SDS. Anti-digoxigenin alkaline phosphatase conjugate and the chemiluminescence substrate CSPD were used for the detection of the hybridized probe.

Data analysis

Fluorescence ratios F340/F380 reflecting $[Ca^{2+}]_i$ were used for statistical evaluation of drug effects. NMDA-induced changes in $[Ca^{2+}]_i$ were calculated as difference between the fluorescence ratio subsequent to agonist application and the basal fluorescence ratio, and referred to as Δ fluorescence ratios. Drug effects on the NMDA-induced increase in $[Ca^{2+}]_i$ were

evaluated by calculating the ratio between the second and the first NMDA response, and are expressed as a percentage of the mean NMDA control ratio (i.e., Δ fluorescence ratios, expressed as per cent of control).

All data are given as arithmetic means $\pm$ s.e.mean. Subsequent to one-way-analysis of variance (ANOVA), the significance of differences between the means of various groups was determined by Student's *t*-test. Statistical analysis of concentration response curves was performed using a 4-parameter logistic function (GraFit, Erithacus Software Ltd., U.K.) to generate estimates of EC_{50} and IC_{50} values.

Drugs and chemicals

DL-2-amino-5-phosphonopentanoic acid (AP5), 5,7-dichlorokynurenic acid, ifenprodil tartrate, nitrendipine, tetrodotoxin citrate (TTX) (Biotrend, Köln, Germany); fura-2 acetoxy-methyl ester, glycine, ionomycin calcium salt, NMDA (Sigma, Deisenhofen, Germany). Memantine HCl was kindly donated by Dr G. Quack (Merz & Co., Frankfurt/Main, Germany). Stock solutions (10 mM) of nitrendipine and 5,7-dichlorokynurenic acid were made in dimethyl sulphoxide (DMSO); final concentrations of DMSO did not exceed 0.1% (v/v). AP5 was dissolved at 10 mM in 0.05 N NaOH and diluted appropriately.

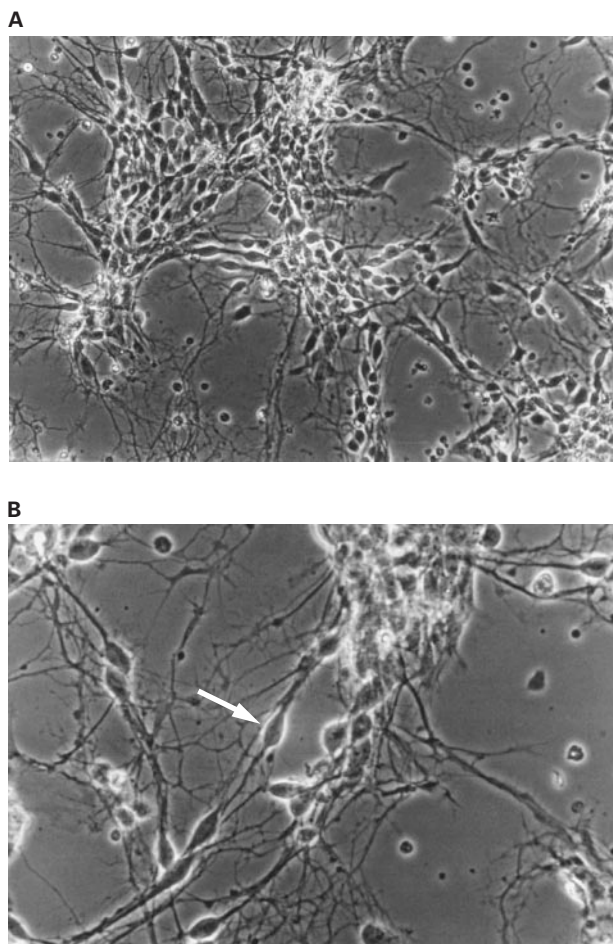


Figure 1 Phase-contrast photomicrographs of dissociated primary cultures of rat ventral mesencephalon after 7 days in culture. (A) overview photograph (magnification 50 fold); (B) arrow-labelled bipolar mesencephalic neurone of typical morphology as used in the fura experiments (magnification 100 fold).

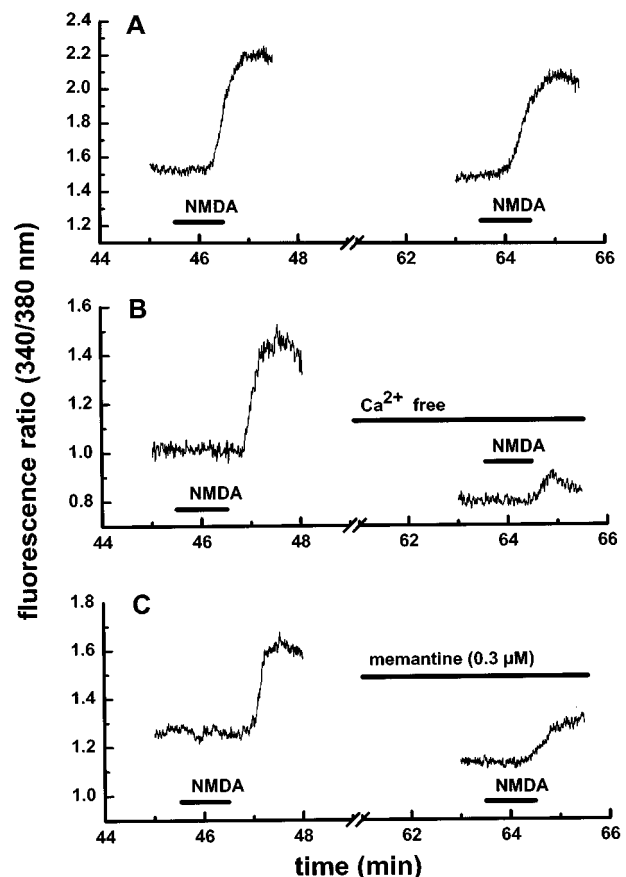


Figure 2 Effect of NMDA stimulation on the intracellular calcium concentration in individual mesencephalic neurones: time course of the experiment. (A) The cultures were superfused with magnesium-free HEPES buffer and stimulated twice with 100 μ M NMDA (plus 10 μ M glycine) for 1 min as described in Methods. (B) The original buffer containing 1.3 mM calcium was replaced by calcium-free buffer from 10 min before the second stimulation onwards. (C) Memantine (0.3 μ M) was present from 10 min before the second stimulation onwards. Representative traces from single experiments are shown.

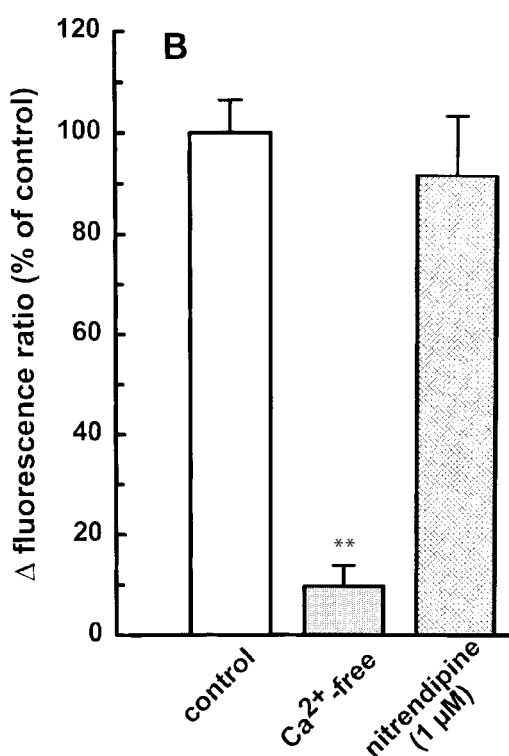
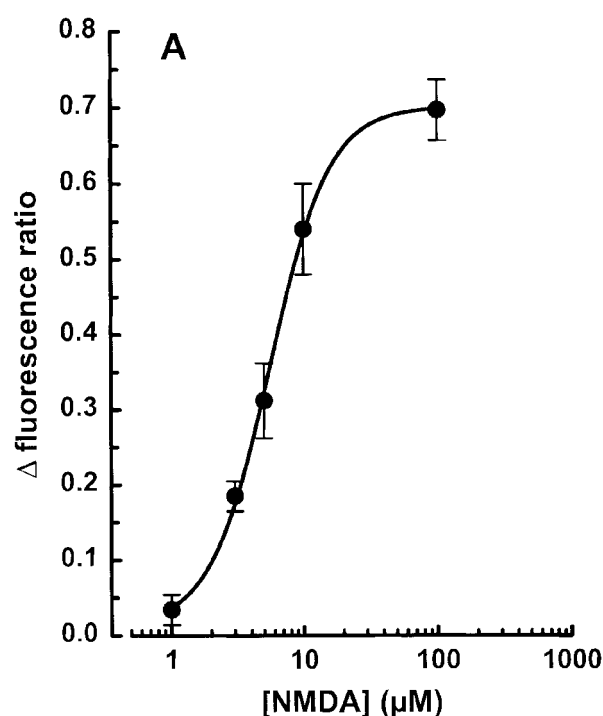


Figure 3 NMDA-induced enhancement of the intracellular calcium concentration in individual mesencephalic neurones: (A) concentration dependence; (B) effects of calcium deprivation and nitrendipine. The cells were superfused and stimulated twice with NMDA (1–100 μM; plus 10 μM glycine) for 1 min at increasing concentrations as described in Methods. Calcium-free buffer was used or 1 μM nitrendipine was present from 10 min before the second stimulation onwards. (A) Δ Fluorescence ratios above baseline were calculated from the difference between the peak response to each NMDA concentration and the corresponding basal value (see Figure 2). As verified in control experiments, responses to two consecutive stimulations with 100 μM NMDA were of similar magnitude. (B) Effects of calcium deprivation and nitrendipine were evaluated by calculating the ratio between the second and the first NMDA response, and expressed as a percentage of the mean NMDA control

Results

NMDA receptor function: intracellular Ca^{2+} levels

[Ca^{2+}]_i was determined in the cell bodies of bipolar rat mesencephalic neurones (DIV 7–11; Figure 1) incubated in magnesium-free HEPES buffer by means of the fura-2 method. Basal calcium concentrations averaged 95.6 ± 13.4 nM ($n = 11$). Application of NMDA (100 μM) plus glycine (10 μM) induced a rapid increase in [Ca^{2+}]_i which was reversible within 5 min and usually reproducible after an additional 15-min washout period (Figure 2A). The concentration dependence of the NMDA response (1–100 μM) is given in Figure 3A; the EC_{50} value was 5.7 μM. The maximal effect observed at 100 μM NMDA corresponded to an increase of [Ca^{2+}]_i to 320.5 ± 28.6 nM ($n = 13$). Two thirds of the neurones kept in culture for 4 days and longer were responsive to NMDA, whereas only one out of seven neurones exhibited a measurable response to NMDA after 2 DIV (Figure 4). The NMDA-induced increase in [Ca^{2+}]_i remained unchanged in the presence of 0.3 μM TTX (Δ fluorescence ratios above baseline were 0.71 ± 0.18 ($n = 7$) and 0.78 ± 0.13 ($n = 9$) with and without TTX, respectively), in contrast to that evoked by electrical field

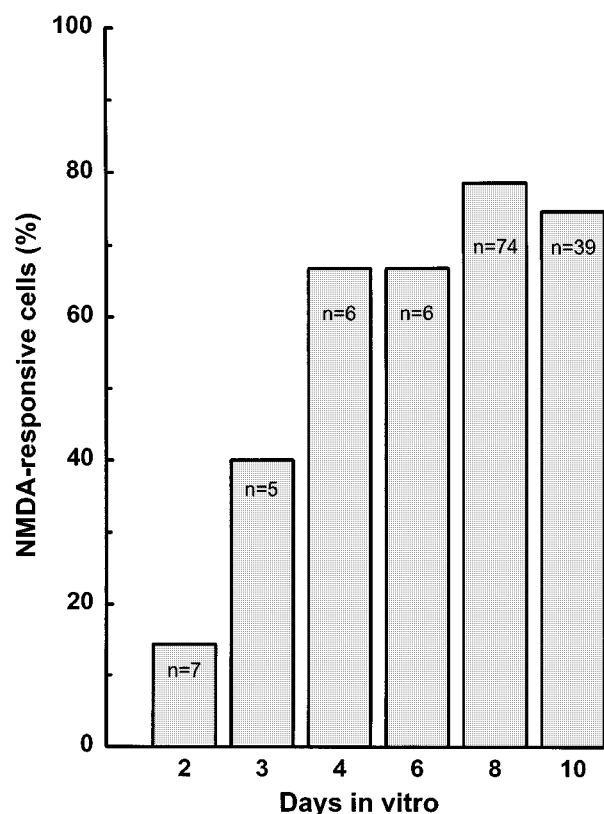


Figure 4 Development of NMDA receptor function in individual mesencephalic neurones. Cultures (2–10 DIV) were superfused and stimulated twice with 100 μM NMDA (plus 10 μM glycine) for 1 min. Δ Fluorescence ratios above baseline were calculated from the difference between the peak response to NMDA and the corresponding basal value. The percentage of cells exhibiting a Δ fluorescence ratio > 0.6 is given.

ratio. Means $\pm$ s.e. mean of 4–24 (A) and 3–8 (B) observations. ** $P < 0.01$ vs control.

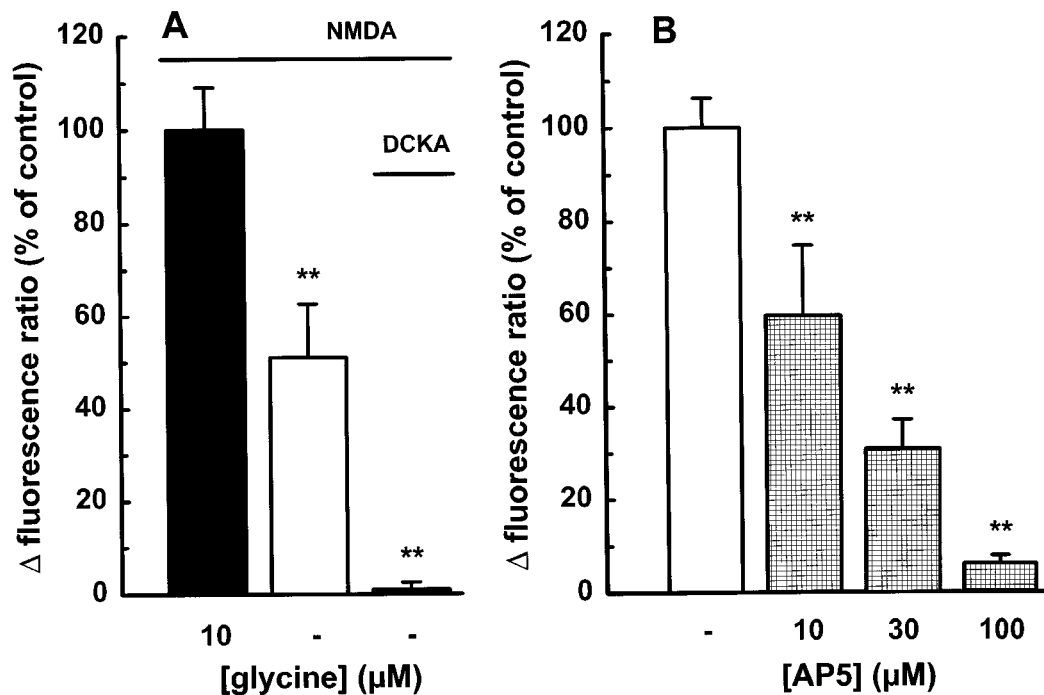


Figure 5 NMDA-induced enhancement of the intracellular calcium concentration in individual mesencephalic neurones: effect of (A) glycine and (B) AP5. The cells were superfused and stimulated twice with 100 μ M NMDA in the (A) absence or presence of 10 μ M glycine for 1 min. In some experiments, 5,7-dichlorokynurenic acid (DCKA; 10 μ M) was added. (B) AP5 (10–100 μ M) was present from 10 min before the second stimulation. Drug effects were evaluated by calculating the ratio between the second and the first NMDA response, and are expressed as a percentage of the mean NMDA control ratio (100 μ M plus 10 μ M glycine). Means $\pm$ s.e. mean of 3–8 observations. ** P < 0.01 vs control.

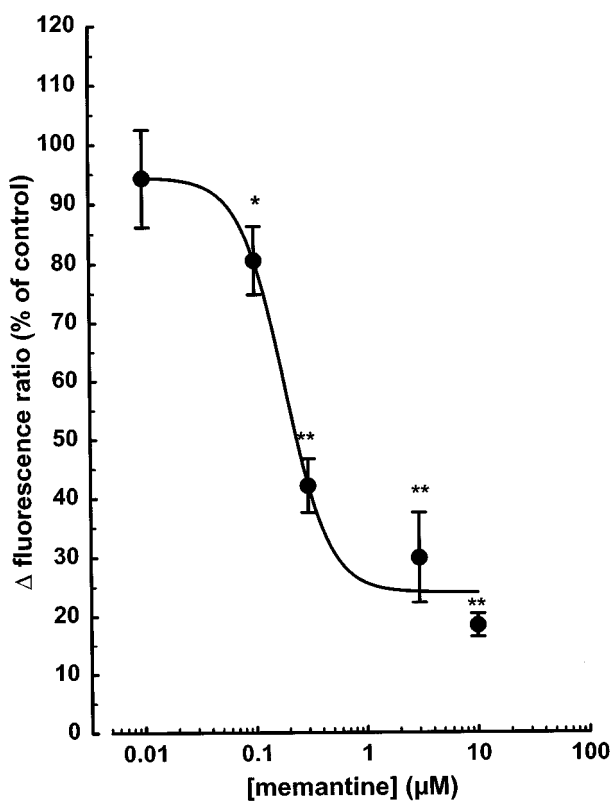


Figure 6 NMDA-induced enhancement of the intracellular calcium concentration in individual mesencephalic neurones: effect of memantine. The cells were superfused and stimulated twice with 100 μ M NMDA (plus 10 μ M glycine) for 1 min. Memantine (0.01–10 μ M) was present from 10 min before the second stimulation. Drug effects were evaluated by calculating the ratio between the second and the first NMDA response, and are expressed as a percentage of the

stimulation which was abolished by blockade of voltage-dependent Na^+ channels. The effect of NMDA was prevented in the absence of extracellular Ca^{2+} (Figure 2B and 3B) but was virtually insensitive to nitrendipine (1 μ M), a blocker of voltage-dependent L-type Ca^{2+} channels (Figure 3B).

In the absence of exogenous glycine, NMDA (100 μ M) caused less increase in $[\text{Ca}^{2+}]_i$ than that observed in the presence of the co-agonistic amino acid (Figure 5A). 5,7-dichlorokynurenic acid (10 μ M), a specific antagonist at the glycine site on the NMDA receptor, abolished the NMDA response (Figure 5A).

Addition of AP5 (10–100 μ M), a competitive NMDA receptor antagonist, attenuated the elevation of $[\text{Ca}^{2+}]_i$ in a concentration-dependent manner (Figure 5B); the NMDA response was virtually abolished at 100 μ M AP5. Memantine, an open-channel blocker antagonized the NMDA response with an IC_{50} value of 0.17 μ M (Figure 2C and 6). Exposure to 50 and 100 mM ethanol for 10 min caused about 25% and 45%-inhibition, respectively (Figure 7A). Ifenprodil, a preferential non-competitive NR1/NR2B receptor antagonist, significantly diminished the effect of NMDA at a concentration as low as 0.1 μ M; at 10 μ M the inhibition amounted to approximately 75% (Figure 7B). Half-maximal inhibition occurred at $\sim$ 1 μ M ifenprodil.

mRNA expression of NMDA receptor subunits

NR1 splice forms were detected by RT-PCR from mRNA of cultured mesencephalic neurones (8 DIV) using primer pairs

mean NMDA control ratio. Means $\pm$ s.e. mean of 3–8 observations. Significant differences vs control: * P < 0.05; ** P < 0.01.

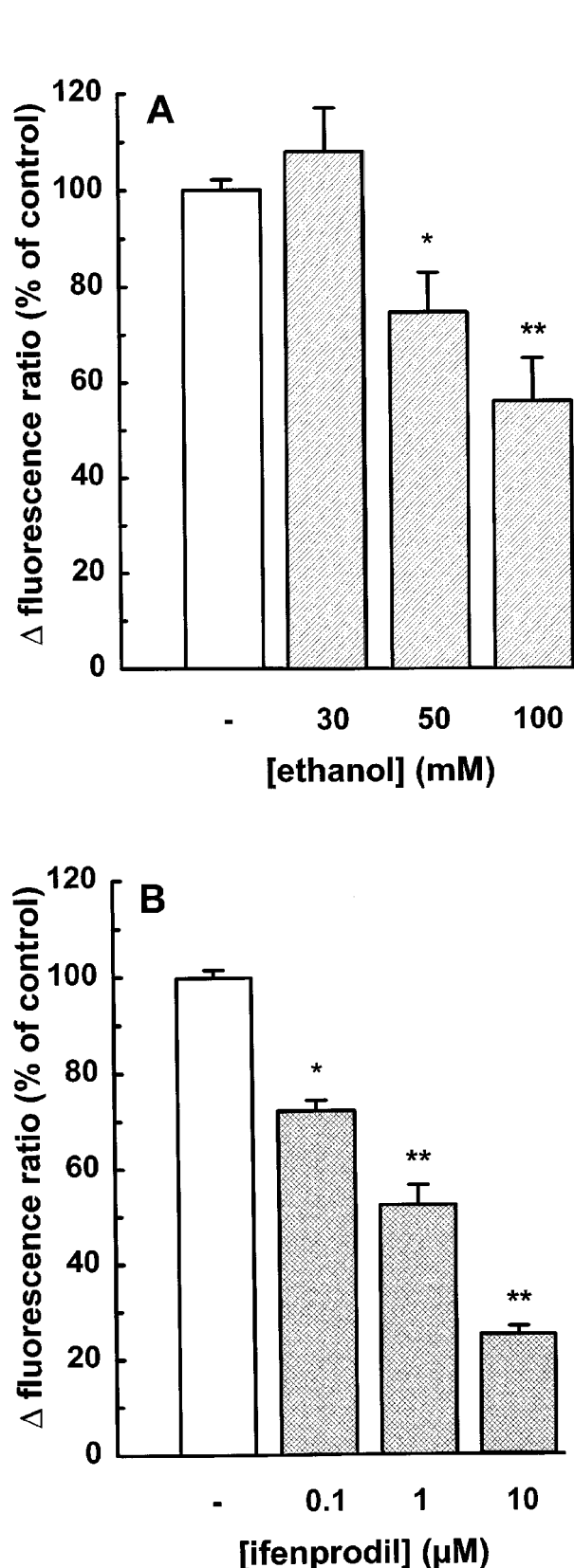


Figure 7 NMDA-induced enhancement of the intracellular calcium concentration in individual mesencephalic neurones: effect of (A) ethanol and (B) ifenprodil. The cells were superfused and stimulated twice with 100 μM NMDA (plus 10 μM glycine) for 1 min. Ethanol (30–100 mM) or ifenprodil (0.1–10 μM) was present from 10 min before the second stimulation. Drug effects were evaluated by calculating the ratio between the second and the first NMDA response, and are expressed as a percentage of the mean NMDA control ratio. Means ± s.e.mean of 4–10 observations. Significant differences vs control: * $P < 0.05$; ** $P < 0.01$.

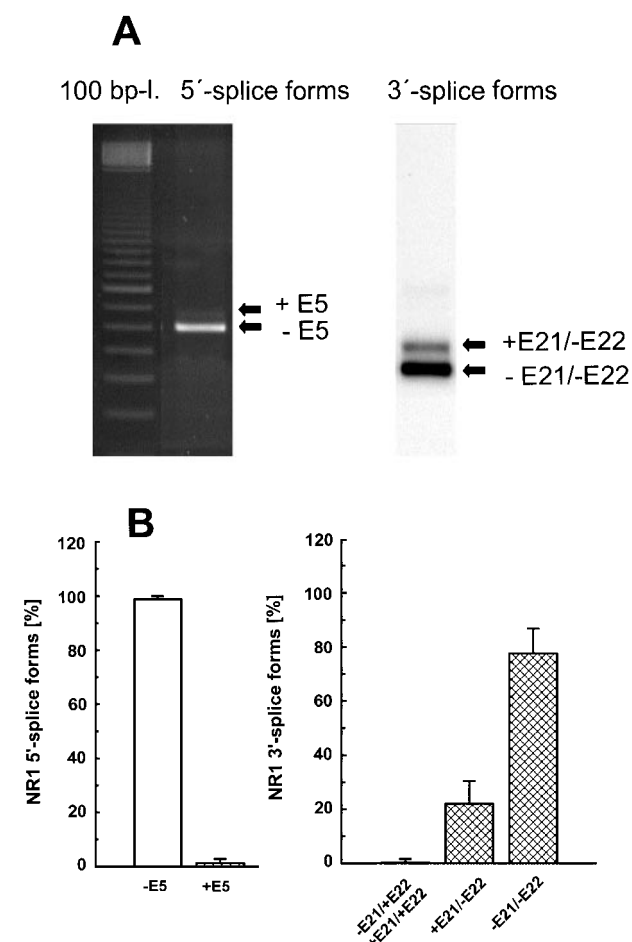


Figure 8 NR1 subunit mRNA expression in cultures of mesencephalic neurones (8 DIV). Subsequent to total mRNA extraction and RT-PCR amplification, cDNA products were analysed by agarose gel electrophoresis. NR1 fragments were detected using primer pairs flanking E5 (5'-splice forms) or E21/E22 (3'-splice forms). The ratios of the 5'- and 3'-splice forms were estimated after ethidium bromide staining and Southern hybridization, respectively. (A) Representative agarose gel electrophoresis and Southern blot. (B) Relative percentages of expression of each NR1 splice variant calculated from the corresponding total expression values (means ± s.d. of three individual experiments).

flanking E5 and E21/E22, respectively. Estimation of the ethidium bromide fluorescence intensities subsequent to agarose gel analysis of the PCR products representing the 5' splice variants ($\pm$ E5), revealed a virtually exclusive expression of the -E5 variant (Figure 8). Southern hybridization followed by CSPD chemiluminescence detection was used for semi-quantification of the 3' splice variants. The -E21/-E22 isoform exceeded +E21/-E22 by approximately 4 fold (Figure 8). Only traces of the -E21/+E22 and +E21/+E22 isoforms were detectable.

Digestion of amplified NR2A-C fragments was analysed by agarose gel electrophoresis (Figure 9). The relative amount of the digestion products of NR2A, 2B and 2C corresponded to a ratio of approximately 1:2:1, respectively, as estimated from their ethidium bromide fluorescence intensities (Figure 9B). The occurrence of mRNA coding for the NR2D subunit was demonstrated by separate RT-PCR analysis (Figure 9C).

In primary cultures of glial cells prepared for control purposes from mesencephalic tissue of rats at embryonic day 14, NR1 or NR2A-C mRNA expression was not observed (data not shown).

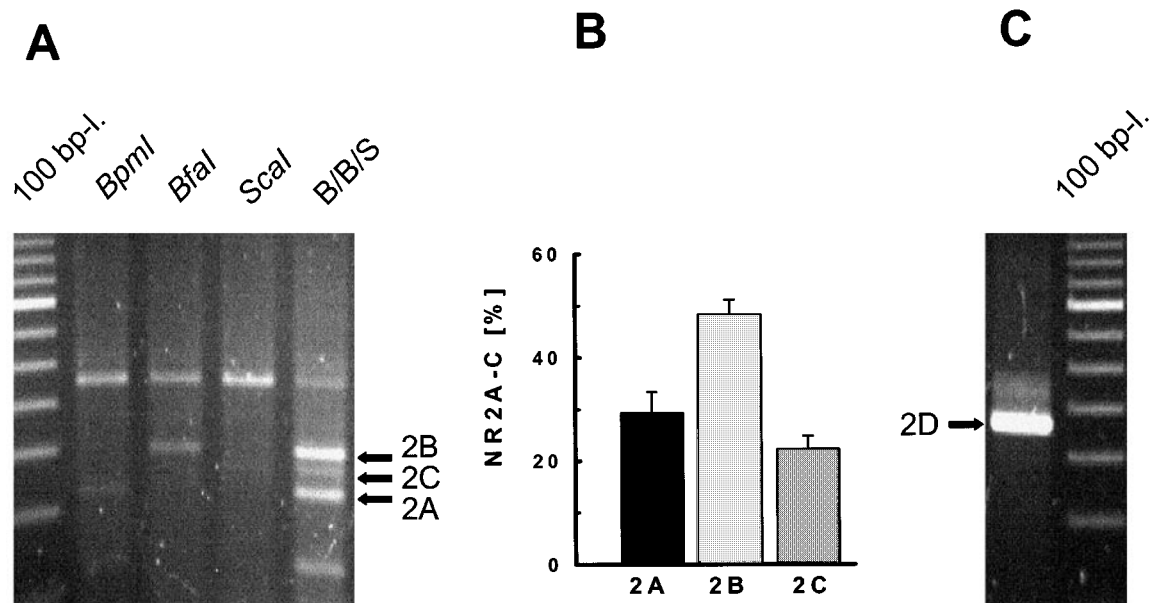


Figure 9 NR2A-D subunit mRNA expression in cultures of mesencephalic neurones (8 DIV). Subsequent to total mRNA extraction and RT-PCR amplification, cDNA products were analysed by agarose gel electrophoresis. (A) Restriction analysis of a NR2A-C amplification product with *BpmI* (lane 2), *BfaI* (lane 3), and *ScaI* (lane 4) which selectively cut the NR2A, 2B and 2C fragments, respectively; lane 5 (B/B/S) corresponds to the simultaneous digestion by the three enzymes; 100 bp ladder (lane 1). (B) Relative percentages of expression of each NR2A-C subunit calculated from the corresponding total expression values (means $\pm$ s.d. of three individual experiments). (C) Representative agarose gel electrophoresis of the 2D mRNA RT-PCR amplification product.

Discussion

Primary cultures of mesencephalic neurones were prepared from the ventral brainstem of Wistar rat foetuses. At gestational day 14, this area contains 90% of mesencephalic dopaminergic neurones representing about 20% of the total number of neurones (Shimoda *et al.*, 1992). These observations have been corroborated for the present preparation by detecting tyrosine hydroxylase (TH) immunoreactivity by using a mouse monoclonal TH antibody and subsequently the Vectastain ABC kit in combination with DAB reagents or goat anti-mouse IgG-Cy3 as secondary antibody. About 50% of the TH-positive cells in the mature culture (DIV 7) are bipolar, and spindle to oval shaped (Shimoda *et al.*, 1992). The cultures additionally comprise GABAergic and glutamatergic neurones (Rohrbacher *et al.*, 1995). Astrocytes did not significantly contribute to total cell counts up to DIV 11 (Shimoda *et al.*, 1992; own observations).

The NMDA-induced elevation of $[Ca^{2+}]_i$ was studied in the cell bodies of bipolar mesencephalic neurones. Although it was not possible to determine the phenotype of each individual cell under investigation, it may be assumed that dopamine neurones contribute with a considerable proportion to the total number of measured cells. In response to a given NMDA concentration no larger differences between various neurones were observed. Seven to 11-day old neurones were used, since the fraction of responsive cells increased with time, reaching a plateau at about 70% from DIV 6 onwards.

Glycine has repeatedly been shown to act as a co-agonist at a strychnine-insensitive site of the NMDA receptor complex different from that at which glutamate binds (Kleckner & Dingledine, 1988; Kemp & Leeson, 1993). Since 5,7-dichlorokynurenic acid abolished the NMDA response, the NMDA receptor apparently required the occupation of both NMDA and glycine, in order to be activated. The effect of NMDA in

the absence of exogenous glycine, however, may suggest the presence of endogenous glycine in the preparation investigated.

The response to NMDA was abolished by deprivation of extracellular Ca^{2+} , but remained virtually unaffected in the presence of nitrendipine, a blocker of L-type voltage-dependent Ca^{2+} channels indicating that this type of Ca^{2+} channel does not contribute to the NMDA effect. A role of voltage dependent Na^{+} influx can also be ruled out, since TTX ($0.3 \mu M$) had no significant effect on the NMDA response.

NMDA antagonists strongly reduced the increase in $[Ca^{2+}]_i$ evoked by $100 \mu M$ NMDA in mesencephalic neurones. AP5, a selective NMDA receptor antagonist which competes with NMDA at the agonist binding site, significantly decreased the NMDA response at $10 \mu M$ and more. The IC_{50} value of memantine, an open channel blocker of NMDA receptors with anti-parkinsonian and neuroprotective properties (Chen *et al.*, 1992; Parsons *et al.*, 1993; Danysz *et al.*, 1997) was $0.17 \mu M$ in the present study. This value resembles the IC_{50} value observed previously in cultured cerebellar neurones ($0.2 \mu M$) with the fura-2 method (Danysz *et al.*, 1997). In cortical neurones, however, memantine was approximately 25 times less potent (IC_{50} of $4.1 \mu M$; Black *et al.*, 1996) than in mesencephalic or cerebellar neurones. The high potency of memantine observed in the present study may be due to a particularly high affinity of memantine to NMDA receptors comprising the NR2C subunit (Danysz *et al.*, 1997). The highest levels of the NR2C subunit are expressed in the cerebellum (Nakanishi, 1992), but the present results show a significant expression also in mesencephalic neurones.

Ethanol, when administered acutely, impaired NMDA receptor function in intact brain tissue as well as in cultured neurones (Hoffman, 1995). For example, short-term *in vitro* exposure to ethanol inhibited NMDA-activated Ca^{2+} currents in hippocampal neurones (Lovinger *et al.*, 1989), $^{45}Ca^{2+}$

uptake in cerebellar granule cells (Hoffman *et al.*, 1989), and the elevation of $[Ca^{2+}]_i$ in cortical neurones (Lustig *et al.*, 1992; Hu & Ticku, 1995; Bhavé *et al.*, 1996). In the latter preparation (Bhavé *et al.*, 1996) and in cerebellar granule cells (Bhavé & Hoffman, 1997), ethanol inhibited the NMDA response in a concentration-dependent manner with a threshold concentration determined at 30 mM; a maximum effect of 40 and 70% in cortical neurones and cerebellar granule cells, respectively, was observed at 100 mM. In the present study on mesencephalic neurones, the inhibition of the NMDA-induced increase in $[Ca^{2+}]_i$ caused by 100 mM ethanol amounted to 45%.

The mechanism of the acute inhibitory effect of ethanol on NMDA receptor function is still unclear. Considerable discrepancies occur between various studies. Since glycine reversed the inhibitory effect of ethanol on NMDA receptor function in striatal slices (dopamine release; Woodward & Gonzales, 1990), dissociated cells of neonatal brain (elevation of $[Ca^{2+}]_i$; Dildy-Mayfield & Leslie, 1991), and cerebellar granule cells (elevation of $[Ca^{2+}]_i$; Bhavé & Hoffman, 1997), the glycine binding site was postulated as site of the action of ethanol. However, studies on cultured hippocampal neurones (Peoples & Weight, 1992), cortical and hippocampal tissue (Gonzales & Woodward, 1990; Woodward, 1994), and cultured cerebellar granule cells (glycine $\leq 100 \mu M$; Cebers *et al.*, 1996) failed to demonstrate a reversal of the effect of ethanol by glycine. The reason for the discrepancies observed are not completely understood. The sensitivity to ethanol and glycine may partly depend on the NMDA receptor subunit composition. Studies performed on recombinant NMDA receptors suggest that ethanol preferentially affects heteromers containing the NR2A or NR2B subunit (Kuner *et al.*, 1993; Sucher *et al.*, 1996). In addition, it was reported that glycine reversal of ethanol inhibition was more pronounced when NR1/NR2A subunits were expressed, compared with NR1/NR2B (Buller *et al.*, 1995).

Ifenprodil, an agent with potential anti-ischæmic properties, can be used to detect NMDA receptors comprising the NR2B subunit. Ifenprodil is a non-competitive NMDA receptor antagonist initially assumed to act at the polyamine binding site (Carter *et al.*, 1989). However, at low concentrations ifenprodil promoted a non-conducting state of the receptor that did not involve the polyamine site on NMDA receptors (Legendre & Westbrook, 1991). According to recent data, ifenprodil binds to a site close to but distinct from the polyamine binding site so that the mechanism of interaction is presumably due to anti-cooperativity (Trube & Malherbe, 1998). The racemic form of ifenprodil which has been commonly used exhibited a potency for the NR1/NR2B receptor exceeding that for the NR1/NR2A receptor by more than 400 fold (Lovinger, 1995; Williams, 1993; Lovinger & Ziegelgänsberger, 1996). Similar results were observed with the pure stereoisomers of ifenprodil (Avenet *et al.*, 1996). In rat cultured hippocampal pyramidal neurones, ifenprodil attenuated the NMDA-induced elevation of $[Ca^{2+}]_i$ with an IC_{50} -value of $0.7 \mu M$ (Church *et al.*, 1994). In mesencephalic neurones, ifenprodil exhibited a similar potency indicating that the NMDA response in each preparation is mediated by NMDA receptors comprising the NR2B subunit.

Expression of NR subunit mRNA levels has been investigated in detail in the adult rat CNS using *in situ* hybridization or solution hybridization/RNase protection assays (Laurie & Seeburg, 1994; Laurie *et al.*, 1995; Zhong *et al.*, 1995). The NR1 mRNA is abundantly expressed in every neuronal type examined. In the cortex and hippocampus, the predominant splice variants were those without E5 and with or without both E21 and E22. In cerebellum, however, the major

NR1 variants were those lacking E5 and both 3' inserts (Zhong *et al.*, 1995). Little is known on the NR1 splice variant mRNA expression in mesencephalic structures. In the substantia nigra pars compacta, a significant expression of only the splice forms lacking E5 and both E21 and E22 has been found (Laurie *et al.*, 1995). Similarly, the present study on cultured mesencephalic neurones using RT-PCR reveals an almost exclusive expression of the low molecular splice variants (i.e. lacking E5, E22).

Studies on recombinant NMDA receptors expressed in *Xenopus* oocytes and in mammalian cells revealed distinct functional properties of the different NR1 isoforms (Zukin & Bennett, 1995; Sucher *et al.*, 1996). For example, NR1 splice variants containing E5 had approximately five-times lower affinity for the agonists glutamate and NMDA, a higher affinity for antagonists, a virtual loss of potentiation by polyamines, and altered allosteric modulation by Mg^{2+} . The functional significance of alternative splicing of the C-terminal region of NR1 subunits has yet to be established. E21 was shown to contain most of the target sites for protein kinase C. Consequently, subcellular distribution of the NR1 subunit and NMDA channel properties such as open times and desensitization may be regulated by alternative splicing of E21 (Laurie *et al.*, 1995; Sucher *et al.*, 1996).

In contrast to NR1 mRNA expression, the mRNAs encoding the NR2 subunits are expressed more restrictedly, both spatially and in abundance. The NR2A mRNA is detectable throughout the brain, NR2B is confined to forebrain structures, NR2C is primarily expressed in cerebellar granule cells, and NR2D mRNA is expressed in lower abundance in structures such as midline thalamus and dentate gyrus (Monyer *et al.*, 1994). The cell-specific and developmental regulation of NR2 subunit expression additionally accounts for the heterogeneity of NMDA receptor properties observed in different neuronal populations. If the heteromeric NR1/NR2 receptor contains the subunit 2A or 2C, potentiation by spermine will not be observed. In contrast, NR2B is permissive for potentiation by spermine. NR1/NR2A receptors exhibit a much stronger block by Mg^{2+} than NR1/NR2C channels do. Receptors consisting of NR1/NR2C subunits were less sensitive to inhibition by ethanol compared to receptors consisting of NR1/NR2A or NR1/NR2B, while there was only a minor difference within the sensitivity of the latter two types of receptors to ethanol (Kuner *et al.*, 1993; Buller *et al.*, 1995). In the present study on mesencephalic neurones, NR2A, NR2B, and NR2C subunit expression was determined at a ratio of approximately 1:2:1, respectively, by RT-PCR. In addition, the occurrence of mRNA coding for the NR2D subunit was demonstrated. The question whether the NMDA subunits are expressed in dopaminergic or GABAergic neurones remains as yet open. The answer can be presumably given by carrying out single cell RT-PCR experiments. An immunohistochemical and *in situ* hybridization study performed in midbrain tissue failed to detect NR2A/B subunits in dopaminergic neurones of the substantia nigra pars compacta or VTA (Paquet *et al.*, 1997).

In conclusion, the present data indicate that mesencephalic neurones bear ethanol sensitive NMDA receptors which might be implicated in the development of ethanol dependence and withdrawal. The high affinity of NMDA and the sensitivity to ifenprodil may suggest that the NMDA receptor comprises the NR1 splice variant lacking E5, and the NR2B subunit. Due to the high potency of memantine, the NMDA receptor may additionally contain the NR2C subunit, which is significantly expressed in mesencephalic neurones.

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Two affinities for a single antagonist at the neuronal NK₁ tachykinin receptor: evidence from quantitation of receptor endocytosis

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1 In smooth muscle contractility assays, many NK₁ receptor (NK₁r) antagonists inhibit responses to the neurotransmitter, substance P (SP), and its analogue, septide, with markedly different potency, leading to the proposal that there is a septide-preferring receptor related to the NK₁r.

2 We used fluorescence immunohistochemistry and confocal microscopy to visualize agonist-induced NK₁r endocytosis and analyse agonist/antagonist interactions at native NK₁r in neurons of the myenteric plexus of guinea-pig ileum.

3 SP and septide gave sigmoid log concentration-response curves and were equipotent in inducing NK₁r endocytosis.

4 The NK₁r antagonists, CP-99994 (2S,3S)-3-(2-methoxybenzyl)amino-2-phenylpiperidine dihydrochloride and MEN-10581, cyclo(Leu/[CH₂NH]Lys(benzyloxycarbonyl)-Gln-Trp-Phe-βAla) were both more potent in inhibiting endocytosis (50× and 8× greater respectively) against septide than against SP.

5 The results suggest that SP and septide interact differently with the NK₁r, and that a single antagonist can exhibit different affinities at a single NK₁r population, depending on the agonist with which it competes. Thus it may not be necessary to posit a separate septide-preferring tachykinin receptor.

Keywords: Confocal microscopy; CP-99994; MEN-10581; NK₁ receptor; receptor endocytosis; septide; substance P; tachykinin

Abbreviations: FITC, fluorescein isothiocyanate; HBSS, Hanks balanced salt solution; NK₁r, neurokinin 1 receptor; NK₁r-IR, NK₁ receptor immunoreactivity; PBS, sodium phosphate buffered saline; PSS, physiological salt solution; SP, substance P

Introduction

Differences in pharmacological profiles between the natural ligand for the NK₁r, SP, and its analogue, septide, (pGlu⁶,Pro⁹]-SP (6–11), have been revealed in organ bath and receptor-binding studies. Septide contracts a number of smooth muscle preparations, including the guinea-pig ileum, with similar potency to SP (EC₅₀, 1–9 nM), whereas it competes with low affinity for SP binding (Laufer *et al.*, 1988; Petit *et al.*, 1992; Carruette *et al.*, 1992; Pradier *et al.*, 1993; Glowinski, 1995; Nguyen-Le *et al.*, 1996). Many NK₁r antagonists inhibit responses of isolated tissues to septide with markedly greater potency (higher pA₂ values) than they inhibit responses to SP, when both agonists are active at nanomolar concentrations, and this led to the suggestion that septide may interact with a 'septide-preferring' tachykinin receptor, distinct from the NK₁r (Petit *et al.*, 1992; Longmore *et al.*, 1994; Zeng & Burcher, 1994; Patacchini *et al.*, 1995; Glowinski, 1995; Nguyen-Le *et al.*, 1996). Receptor cloning has failed to identify such a receptor (Maggi & Schwartz, 1997). However, in transfected cells that express only the NK₁r, homologous binding data indicate that septide and SP bind to the receptor with similar nanomolar affinity (Hastrup & Schwartz, 1996). In addition to the nanomolar binding of SP and septide, a subnanomolar affinity binding that is selective for SP has been identified (Pradier *et al.*, 1993; Hastrup & Schwartz, 1996).

Maggi & Schwartz (1997) postulated that the pharmacological and binding data can be explained if there are two sites or conformations of a single NK₁r, one (the general tachykinin conformer) at which SP and septide both bind with nanomolar affinity (K_D , 0.5 nM) and one (the SP-preferring conformer) which binds SP at subnanomolar affinity (K_D , 0.05 nM) and septide at relatively low affinity (K_D , 50 nM). If this is true, there may be no separate septide receptor; however, the differential effects of antagonists against nanomolar concentrations of SP and septide remain unexplained.

The aim of the present study was to clarify whether SP and septide activate the same tachykinin receptor and determine whether selective NK₁r antagonists differentially inhibit responses to each agonist when acting at the same immunohistochemically identified receptor. The NK₁r is a G-protein coupled receptor that undergoes endocytosis into cytoplasmic organelles when activated by agonists. We have previously shown that confocal images can provide quantitative data on trafficking of the NK₁r in enteric neurons (Southwell *et al.*, 1998a,b). In this study we have used this technique for pharmacological analysis of activation of immunohistochemically identified receptors on individual cells in intact tissue. We have examined SP- and septide-induced endocytosis of native NK₁r on enteric neurons and investigated the effects of the selective NK₁r antagonists CP-99994 and MEN-10581 on this endocytosis. Both CP-99994 and MEN-10581 have previously been shown to have different potencies when acting against SP and septide in smooth muscle

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contractility studies (Patacchini *et al.*, 1995; Nguyen-Le *et al.*, 1996).

Methods

Experimental protocol

NK₁r endocytosis in enteric neurons in the guinea-pig ileum was studied using methods that have been characterized previously (Grady *et al.*, 1995; Southwell *et al.*, 1996, 1998a). Guinea-pigs of either gender (250–350 g) were killed by a blow to the head and exsanguination. One cm segments of ileum were isolated and incubated for 1 h at 37°C in modified physiological salt solution (PSS; composition in mM: NaCl 118; KCl 4.8; NaHCO₃ 25; NaH₂PO₄ 1.0; MgSO₄ 1.2, glucose 11.1; CaCl₂ 2.5; containing 3 µM nicardipine and 0.3 µM tetrodotoxin; bubbled with 95% O₂ and 5% CO₂), to allow receptor to locate on the cell surface (Southwell *et al.*, 1996). Tissues were rinsed in ice-cold Hanks balanced salt solution (HBSS; containing 3 µM nicardipine, 1% bovine serum albumin, 50 µM monensin, and the protease inhibitors 4 µg ml⁻¹ chymostatin, 40 µg ml⁻¹ bacitracin and 4 µg ml⁻¹ leupeptin), and incubated for 1 h at 4°C in HBSS in the absence or presence of SP (10 pM–10 µM) or septide (10 pM–10 µM). At 4°C the agonists are able to diffuse into the tissue and bind receptors, but membranes are solidified and so receptor endocytosis cannot occur. Tissues were then rinsed in warm PSS and incubated in PSS containing 50 µM monensin for 20 min at 37°C. Monensin blocks the exchange of H⁺ ions across endosomal membranes (Maxfield, 1982; Pless & Wellner, 1996). One effect of this block is to inhibit the recycling of NK₁r without preventing their endocytosis (Grady *et al.*, 1995; Southwell *et al.*, 1998a). We have previously shown that maximum endocytosis of NK₁r occurs after 20 min warming (Southwell *et al.*, 1996). Endocytosed NK₁r is recycled to the cell surface within 30 min of endocytosis (Southwell *et al.*, 1996; 1998a), and so monensin was used in the present study to prevent NK₁r recycling.

To investigate the effects of the NK₁r antagonists CP-99994, (2S,3S)-3-(2-methoxybenzyl)amino-2-phenylpiperidine dihydrochloride (10 pM–10 nM) or MEN-10581, cyclo (Leuψ[CH₂NH]Lys(benzyloxycarbonyl)-Gln-Trp-Phe-βAla) (10 nM–3 µM) on agonist-induced NK₁r endocytosis, tissue was incubated with antagonist for 1 h at 37°C in PSS without agonist, prior to incubation for 1 h at 4°C in HBSS with antagonist and with or without agonist. The antagonists, but not agonists, were also included in the final 20 min incubation in PSS at 37°C.

At the end of the incubations, tissues were transferred to ice cold PBS (0.9% NaCl in 0.01 M sodium phosphate buffer, pH 7.0) containing 3 µM nicardipine, opened along the mesenteric attachment, stretched and pinned to balsa board and fixed overnight in 2% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.0.

Immunohistochemistry

Fixed segments of ileum were dehydrated in dimethylsulphoxide (3 × 10 min) and rehydrated in PBS (3 × 10 min). Wholmount preparations of myenteric plexus were incubated for 1 h in PBS containing 1% Triton X-100 and 10% normal horse serum to block non-specific binding. Gut was incubated overnight at room temperature in hypertonic PBS containing antisera against the rat NK₁r (rabbit polyclonal, code 94168, to the intracellular 15 C-terminal amino acids of the rat NK₁r;

(Grady *et al.*, 1996); diluted 1 : 500) and against synaptophysin (mouse monoclonal, Dako Laboratories, code M776; 1 : 100). This NK₁r antibody binds to the full length NK₁r, but does not bind to the NK₂ or NK₃ tachykinin receptors, or to the C-terminus truncated form of the NK₁r (Grady *et al.*, 1996). Synaptophysin is a synaptic vesicle protein. It is in high concentrations in varicosities that surround nerve cells and was used to indicate the location of nerve cell surfaces. Tissues were washed in PBS (3 × 10 min) then incubated for 2 h at room temperature in PBS containing biotinylated horse anti-mouse antiserum (Vector Laboratories, code H0508; 1 : 100). Preparations were again washed in PBS (3 × 10 min), and incubated for 90 min in PBS containing streptavidin-Texas red complex (Amersham, Australia, batch No 31; 1 : 50) and sheep anti-rabbit antiserum coupled to fluorescein isothiocyanate (FITC) (Wellcome Laboratories, Australia, code K0722; 1 : 200). Tissue was washed in PBS (3 × 10 min) and mounted on microscope slides in buffered glycerol (pH 8.6).

Confocal microscopy

Samples were viewed on a Biorad MRC1024 confocal scanning laser system with a krypton/argon laser, installed on a Zeiss Axioplan 2 microscope. Fluorophores were visualized using a 488 nm excitation filter and 522–535 nm emission filter for FITC and a 568 nm excitation filter and 605–632 nm emission filter for Texas red. Single confocal images (optical section, 0.5 µM), that included the nucleus and a large area of cytoplasm, were taken through the centre of NK₁r-immunoreactive neurons chosen at random. Light emitted as green (NK₁r-IR) or red (synaptophysin) fluorescence was collected simultaneously using a 100 × objective (NA 1.4) and 1.4 zoom. Images of 512 × 512 pixels were obtained and processed using Confocal Assistant, Adobe Photoshop and Corel Draw software.

Fluorescence quantitation

Subcellular distribution of NK₁r-IR was quantified in imaged neurons, using NIH-Image software (<http://rsb.info.nih.gov/nih-image/>) as previously described (Southwell *et al.*, 1998a,b). Nuclear fluorescence was measured and this value was set as a threshold for measuring specific staining. Total cell and cytoplasmic FITC fluorescence were measured by counting the number of pixels with intensity above threshold in the whole cell and then in the cytoplasm only, and the percentage of fluorescence attributable to endocytosed NK₁r-IR was calculated.

Data analysis

Ten cells were imaged per tissue and each experiment was performed 3–4 times on tissue from different animals. Differences between groups were compared by two-way ANOVA followed by Student-Newman-Keuls test using Sigma Stat software. A *P* value < 0.05 was taken as significant. Antagonist activities were quantified as pA₂ values determined by the intercepts on the abscissa of Schild plots (Arunlakshana & Schild, 1959). The values determined are described as apparent pA₂ values as only two data points are used for each calculation.

Drugs

Bacitracin (Calbiochem, Australia); chymostatin (Sigma, U.S.A.); cyclo (Leuψ[CH₂NH]Lys(benzyloxycarbonyl)-Gln-

Trp-Phe- β Ala) (MEN-10581; Menarini, Italy); leupeptin (Auspep, Australia); (2S,3S)-3-(2-methoxybenzyl)amino-2-phenylpiperidine dihydrochloride (CP-99994; Pfizer, U.S.A.); monensin (Sigma, U.S.A.); nicardipine (Sigma, U.S.A.); septide (Auspep, Australia); substance P (SP; Auspep, Australia); tetrodotoxin (Alomone, Israel).

Results

Agonist-induced endocytosis of NK₁r

In tissues not exposed to agonist, $80.9 \pm 1.6\%$ of NK₁r-IR was present as a smooth band on the plasma membrane of a subpopulation of nerve cells in myenteric ganglia and the residual $19.1 \pm 1.6\%$ was located in small aggregations throughout the cytoplasm (Figure 1A). Both SP and septide caused concentration-dependent endocytosis of NK₁r-IR in the nanomolar range (Figure 2). As agonist concentration was increased, the proportion of NK₁r-IR on cell membranes was progressively reduced with a concomitant increase in the number of NK₁r-IR aggregations in the cytoplasm (Figure 1B–E, Figure 2). At concentrations of SP or septide of $1 \mu\text{M}$ or higher, most NK₁r-IR was present in aggregations distributed evenly throughout the cytoplasm of the cell; the continuous band of immunoreactivity was no longer evident on the surfaces of cells (Figure 1C and E). Both agonists produced a maximum receptor endocytosis of approximately 75% at $10 \mu\text{M}$, the highest concentration used, with the remaining 25% being present on, or very near, the cell surface (Figure 2). The effects of SP and septide on the subcellular distribution of NK₁r did not differ either qualitatively (Figure 1) or quantitatively (Figure 2). The two agonists induced NK₁r endocytosis with equivalent potency (EC_{50} : $\text{SP} = 5.6 \pm 1.1 \text{ nM}$;

septide = $8.5 \pm 1.9 \text{ nM}$). Nonlinear regression analysis of agonist concentration-response curves produced Hill coefficients that did not differ between SP (0.43 ± 0.10) and septide (0.45 ± 0.11).

Effects of NK₁r antagonists on receptor endocytosis

We investigated the effects of the NK₁r antagonists, CP-99994 and MEN-10581, on agonist-induced receptor endocytosis. Neither CP-99994 (10 pM – 10 nM) nor MEN-10581 (10 nM – $3 \mu\text{M}$) induced NK₁r endocytosis in myenteric neurons (Figure 4A and B), confirming our previous observations that endocytosis is dependent on the activation of receptor by agonist, but is not induced when antagonists bind to the receptor (Southwell *et al.*, 1996). Both CP-99994 and MEN-10581 markedly inhibited receptor endocytosis induced by either SP or septide (Figure 4C–F), producing rightward shifts in the concentration-response curves to the two agonists (Figure 4). Both antagonists were markedly more potent inhibitors of responses to septide than they were of responses to SP (Figures 3 and 4).

Schild analysis of antagonist activities produced apparent pA_2 values of 10.2 and 11.9 for CP-99994 acting against SP and septide, respectively, and 7.5 and 8.4 for MEN-10581 acting against SP and septide, respectively (Figure 4E and F), indicating that the antagonists were $50 \times$ (CP-99994) and $8 \times$ (MEN-10581) more potent against septide than against SP. Slopes of the Schild plots were close to unity: for CP 99994, -0.96 versus SP and -1.02 versus septide; for MEN-10581, -1.15 versus SP and -0.94 versus septide. These slopes are consistent with agonists and antagonists interacting competitively. Both antagonists have been shown in smooth muscle contractility studies to be competitive antagonists at the NK₁r (Longmore *et al.*, 1994; Zeng & Burcher, 1994; Patacchini *et al.*, 1995; Nguyen-Le *et al.*, 1996). However, in the present study the agonist concentration-response curves became steeper in the presence of MEN-10581, suggesting an allosteric interaction may occur for this antagonist.

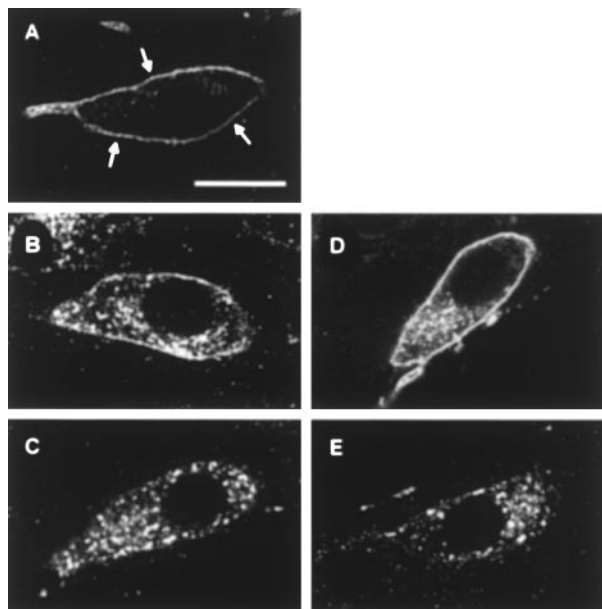


Figure 1 Agonist-induced endocytosis of NK₁ receptor immunoreactivity (NK₁r-IR) in myenteric neurons from guinea-pig ileum. Each micrograph is a single $0.5 \mu\text{m}$ thick optical section obtained by confocal microscopy. The dark areas are the cell nuclei. Neurons were incubated with: (A), no agonist; (B), 10 nM substance P (SP); (C), $1 \mu\text{M}$ substance P; (D), 10 nM septide; and (E), $1 \mu\text{M}$ septide. Prior to agonist exposure, NK₁r-IR is at the cell surface (arrows). Both substance P and septide induced a concentration-dependent endocytosis of NK₁ receptors. Scale bar, $20 \mu\text{m}$.

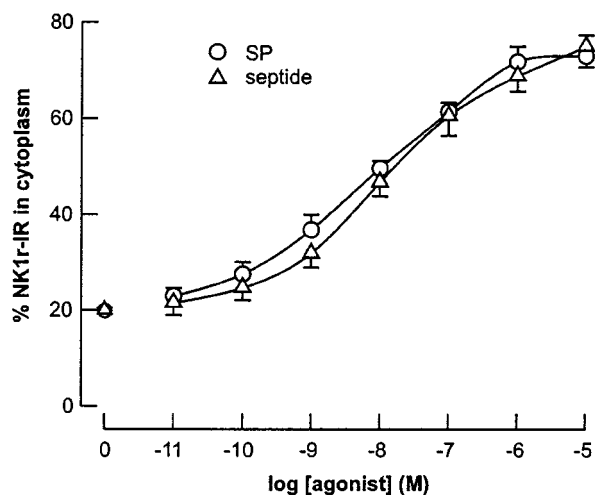


Figure 2 Concentration-response curves for NK₁ receptor endocytosis induced by substance P (SP) and septide in myenteric neurons from guinea-pig ileum. The subcellular distribution of NK₁ receptor immunoreactivity in imaged neurons was measured using quantitative confocal microscopy. Both substance P and septide induced a concentration-dependent endocytosis of NK₁ receptors. The effects of substance P and septide did not differ from each other ($P > 0.05$). Values are means $\pm$ s.e.mean. ($n = 3$; 30 cells, three experiments, ten cells were imaged for each experiment).

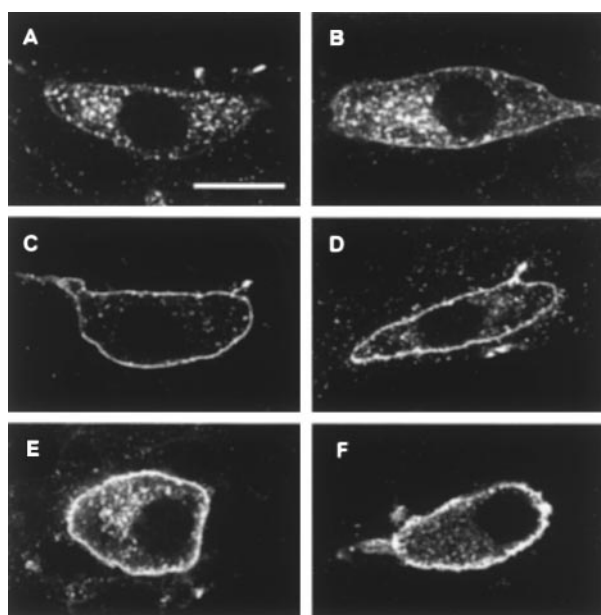


Figure 3 Confocal images (single 0.5 μ m optical sections) showing the effects of the NK₁ receptor antagonists CP-99994 and MEN-10581 on endocytosis of NK₁ receptor immunoreactivity (NK₁r IR) induced by substance P (SP) or septide in myenteric neurons from guinea-pig ileum. Neurons were incubated with: (A), 100 nM SP; (B), 100 nM septide; (C), 100 nM SP and 10 nM CP-99994; (D), 100 nM septide and 100 pM CP-99994; (E) 100 nM SP and 3 μ M MEN-10581; and (F), 100 nM septide and 100 nM MEN-10581. Both CP-99994 and MEN-10581 inhibited NK₁ receptor endocytosis induced by SP or septide. Both antagonists were markedly more potent when acting against septide than against SP. Scale bar, 20 μ m.

As NK₃ receptors almost certainly exist on guinea-pig myenteric neurons (Johnson *et al.*, 1998), we tested the possibility that interaction of SP with NK₃ receptors may influence our results. Segments of guinea-pig ileum were incubated with SP in the presence of the NK₃ receptor antagonist SR-142801 to block binding to NK₃ receptor. This did not alter the percentage of NK₁r endocytosed in response to SP ($n=2$, data not shown).

Discussion

The present study investigated ligand-induced endocytosis of NK₁r on myenteric neurons of the guinea-pig ileum and demonstrates that the neurotransmitter, SP, and its artificial analogue, septide, induce endocytosis of the NK₁r with equivalent potency. Endocytosis of the NK₁r resulted from agonist binding to receptors and was not induced by binding of the selective NK₁r antagonists CP-99994 or MEN-10581. Analysis of agonist-induced endocytosis of NK₁r produced sigmoidal log concentration-response curves, equivalent in form to those obtained in conventional pharmacological studies (Figure 2). Moreover, the agonist concentrations producing receptor endocytosis were in the nanomolar range, similar to the concentrations with which these agonists contract smooth muscle preparations (Laufer *et al.*, 1988; Petitet *et al.*, 1992; Hall *et al.*, 1994), including the guinea-pig ileum (Carruette *et al.*, 1992; Nguyen-Le *et al.*, 1996), with which they stimulate inositol phosphate formation in transfected cells (Sagan *et al.*, 1996) and which is observed in homologous binding studies (Hastrup & Schwartz, 1996). As this technique produces agonist concentration-response data

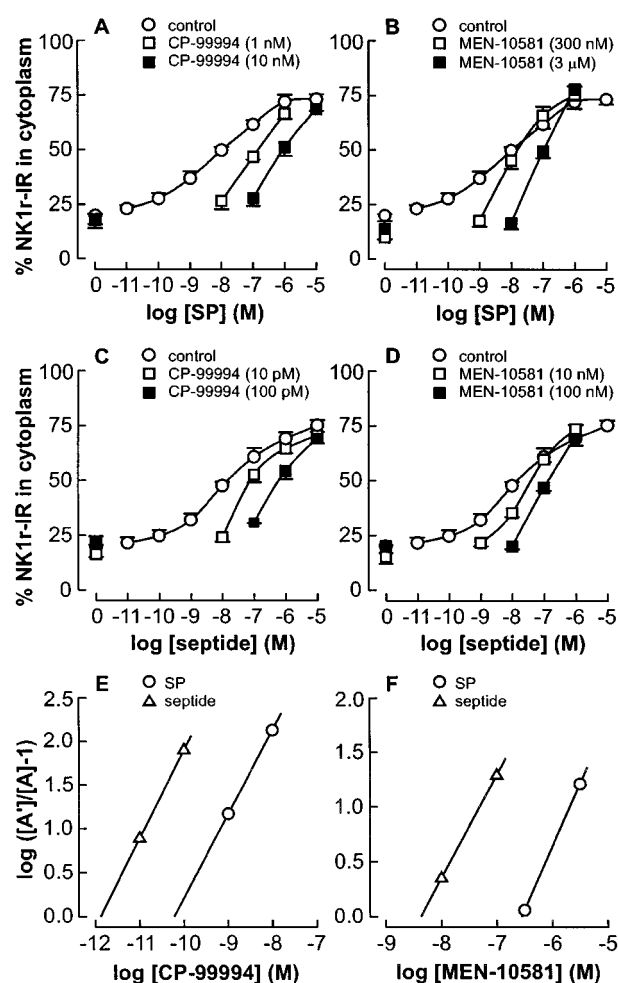


Figure 4 Concentration-response curves for the effects of CP-99994 (A and C) and MEN-10581 (B and D) on endocytosis of NK₁ receptor immunoreactivity (NK₁r IR) induced by substance P (SP) (A and B) or septide (C and D) in myenteric neurons from guinea-pig ileum. Both CP-99994 and MEN-10581 inhibited receptor endocytosis induced by either SP or septide. Both antagonists were more potent inhibitors of NK₁r endocytosis induced by septide than of that induced by SP. Values are means $\pm$ s.e. mean ($n=3-4$; 30 or 40 cells, 3-4 experiments, ten cells were imaged for each experiment). (E and F): Schild plots for the antagonism by CP-99994 (E) and MEN-10581 (F) against septide and SP. Scale on ordinate is log concentration ratio minus 1, where the concentration ratio ($[A']/[A]$) is the ratio of the EC₅₀ concentrations for the agonist in the presence and absence of antagonist.

similar to that obtained by more established methods, we suggest that quantitative confocal microscope analysis of receptor endocytosis provides a useful new method for pharmacological analysis of receptor activation. The current technique has the advantages that the receptor population is visualized by specific antibody probes and the analysis is performed at the single cell level.

The competitive antagonists, CP-99994 and MEN-10581, differentially inhibited endocytosis of the NK₁r induced by nanomolar concentrations of SP or septide. There are two possible explanations for this finding: either the NK₁r antibody recognizes both the NK₁r and the proposed 'septide-sensitive' receptor; or SP and septide bind differently in the nanomolar concentration range to a single NK₁r population. The NK₁r antibody used in this study was raised against the 15 C-terminal amino acids of the rat NK₁r, and binds the full-length NK₁r, but not the NK₂ or NK₃ receptors or the C-terminal

truncated form of the NK₁r (Grady *et al.*, 1996). It is possible that a 'septide-sensitive' receptor exists that differs to a small extent from the NK₁r in a ligand binding domain(s) but not in the C-terminus; however, this seems unlikely as receptor cloning studies have failed to identify such a receptor (Glowinski, 1995; Maggi & Schwartz, 1997). Moreover, homologous binding data from cells transfected with cloned human NK₁r demonstrates that SP and septide bind differently to this receptor (Hastrup & Schwartz, 1996).

The hypothesis that SP and septide bind differently to the NK₁r at nanomolar concentrations is supported by earlier smooth muscle contractility studies, although the earlier studies could not distinguish between there being two receptors or two states for one receptor. The contractility studies demonstrated that NK₁r antagonists are as much as 16 times more potent against septide than against SP when both agonists are used in nanomolar concentrations. Furthermore, septide is about 200 times less effective than SP in displacing (Pro⁹)-SP (an agonist which behaves like SP (Hall *et al.*, 1994; Sagan *et al.*, 1996)). Hastrup & Schwartz (1996) used a homologous binding assay to identify two agonist binding affinities at the NK₁r, subnanomolar binding of SP, but not septide, and nanomolar binding of SP and septide. In view of the present results, we suggest that the NK₁r exhibits three different agonist binding properties: subnanomolar binding of SP, but not septide; nanomolar binding of septide; and nanomolar binding of SP, all of which have been shown to cause receptor activation (Laufer *et al.*, 1988; Petitet *et al.*, 1992; Pradier *et al.*, 1993; Sagan *et al.*, 1996). The last two binding properties are distinguished by the differential potency of antagonists acting against nanomolar concentrations of SP and septide in this study and in previous studies (Petitet *et al.*, 1992; Longmore *et al.*, 1994; Zeng & Burcher, 1994; Patacchini *et al.*, 1995; Nguyen-Le *et al.*, 1996), and by the low potency of septide in displacing nanomolar binding of (Pro⁹)-SP and SP (Petitet *et al.*, 1992; Pradier *et al.*, 1993; Sagan *et al.*, 1996).

Receptor mutagenesis demonstrates that the same amino acid residues are necessary for binding SP and septide to the NK₁r (Huang *et al.*, 1994; 1995). In fact, subnanomolar binding of SP and nanomolar binding of both agonists were completely abolished by either of two point mutations of the receptor (at N85 and N89), demonstrating that all three binding properties of the receptor result from interaction with the same binding site. The observation that subnanomolar SP displaces nanomolar concentrations of septide (Hastrup & Schwartz, 1996) supports this conclusion. However, the relative involvement of other residues (Y92 and N96) in agonist binding varies markedly between SP and septide, suggesting that the two agonists fit differently into this common binding site. Binding domains for small non-peptide antagonists, such as those used in the present work, only partly overlap with the agonist binding region (Huang *et al.*, 1994; 1995). Consequently, the region of overlap between antagonist binding and binding of SP may be different from the overlap between antagonist and septide; this difference in spatial overlap may partly explain differences in pA₂ values for antagonism of septide and SP.

Differences in agonist binding to a shared region may also involve differences in compatibility of the agonists with different conformations of the NK₁r (Huang *et al.*, 1995; Maggi & Schwartz, 1997). This is suggested by experiments on the superior cervical ganglion, where SP and septide have

similar nanomolar potency. At 37°C, the NK₁r antagonist, GR82334, is about 12 times more potent against septide than against SP, whereas at 22°C the difference is only 2 fold (Hawcock *et al.*, 1995). Thus, lower temperature may favour a conformation that increases the interaction of the antagonist with SP binding. Similarly, the effects of stable GTP analogues on SP binding to transfected and native NK₁r is consistent with agonist binding being influenced by receptor conformation (Aharony *et al.*, 1991; Cascieri *et al.*, 1992). In the absence of GTP analogues, SP bound the receptor with single subnanomolar affinity, whereas in their presence the majority of binding was at nanomolar affinity (Aharony *et al.*, 1991; Cascieri *et al.*, 1992).

The present results allow us to extend the proposal of Maggi & Schwartz (1997) that a single site on the NK₁r exhibits two conformations, the subnanomolar SP-preferring conformer and the nanomolar general tachykinin conformer. We suggest that septide and SP fit differently into the general tachykinin conformer. This would explain previous data showing differences in agonist displacement kinetics and antagonist potencies when both agonists are used at nanomolar concentrations. Thus, it is not necessary to posit the existence of the elusive 'septide-preferring' tachykinin receptor.

The pA₂ values calculated for CP-99994 and MEN-10581 in the present study are higher than those previously reported for either antagonist acting at the NK₁r (Longmore *et al.*, 1994; Patacchini *et al.*, 1995; Nguyen-Le *et al.*, 1996). The equilibration period used for antagonists in the present study (60 min) was considerably longer than that used by Patacchini *et al.* (1995) for MEN-10581 (15 min), and by Nguyen-Le *et al.* (1996) for CP-99994 (7–8 min). If the equilibration period used in these previous studies was insufficient to allow the antagonists to reach an equilibrium with the NK₁r prior to addition of agonist, this may help explain why the antagonists appear more potent in the present study. However, Longmore *et al.* (1994) equilibrated CP-99994 for the same time used in the present study, and so this cannot explain why CP-99994 was more potent in this study than in that by Longmore *et al.* (1994).

The concept that the pA₂ value for a particular antagonist acting at a receptor is constant, and is the same against all agonists for the receptor, has been a fundamental tenet of receptor classification (Arunlakshana & Schild, 1959; Bowman & Rand, 1988). In fact, it is often on the basis of different pA₂ values that sub-types of receptor have been identified. The current work suggests that antagonists may exhibit two pA₂ values at the NK₁r. Thus it may not be possible to define the NK₁r by a unique pA₂ value for a given antagonist.

In conclusion, the present study has demonstrated that SP and septide are equipotent agonists at the NK₁r, and that the competitive NK₁r antagonists, CP-99994 and MEN-10581, differentially inhibit receptor endocytosis induced by each agonist. These findings suggest that SP and septide bind differently to the NK₁r in the nanomolar concentration range.

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A comparison of an A₁ adenosine receptor agonist (CVT-510) with diltiazem for slowing of AV nodal conduction in guinea-pig

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1 The purpose of this study was to compare the pharmacological properties (i.e. the AV nodal depressant, vasodilator, and inotropic effects) of two AV nodal blocking agents belonging to different drug classes; a novel A₁ adenosine receptor (A₁ receptor) agonist, N-(3(R)-tetrahydrofuranyl)-6-aminopurine riboside (CVT-510), and the prototypical calcium channel blocker diltiazem.

2 In the atrial-paced isolated heart, CVT-510 was approximately 5 fold more potent to prolong the stimulus-to-His bundle (S–H interval), a measure of slowing AV nodal conduction (EC₅₀ = 41 nM) than to increase coronary conductance (EC₅₀ = 200 nM). At concentrations of CVT-510 (40 nM) and diltiazem (1 µM) that caused equal prolongation of S–H interval (~10 ms), diltiazem, but not CVT-510, significantly reduced left ventricular developed pressure (LVP) and markedly increased coronary conductance. CVT-510 shortened atrial (EC₅₀ = 73 nM) but not the ventricular monophasic action potentials (MAP).

3 In atrial-paced anaesthetized guinea-pigs, intravenous infusions of CVT-510 and diltiazem caused nearly equal prolongations of P–R interval. However, diltiazem, but not CVT-510, significantly reduced mean arterial blood pressure.

4 Both CVT-510 and diltiazem prolonged S–H interval, i.e., slowed AV nodal conduction. However, the A₁ receptor-selective agonist CVT-510 did so without causing the negative inotropic, vasodilator, and hypotensive effects associated with diltiazem. Because CVT-510 did not affect the ventricular action potential, it is unlikely that this agonist will have a proarrhythmic action in ventricular myocardium.

Keywords: Adenosine; adenosine agonist; diltiazem; supraventricular tachycardia; re-entry; AV node; ventricular contractility; coronary vasodilation; action potential

Abbreviations: LVP, left ventricular developed pressure; MABP, mean arterial blood pressure; MAPD₉₀, monophasic action potential duration at 90% repolarization; S–H, stimulus-His-bundle interval

Introduction

Pharmacological and molecular cloning studies have led to the identification of four distinct cell surface adenosine receptor subtypes: A₁, A_{2A}, A_{2B}, and A₃ (Shryock & Belardinelli, 1997). In the heart, mRNAs for all four adenosine receptor subtypes have been detected using the sensitive reverse transcription–polymerase chain reaction, (RT–PCR, Dixon *et al.*, 1996). The mRNA levels for the A_{2B} and A₃ receptors are very low, whereas A₁ and A_{2A} mRNA levels are somewhat higher (Dixon *et al.*, 1996). The localization and/or function(s) of A_{2B} and A₃ receptors in the heart remain to be elucidated. In comparison, it is well established that a number of important cardiac functions are modulated by the A₁ and A_{2A} receptor subtypes (Shryock & Belardinelli, 1997; Belardinelli *et al.*, 1995). The A₁ adenosine receptor (A₁ receptor) mediates the actions of adenosine to (1) slow heart rate (negative chronotropic effect), (2) slow impulse conduction through the AV node (negative dromotropic effect), (3) depress atrial, but not ventricular contractility (negative inotropic effect), and (4) attenuate the cardiac stimulatory effects of catecholamines (anti-β-adrenergic effect). The A_{2A} adenosine receptor (A_{2A}AdoR) mediates

the coronary vasodilation caused by adenosine (Poucher *et al.*, 1995; Belardinelli *et al.*, 1998).

The A₁ receptor-mediated negative dromotropic effect of adenosine is the basis for the use of this nucleoside for the treatment of paroxysmal supraventricular tachycardias (PSVT, Belardinelli & Lerman, 1990; Lerman & Belardinelli, 1991). On the other hand, the A_{2A} receptor-mediated coronary vasodilator effect of adenosine led to its use as a pharmacological coronary vasodilator in combination with myocardial radionuclide imaging for the detection of ischemic heart disease (Verani *et al.*, 1990; Iskandrian *et al.*, 1991). These and a number of other potential beneficial actions of adenosine mediated by A₁ and A_{2A} receptors are in part responsible for the considerable effort devoted by both academia and the pharmaceutical industry to develop potent and selective agonists for these two receptor subtypes.

CVT-510, N-(3(R)-tetrahydrofuranyl)-6-aminopurine riboside (Figure 1), was specifically developed to exploit the A₁ receptor-mediated negative dromotropic effect for the purposes of treating AV nodal re-entrant arrhythmias. In the present report, we describe the pharmacological properties of CVT-510, a new, potent, and selective agonist of the A₁ receptor subtype and compare them to that of the calcium channel blocker diltiazem.

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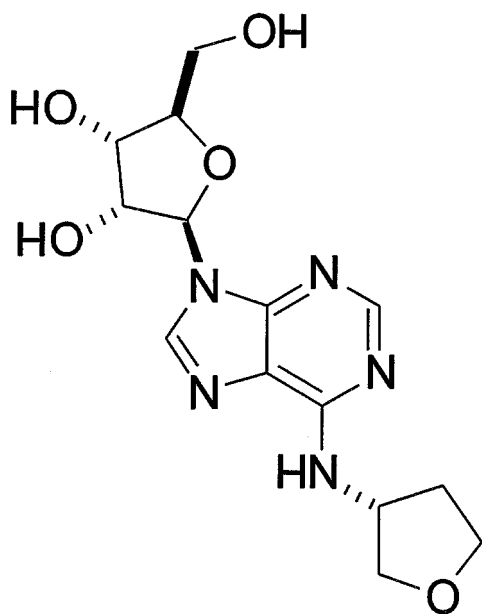


Figure 1 Chemical structure N-(3(R)-tetrahydrofuran-1-yl)-6-aminopurine riboside (CVT-510).

Methods

Isolated perfused heart preparation

Guinea-pigs ($n=46$) of either sex weighing 250–300 g were anaesthetized with methoxyflurane and killed by cervical dislocation. The hearts were quickly removed and rinsed in ice-cold Krebs-Henseleit solution. The aorta was cannulated and the heart retrogradely perfused at a constant flow of 10 ml min^{-1} with modified Krebs-Henseleit solution containing (mM): NaCl 117.9, KCl 4.8, CaCl_2 2.5, MgSO_4 1.18, KH_2PO_4 1.2, Na_2EDTA 0.5, ascorbic acid 0.14, glucose 5.5, pyruvic acid (sodium salt) 2.0, and NaHCO_3 25. The K-H solution was oxygenated with 95% oxygen and 5% CO_2 , pH 7.4, and temperature maintained at 36°C .

Hearts were electrically paced at a cycle length of 290–300 ms (unless otherwise indicated) using a bipolar electrode placed on the right atrium or ventricle. The stimulator, an interval generator (Model 1830, WPI, Sarasota, FL, U.S.A.) delivered stimuli through a stimulus isolation unit (Model 1880, WPI) as square wave pulses of 3 ms in duration and at least twice the threshold intensity. The stimulus-to-His bundle (S–H) interval was used as index of AV nodal conduction time and was measured on-line during constant atrial pacing from the His bundle electrogram using a Gateway 4 DX-33 MHz microcomputer with a DT-208 1A interface board (Axon Instruments, Burlingame, CA, U.S.A.). In some experiments, for convenience, the stimulus-to-ventricle (S–V) interval was measured. Because adenosine and A₁ receptor agonists do not prolong His-bundle-to-ventricle (H–V) interval, prolongation of S–V interval accurately reflects changes in atrium-to-His-bundle (A–H interval), i.e., proximal AV nodal conduction time.

Coronary perfusion pressure was measured using a pressure transducer that was connected to the aortic cannula *via* a T-connector. Coronary perfusion pressure (in mmHg) was monitored throughout an experiment and recorded on a 4-channel Gould strip-chart recorder (Gould RS3400, Cleveland, OH, U.S.A.). Coronary conductance (in $\text{ml min}^{-1} \text{ mmHg}^{-1}$) was calculated as the ratio of coronary perfusion rate

(10 ml min^{-1}) and perfusion pressure (mmHg). Isovolumetric ventricular pressure (LVP) was measured using a saline-filled latex balloon placed in the left ventricle and connected to a pressure transducer (Gould P231D). The volume of the balloon was adjusted and held constant to maintain an LV diastolic pressure of 4–8 mmHg. Pressures were recorded on a 4-channel strip chart recorder. The $\text{dP dt}^{-1}_{\text{max}}$ was measured offline by using the Snapshot storage program (Version 3.21D, HEM Data Corp., Southfield, MI, U.S.A.) and taken as an index of left ventricular contractility.

Monophasic action potentials (MAP) were recorded using a pressure contact silver-silver chloride electrode (Langendorff Probe, EP Technologies, Inc., Sunnyvale, CA, U.S.A.) placed on the surface of the left atrium and/or left ventricle. The signals were amplified and filtered by an isolated biological amplifier (IsoDam, WPI) and displayed in real time on a digital oscilloscope (2201 Tektronix, Inc., Beaverton, OR, U.S.A.). Signals were considered adequate if they were stable for at least 5 min and the amplitudes of the MAPs exceeded 10 mV. The data were digitized at 2 kHz by a DT-2801A digitizing board (Data Translation, Marlboro, MA, U.S.A.) and stored using the Snapshot data acquisition program (Snapshot Storage Scope, HEM Data Corp., Southfield, MI, U.S.A.) for later analysis. The durations of the atrial and ventricular monophasic action potentials were measured at 90% repolarization (MAPD_{90}), using the Snapshot program. After completion of dissection and instrumentation, the hearts were allowed to equilibrate for 30 min before the experiments were begun. Whenever the baseline and post-intervention (washout) values differed by more than 15%, the data were discarded. Approximately 60–70% of the experiments conformed with this criterion.

Anaesthetized guinea-pig preparation

Guinea-pigs ($n=25$) of either sex weighing 300–350 g were anaesthetized with 0.75% isoflurane administered through a nose cone, with oxygen making up the balance. After a 20 min induction, a transverse incision was made in the neck region to expose the trachea, jugular veins, and carotid arteries. The trachea was catheterized using a 14-gauge catheter and the animal ventilated with 0.75% isoflurane/oxygen at 60 strokes min^{-1} and a tidal volume of 2.5 ml using a Harvard Apparatus (Southnatic, MA, U.S.A.) rodent ventilator. In previous experiments isoflurane at the concentration used in the present study was found to provide full anaesthesia, assessed by foot pinching and touching of the cornea, without requiring the use of muscle relaxant or any other anaesthetic agent. The left jugular vein and carotid artery were catheterized with 24-gauge catheters for administration of drugs and for recording of arterial blood pressure, respectively. In experiments involving electrical pacing of the atrium or ventricle, the right jugular vein was catheterized with a custom-made electrocatheter. ECG leads were attached to record in the L-2 configuration. A non-depolarizing neuromuscular blocker, vecuronium bromide (0.5 mg), was administered intravenously (i.v.), after all surgical procedures and instrumentation were completed, to prevent muscular fasciculation from interfering with the ECG recording. Anaesthetic depth was determined by observing autonomic responses such as heart rate and blood pressure. The L-2 of the ECG and arterial blood pressure were monitored throughout the experiments and recorded on a 2-channel Gould strip-chart recorder. After completion of surgery and instrumentation, a 20-min equilibration period was allowed before the experimental protocols were commenced. During the surgical and experi-

mental procedure, the animal temperature was maintained at 37°C using a warm water blanket. Identical to the isolated heart experiments, whenever the baseline and post-intervention (washout) values differed by more than 15%, the data were discarded. Approximately 75% of this series of experiments met this criterion.

All experimental procedures and protocols for handling animals were approved by the University of Florida Institutional Animal Care and Use Committee, and conform with NIH guidelines for the use of animals.

Binding studies

Membrane preparation Porcine brain (forebrain and striatum) were minced and then homogenized in ice cold Tris-HCl buffer (50 mM), pH 7.4. Homogenates were filtered through cotton gauze and centrifuged at $48,000 \times g$ for 15 min. The membrane pellets were washed twice by resuspension in fresh buffer and centrifugation. Final pellets were resuspended in Tris-HCl buffer (50 mM), pH 7.4, and frozen at -80°C until use.

Binding assays The effect of CVT-510 on binding to A₁ and A_{2A}-adenosine receptors of porcine forebrain and striatum membranes, respectively, were determined. Assays for A₁ and A_{2A} receptors were carried out by using the A₁ receptor antagonist [³H]CPX and the A_{2A} receptor agonist [³H]CGS 21680. Membranes were treated with adenosine deaminase (2 U ml^{-1}) for 20 min at room temperature prior to and during radioligand binding assays. Membranes (0.2–0.7 mg), adenosine deaminase, and the indicated radioligand were incubated for 3 h in a 300 μl volume of Tris-HCl buffer (50 mM) (pH 7.4). Assays were carried out in triplicate at room temperature. After the incubation period, bound and free radioligand were diluted by the addition of ice-cold Tris-HCl buffer (5 ml), and immediately separated by vacuum filtration of assay contents onto Whatman GF/C filters and washing of trapped membranes with Tris-HCl buffer (20 ml). Filter disks containing membrane-bound radioactivity were placed in 4 ml Scintiverse (Fisher Scientific, Pittsburgh, PA, U.S.A.), and the radioactivity was quantified by a liquid scintillation counter. Specific binding of [³H]CPX and [³H]CGS 21680 was defined as membrane binding displaced in the presence of CPT ($10 \mu\text{M}$) and R-PIA ($10 \mu\text{M}$), respectively.

The apparent potencies of CVT-510 to compete with the specific binding of [³H]CPX (1 nM) to forebrain membranes, and of [³H]CGS 21680 (2 nM) to striatal membranes were determined. Membranes were incubated with the indicated concentrations of radioligand and increasing concentrations of the A₁ receptor agonist CVT-510 for 3 h in a total volume of 300 μl . Assays were terminated as described above. Binding parameters (e.g., IC_{50} , K_i , Hill Coefficients) from the competition assays were determined with the computer program LIGAND (Biosoft, Cambridge, U.K.). The K_i for displacement of each labelled ligand by CVT-510 was calculated using the Cheng/Prusoff equation: $K_i = \text{IC}_{50} \cdot \text{Agonist} / (1 + [\text{Ligand}] / K_d, \text{ligand})$.

Experimental protocols

Negative dromotropic and coronary vasodilator effects of CVT-510 and diltiazem in the guinea-pig isolated heart - The effects of progressively higher concentrations of CVT-510 (0.5 – 3000 nM) and diltiazem (0.001 – $30 \mu\text{M}$) to prolong the S–H interval (A₁ receptor-mediated effect), and to increase coronary conductance (A_{2A} receptor-mediated

effect) were determined. The data were used to obtain concentration-response relationships for the S–H interval prolongations and for the increases in coronary conductance caused by CVT-510 and diltiazem. In these hearts, coronary perfusion pressure and S–H intervals were measured simultaneously.

In a separate series of experiments, the effects of the A₁ receptor antagonist CPX (60 nM) and of the A_{2A} receptor antagonist ZM241,385 (100 nM) on the S–H interval prolongation and coronary vasodilator effects of CVT-510 were determined. Hearts were exposed to single concentrations of CVT-510 for up to 30 min. Once steady-state effects to CVT-510 were achieved (5 – 10 min after onset of infusion), either CPX or ZM241,385 was infused into the perfusate for 15 – 20 min , and the effects of the antagonists on the responses of the hearts to CVT-510 recorded. This was followed by washout (40 – 60 min) of all drugs, and the post-intervention measurements were made.

The effects of CVT-510 and diltiazem on left ventricular contractility Isolated perfused hearts paced at a constant atrial cycle length of 300 ms were instrumented for continuous recording of LVP, $\text{dP dt}^{-1}_{\text{max}}$, S–V interval (i.e., negative dromotropic effect) and coronary perfusion pressure. After an equilibration period of 30 min, control measurements were obtained. CVT-510 or diltiazem was infused at rates (based on concentration-response curves) that caused equal prolongations of the S–V interval (perfusate concentrations of 40 nM and $1 \mu\text{M}$, respectively). The steady-state effects of CVT-510 and diltiazem on LVP, $\text{dP dt}^{-1}_{\text{max}}$, and coronary conductance were determined.

Effect of CVT-510 on atrial and ventricular monophasic action potential (MAP) Isolated perfused hearts were instrumented for simultaneous recording of atrial and ventricular MAP. Concentration-response relationships for CVT-510 to shorten atrial and ventricular MAP were determined. The protocol was similar to that described above for negative dromotropic effect and increase in coronary conductance of CVT-510. After a 30-min equilibration period, control measurements were made of atrial and ventricular MAPD_{90} . CVT-510 was infused into the perfusion line to achieve the desired perfusate concentrations (1 – 3000 nM). The effect of each concentration of CVT-510 on atrial and ventricular MAPD_{90} was measured once the response had reached steady state (5 – 10 min after onset of infusion).

Prolongation of P–R interval in response to i.v. boluses of CVT-510 or adenosine in anaesthetized guinea-pigs After completion of surgery and instrumentation, a 20-min equilibration time was allowed before starting the administration of CVT-510 (0.1 – 1.5 mg kg^{-1}) or adenosine (25 – $100 \mu\text{g kg}^{-1}$). The ECG (L-2) was monitored throughout the experiments. After obtaining a baseline recording of the ECG, boluses of CVT-510 or adenosine were injected into the left jugular vein and followed by saline flushes. Increasing doses of CVT-510 and adenosine were given 15 and 5 min apart, respectively. The longest P–R intervals (peak effect) were determined after each bolus injection of agonist.

Effect of CVT-510 and NECA on mean arterial blood pressure in ventricle-paced anaesthetized guinea-pigs After post-surgical equilibration and recording of baseline ECG and mean arterial blood pressure (MABP), each animal in this series received an i.v. infusion of either CVT-510 ($5.3 \mu\text{g kg}^{-1} \text{ min}^{-1}$) or NECA ($2.6 \mu\text{g kg}^{-1} \text{ min}^{-1}$). Once a steady-

state 2:1 AV block was achieved, the right ventricle was intermittently paced at a rate approximately equal to the intrinsic baseline atrial rate using an electrocatheter positioned in the right ventricle. Intermittent pacing was used to demonstrate the reproducibility of the effect of ventricular pacing on blood pressure during the 2:1 A-V block caused by either CVT-510 or NECA. Ventricular rates and arterial blood pressures were monitored throughout an experiment.

Effect of continuous infusions of CVT-510 and diltiazem on P–R interval, heart rate, and mean arterial blood pressure in anaesthetized guinea-pigs After post-surgical equilibration and recording of baseline ECG and MABP, each animal in this series received an i.v. infusion of either CVT-510 (0.75–1.0 $\mu\text{g kg}^{-1} \text{min}^{-1}$) or diltiazem (200–400 $\mu\text{g kg}^{-1} \text{min}^{-1}$). For both CVT-510 and diltiazem, a rate of infusion was chosen that did not cause second-degree AV block, but was sufficient to cause a significant prolongation of the P–R interval. In this series of experiments, hearts were allowed to beat spontaneously.

Data analysis

All values are reported as means $\pm$ s.e.mean. Significance of differences among values in experiments with multiple treatment groups was determined by analysis of variance followed by Student-Newman-Keuls testing. A value of $P < 0.05$ was considered to indicate a statistically significant difference. Coronary conductance and MAP concentration-response data were fitted to a 4-parameter logistic dose response equation by use of nonlinear regression (Table Curve, Jandel Scientific Software, Corp., Chicago, IL, U.S.A.): $y = a + b / (1 + [(x/c)^d])$, where y , x , a , b , c , and d denote level of response, concentration of agonist, extrapolated minimum response, extrapolated maximum response, EC₅₀ value, and the apparent Hill coefficient, respectively. Concentration-response data for S–H and P–R interval prolongation were analysed as previously described (Dennis *et al.*, 1992). The value of the longest stable S–H or P–R interval immediately prior to the onset of AV block was considered the maximum negative dromotropic effect and used for calculation of EC₅₀ values. For all variables (e.g., coronary conductances, S–H interval prolongation, etc.), the EC₅₀ values were obtained for each individual concentration-response curve, and were used for subsequent statistical analysis.

Chemicals

6-(N-3'-(R) tetrahydrofuryl)-amino-purine ribose adenosine (CVT-510) was provided by CV Therapeutics (Palo Alto, CA, U.S.A.). 8-cyclopentyl-1,3-dipropylxanthine (CPX), 5'-N-ethylcarboxamidoadenosine (NECA), and diltiazem were purchased from Research Biochemicals (Natick, MA, U.S.A.). ZM241,385 (4-(2-[7-amino-2-(2-furyl[1,2,4]-triazolo[2,3-a[1,3, 5]triazin-5-yl-aminoethyl]phenol) was a gift from Dr Simon M. Poucher (Zeneca Pharmaceuticals, Cheshire, U.K.). Stock solutions of CVT-510, CPX, NECA, and ZM241,385 were prepared in dimethylsulfoxide (DMSO, Sigma Chemical Company, St. Louis, MO, U.S.A.) and diluted with saline immediately prior to experiments. Diltiazem was dissolved in saline. Isoflurane was purchased from Ohmeda Caribe, Inc. (Guayama, PR, U.S.A.), and vecuronium bromide from Marsam Pharmaceuticals (Cherry Hill, NJ, U.S.A.).

Results

Negative dromotropic and coronary vasodilatory effect of CVT-510 and diltiazem

In isolated perfused hearts ($n = 4–6$) paced at a constant atrial cycle length of 290–300 ms, CVT-510 and diltiazem caused a concentration-dependent increase of the S–H interval and the coronary conductance (Figures 2 and 3, respectively). The potencies for CVT-510 and diltiazem to prolong the S–H interval and to increase coronary conductance are summarized in Table 1. CVT-510 was ~ 5 fold more potent to prolong the S–H interval than to increase coronary conductance (Figure 2, Table 1). In comparison, the calcium channel blocker diltiazem showed very little selectivity between prolonging the S–H interval and increasing coronary conductance (Figure 3, Table 1). In addition, as illustrated in Figure 4, the prolongation of S–H interval ($[\text{CVT-510}] = 60 \text{ nM}$), but not the increase in coronary conductance ($[\text{CVT-510}] = 1 \mu\text{M}$), was completely blocked by the A₁ receptor-selective antagonist CPX (60 nM),

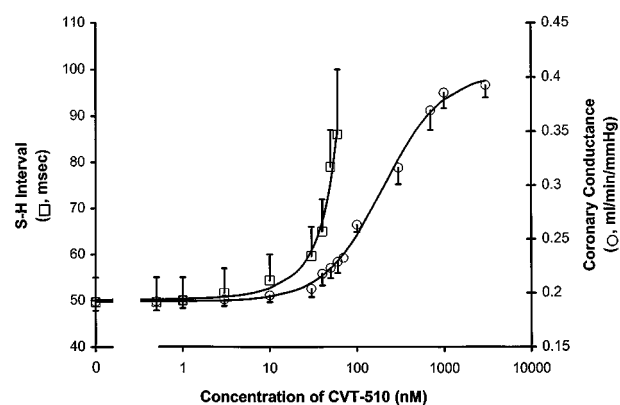


Figure 2 Concentration-response relationship for the A₁ receptor-mediated negative dromotropic (S–H interval prolongation) and the A_{2A} receptor-mediated coronary vasodilator (increase of coronary conductance) effects of CVT-510 in guinea-pig isolated perfused hearts. Symbols and error bars indicate means $\pm$ s.e.mean of single determinations from each of four hearts. Second-degree AV block occurred in all hearts at a concentration of CVT-510 $\geq 40 \text{ nM}$.

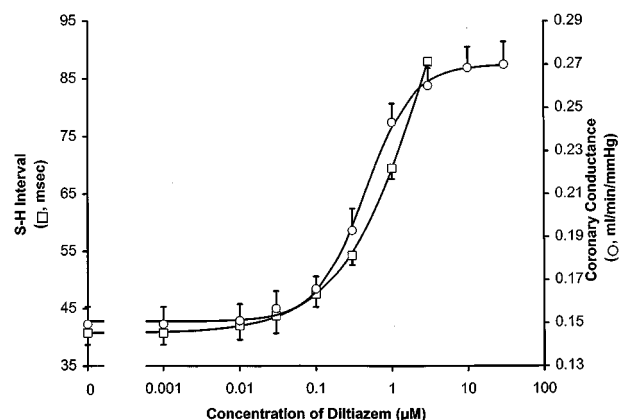


Figure 3 Concentration-response relationship for the calcium channel blocker diltiazem to prolong the S–H interval and increase coronary conductance in guinea-pig isolated perfused hearts. Symbols and error bars indicate means $\pm$ s.e.mean of single determinations from each of 4–6 hearts.

whereas the increase in coronary conductance caused by CVT-510, but not the prolongation of S–H interval, was completely blocked by ZM241,385 an A_{2A} receptor-selective antagonist ($n=4$).

The effects of CVT-510 and diltiazem on left ventricular contractility

In this series of experiments ($n=4$), the effects of CVT-510 on left ventricular contractility and coronary artery conductance were determined and compared to those of diltiazem. Isolated

Table 1 The potencies of CVT-510 and diltiazem to prolong the S–H interval and increase coronary conductance

	S–H interval	Coronary conductance
CVT510	40.6 nM (7.39 ± 0.02) $n=4$	200.5 nM (6.70 ± 0.03) $n=4$
Diltiazem	708 nM (6.16 ± 0.04) $n=6$	427.8 nM (6.38 ± 0.04) $n=4$

Potencies are EC₅₀ values to prolong the stimulus-to-His-bundle interval (S–H interval) and to increase coronary conductance in atrial-paced guinea-pig isolated perfused hearts. Numbers in parenthesis are mean $pD_2 \pm s.e.$ mean for the number of hearts (n).

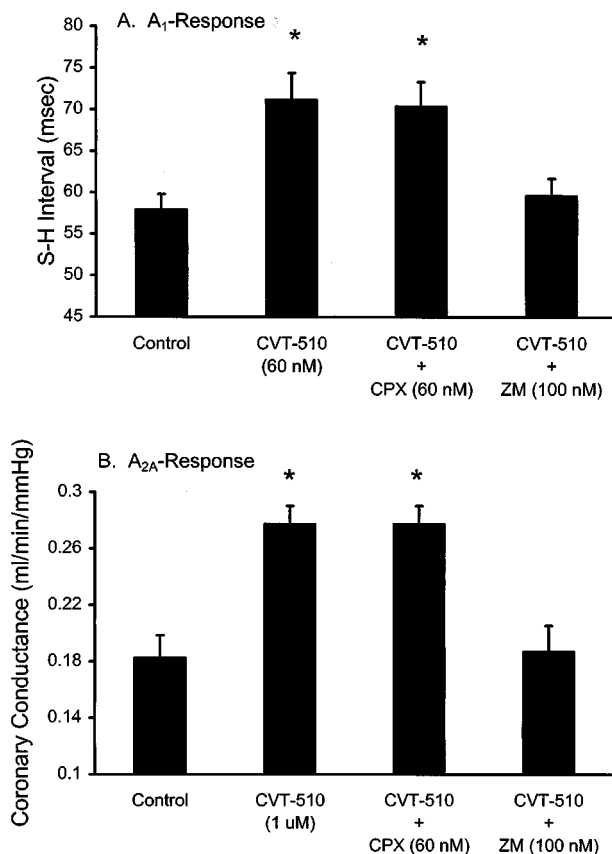


Figure 4 The antagonism of the A₁-receptor-mediated increase in S–H interval (A) and the A_{2A} receptor-mediated increase in coronary conductance (B) by the A₁ receptor-selective antagonist CPX (60 nM) and by the A_{2A} receptor-selective antagonist ZM241,385 (100 nM), respectively, during infusion of CVT-510 (60 nM) in the guinea-pig isolated perfused heart. Bars represent the means $\pm$ s.e. mean of single determinations from each of four hearts. *Denotes a significant difference from control ($P < 0.05$).

perfused hearts were exposed to concentrations of CVT-510 (40 nM) or diltiazem (1 μM) that caused near equal prolongations of stimulus to ventricle (S–V) interval. Both CVT-510 and diltiazem significantly prolonged the S–V interval (i.e. negative dromotropic effect), but only diltiazem reduced LVP and $dP dt^{-1}_{max}$ (i.e. negative inotropic effect). Figure 5 summarizes the results of four such experiments. CVT-510 (40 nM) significantly increased the S–V interval from a control of 47 ± 2 to 59 ± 2 ms but did not affect left ventricular pressure, (LVP), $dP dt^{-1}_{max}$ or coronary conductance (Figure 5A and B). In the same hearts, diltiazem (1 μM) prolonged the S–V interval by 12 ± 2 ms, an increase not significantly different from that caused by CVT-510 (11 ± 1 ms). However, in contrast to CVT-510, the increase in S–V interval caused by diltiazem was accompanied by a $45 \pm 3\%$ reduction in LVP and an increase in coronary conductance of $36 \pm 3\%$ (Figure 5A and B). Thus, for the same magnitude of slowing of AV nodal conduction, CVT-510 had no effect on left ventricular contractility or coronary conductance whereas diltiazem significantly depressed left ventricular contractility and increased coronary conductance.

Shortening of atrial but not ventricular monophasic action potential duration by CVT-510

This series of experiments was carried out to determine the effects of CVT-510 on the atrial and ventricular MAP of

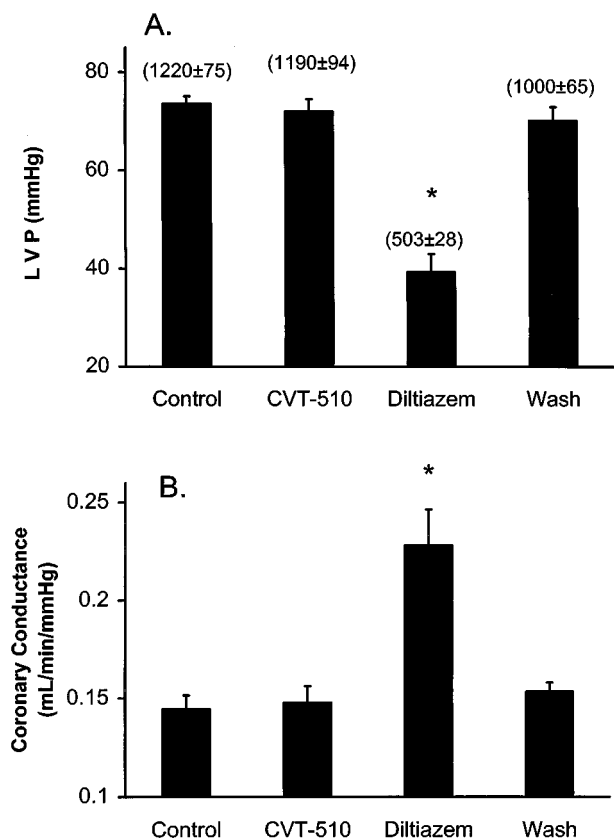


Figure 5 Comparison of the effects of CVT-510 (40 nM) and diltiazem (1 μM) on left ventricular developed pressure (LVP, A) and coronary conductance (B) in guinea-pig isolated perfused heart for the same prolongation of the S–V interval (~ 10 ms). Bars represent the means $\pm$ s.e. mean of single determinations from each of four hearts. Numbers above bars are means $\pm$ s.e. mean of $dP dt^{-1}_{max}$ for each data set. *Denotes significant differences from both 2:1 A–V Block and Washout ($P < 0.05$).

guinea-pig isolated hearts ($n=11$ and 5 for atrial and ventricular MAP, respectively). The baseline atrial and ventricular MAPD₉₀ values were 48 ± 3 and 155 ± 2 ms, respectively. CVT-510 shortened the atrial MAP in a concentration-dependent manner (Figure 6), but had no effect on ventricular MAP. CVT-510 maximally shortened the atrial MAP to 21 ± 1 ms. The concentrations that caused 50% (EC₅₀ value) and maximal shortening (E_{max} value) of the atrial MAP were 126 and 300 nM, respectively. In contrast, concentrations as high as 300 nM caused no shortening of the ventricular MAP.

Prolongation of P–R interval in response to i.v. boluses of CVT-510 or adenosine in anaesthetized guinea-pigs

In anaesthetized closed-chest guinea-pigs atrial paced at a rate of 330–360 beats min⁻¹, i.v. boluses of CVT-510 or adenosine caused dose-dependent prolongations of the P–R interval (Figure 7). The potencies (EC₅₀ values) for CVT-510 and adenosine to prolong the P–R interval were 0.9 ± 0.1 ($n=3$)

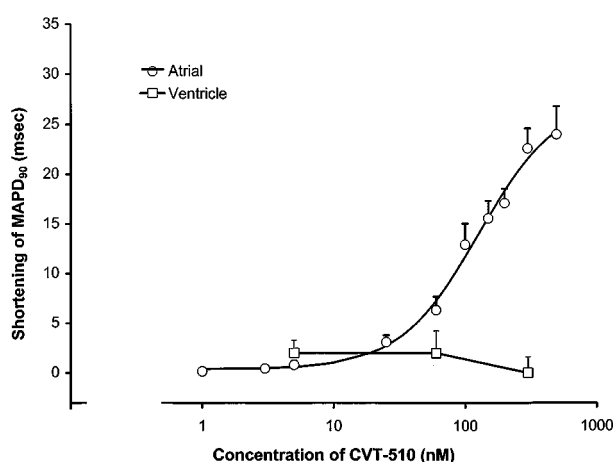


Figure 6 Differential effect of CVT-510 on atrial and ventricular monophasic action potential durations (MAPD). CVT-510 (1–300 nM) shortened the atrial MAPD in a concentration-dependent manner but had no effect on the ventricular MAPD. Values are means $\pm$ s.e. mean of each of 5–11 hearts. Atrial and ventricular MAPD were measured at 90% of repolarization (MAPD₉₀).

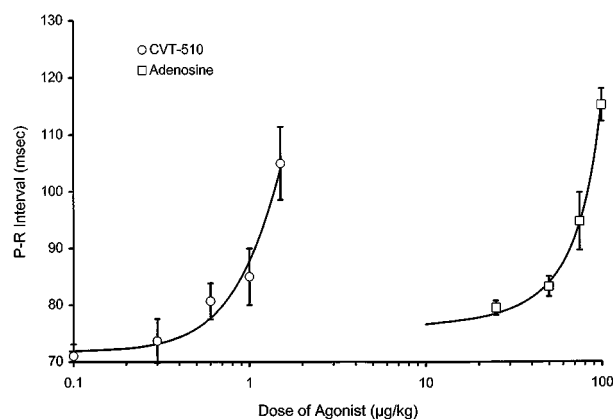


Figure 7 Dose-response relationship for the effect of intravenous injections of CVT-510 or adenosine to prolong the P–R interval in atrial-paced (330–360 beats min⁻¹) anaesthetized guinea-pigs. Symbols and error bars represent means $\pm$ s.e. mean of single determinations from each of 3–4 animals.

and 75 ± 4 ($n=4$) $\mu\text{g kg}^{-1}$, respectively. The longest P–R interval recorded before second-degree AV block occurred was 105 ± 6 ms for CVT-510, and 115 ± 3 ms for adenosine.

Effect of CVT-510 and NECA on mean arterial blood pressure

To determine if the decrease in mean arterial blood pressure (MABP) observed during second-degree AV block caused by CVT-510 is due to peripheral vasodilation or due to its negative dromotropic action, the effect of CVT-510 on MABP was measured in anaesthetized guinea-pigs during intermittent right ventricular pacing. In these experiments, hearts were allowed to beat spontaneously or were subjected to ventricular pacing at the same rate as the control atrial rate to overcome the slowing of ventricular rate caused by the second-degree AV block. As illustrated in Figure 8, a continuous intravenous infusion of CVT-510 ($5.3 \mu\text{g kg}^{-1} \text{ min}^{-1}$) caused second-degree AV block and a marked reduction in ventricular rate which was accompanied by a decrease in MABP. During continuous infusion of CVT-510, immediately upon initiation of right ventricular pacing, the MABP returned to control levels. Upon cessation of ventricular pacing, the MABP markedly fell again. Similar results were obtained in three additional experiments, the data for which are summarized in Figure 9. CVT-510 ($5.3 \mu\text{g kg}^{-1} \text{ min}^{-1}$) caused a stable 2:1 AV block that resulted in a reduction of ventricular rate from a control of 302 ± 6 to 102 ± 4 beats min⁻¹ (Figure 9A). The decrease in ventricular rate was accompanied by a decrease in MABP from a control of 52 ± 3 to 30 ± 2 mmHg. A value of 53.1 ± 4.2 mmHg for MABP of resting, awake guinea-pigs was reported by Brown *et al.* (1989). Thus, the control values of MABP recorded in the present study are within the normal range for this species. Regardless, ventricular pacing completely reversed the decrease in MABP, raising it to 58 ± 3 mmHg. In contrast, the hypotension observed during second-degree AV block caused by the nonselective adenosine receptor agonist NECA was not fully reversed by ventricular pacing. NECA ($2.6 \mu\text{g kg}^{-1} \text{ min}^{-1}$) caused a decrease in the ventricular rate from a control of 303 ± 3 to 86 ± 5 beats min⁻¹ (Figure 9B). The decrease in ventricular rate was accompanied by a decrease in MABP from a control of 71 ± 3 to 32 ± 2 mmHg. In contrast to CVT-510, ventricular pacing failed to reverse the decrease in MABP caused by NECA.

Effects of CVT-510 and diltiazem on P–R interval, heart rate, and arterial blood pressure

In this series of experiments in anaesthetized guinea-pigs ($n=4$), both CVT-510 (0.75 – $1 \mu\text{g kg}^{-1} \text{ min}^{-1}$) and diltiazem (200 – $400 \mu\text{g kg}^{-1} \text{ min}^{-1}$) slowed the heart rate from a control of 290 ± 9 to 193 ± 6 and 185 ± 9 beats min⁻¹, respectively, and prolonged the P–R interval from a control of 66 ± 3 to 94 ± 2 and 84 ± 2 ms, respectively. However, for similar prolongations of P–R interval, diltiazem, but not CVT-510, caused a significant decrease in MABP (Figure 10). Whereas CVT-510 tended to slightly elevate MABP, diltiazem reduced MABP from 50 ± 1 to 27 ± 4 mmHg (Figure 10).

Binding of CVT-510 to A₁- and A_{2A}-adenosine receptors

Competition by CVT-510 of both [³H]CPX (1 nM) and [³H]CGS21,680 (4 nM) specific binding to pig forebrain (A₁ receptor) and striatum (A_{2A} receptor), respectively, was concentration-dependent and complete. However, CVT-510 was 356 fold more potent in competing with the binding of the

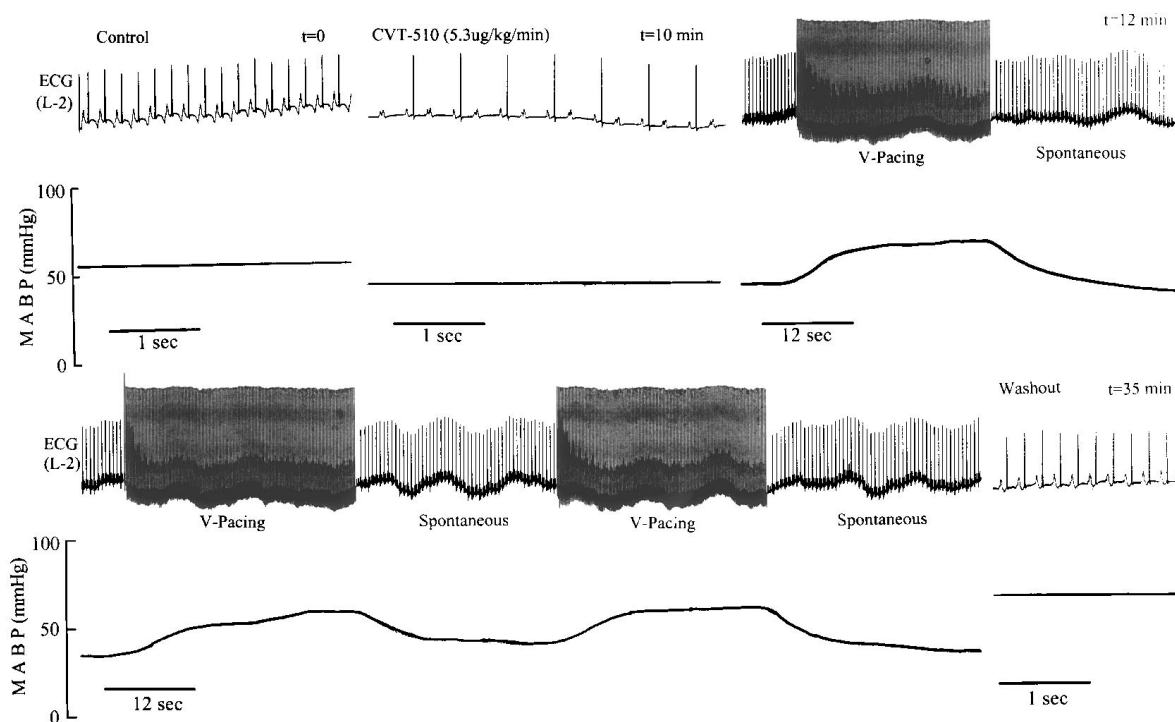


Figure 8 Representative electrocardiogram (ECG lead 2) and mean arterial blood pressure (MABP) from an anaesthetized guinea-pig heart recorded before (control, $t=0$), during, and after (washout, $t=35$ min) a continuous intravenous infusion of CVT-510. The heart was allowed to either beat spontaneously ('Spontaneous') or was subjected to right ventricular pacing (V-pacing), at the same rate as the control atrial rate. At 10 min of infusion of CVT-510 ($t=10$ min), a stable 2:1 atrioventricular (AV) block occurred and this was associated with a decrease in MABP. Subsequently, ($t=12$ min and onward) during intermittent V-pacing, the MABP rose to control levels.

A₁ receptor radioligand [³H]CPX than with the binding of the A_{2A} receptor radioligand [³H]CGS21,680. The binding affinities (K_i , dissociation constant) for CVT-510 were 6.5 nM and 2315 nM for the forebrain A₁ receptor and striatum A_{2A} receptor, respectively ($n=2$).

Discussion

The most important finding of this study is that CVT-510 is a potent agonist of the A₁ adenosine receptor with sufficient functional selectivity to cause slowing of AV nodal conduction (i.e. negative dromotropic effect) at concentrations that do not cause significant coronary vasodilation, depress left ventricular function (i.e. negative inotropic effect), or cause systemic hypotension. In this report, the selectivity of CVT-510 was shown both at the receptor (binding studies) and at the functional level (guinea-pig isolated hearts and anaesthetized guinea-pigs). In guinea-pig isolated perfused hearts, CVT-510 caused vasodilation at concentration 5 fold greater than those required to cause slowing of AV nodal conduction (Figure 2). In anaesthetized guinea-pigs, CVT-510 had no effect on mean arterial blood pressure (MABP) when given at a dose that caused first degree AV block (Figure 10) or even at a dose that caused second degree AV block (Figures 8 and 9). On the other hand, the calcium channel blocker diltiazem, at concentrations that cause equal negative dromotropic effects as CVT-510, significantly increased coronary conductance and decreased left ventricular pressure in the isolated heart (Figure 5), and decreased MABP in anaesthetized guinea-pigs (Figure 10). Thus, in comparison to diltiazem, CVT-510, and presumably other A₁AdoR-selective agonists, may offer advantages over

calcium channel blockers in the treatment of supraventricular tachycardias (SVT) in which the AV node is part of the re-entrant circuit.

Selectivity of CVT-510

The actions of CVT-510 to slow AV nodal conduction and cause coronary vasodilation (albeit at higher concentrations) are most likely mediated by A₁ and A_{2A} receptors, respectively. This conclusion is based on the following findings: (1) the negative dromotropic effect of CVT-510 is attenuated by the A₁ receptor-selective antagonist CPX, but not by the A_{2A} receptor-selective antagonist ZM241,385; (2) the coronary vasodilation caused by CVT-510 is attenuated by the A_{2A} receptor-selective antagonist ZM241,385, but not by the A₁ receptor-selective antagonist CPX; (3) CVT-510 competed with [³H]CPX binding to pig forebrain membranes; and (4) the binding of the A_{2A} receptor agonist radioligand [³H]CGS21680 to pig striatum was competitively antagonized by CVT-510.

The role of receptor reserve in determining functional selectivity of CVT-510

CVT-510 was found to have a 356 fold greater affinity for A₁ receptors (pig forebrain) than for A_{2A} receptors (pig striatum). Yet in functional assays (i.e., guinea-pig isolated perfused hearts), CVT-510 was approximately 5 fold more potent in prolonging the S-H interval (A₁ receptor response) than for increasing coronary conductance (A_{2A} receptor response). The difference between binding and functional selectivity can be explained by the presence of a large receptor reserve for A_{2A} receptor-mediated coronary vasodilation relative to the

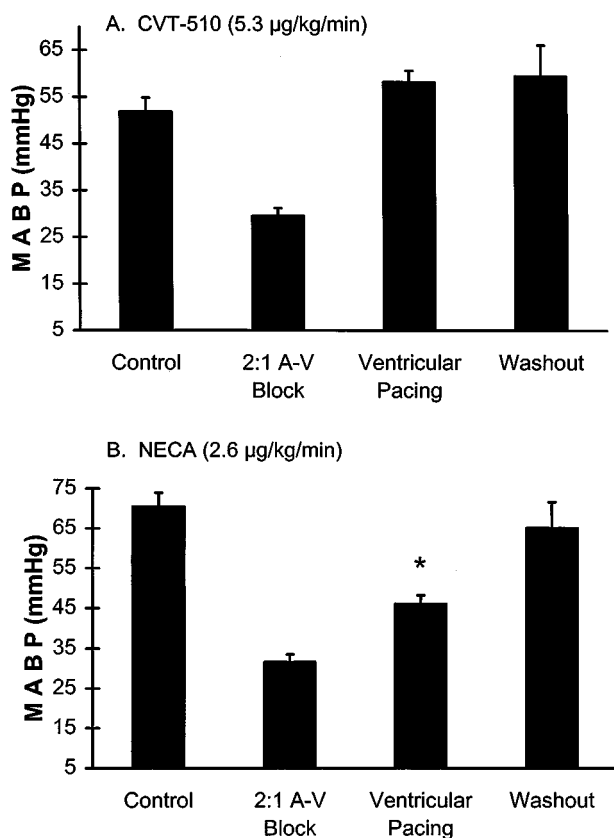


Figure 9 (A) Complete reversal by ventricular pacing of hypotension associated with 2:1 AV block caused by CVT-510 in anaesthetized guinea-pigs. Summary of data showing that 2:1 AV block caused by a continuous intravenous infusion of CVT-510 is associated with a significant reduction in mean arterial blood pressure (MABP). Pacing of the right ventricle (Ventricular Pacing) at the same rate as the control atrial rate, reversed the decrease in MABP observed during CVT-510-induced 2:1 AV block. At 30–40 min after stopping the infusion of CVT-510 (Washout), MABP was not different from baseline. Values are means $\pm$ s.e. mean of single determinations from each of four guinea-pigs. (B) Partial reversal by ventricular pacing of hypotension associated with 2:1 AV block caused by the nonselective adenosine receptor agonist NECA in anaesthetized guinea-pigs. Summary of data showing that 2:1 AV block caused by continuous intravenous infusion of NECA is associated with significant reduction of mean arterial blood pressure (MABP). Pacing of the right ventricle (Ventricular Pacing) at the same rate as the control atrial rate only partially reversed the decrease in MABP observed during NECA-induced 2:1 AV block. At 30–40 min after stopping the infusion of NECA (Washout), MABP returned to baseline. Values are means $\pm$ s.e. mean of single determinations from each of three guinea-pigs. *Denotes significant differences from both 2:1 A-V Block and Washout ($P < 0.05$).

receptor reserve present for A₁ receptor-mediated effects (Shryock *et al.*, 1998; Morey *et al.*, 1998). For example, for the adenosine receptor agonist R-PIA to cause half-maximal shortening of atrial action potential duration (A₁ receptor-response, Morey *et al.*, 1998), and to increase coronary conductance (A_{2A} receptor-response, unpublished) the fractional receptor occupancies were 30 and 10%, respectively. That is, there was a greater receptor reserve for R-PIA to cause vasodilation than to shorten the atrial action potential. However, it should be noted that the magnitudes of receptor reserves for A₁ receptor- and A_{2A} receptor-mediated responses are both agonist and tissue-dependent. Therefore, whether the difference in receptor reserves for CVT-510 to cause coronary vasodilation and slow AV nodal conduction is sufficient to explain the difference between binding and functional selectivity remains to be determined.

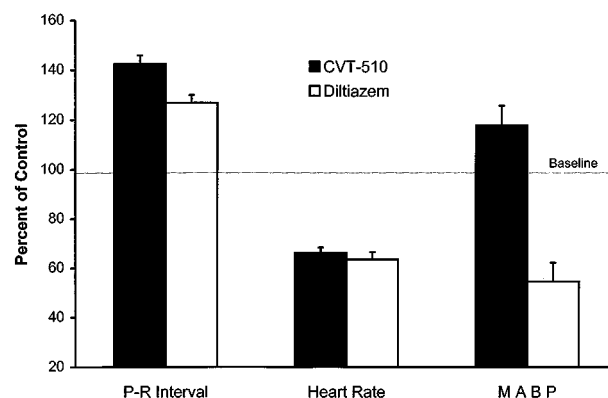


Figure 10 Summary of data comparing the effects of CVT-510 ($0.75\text{--}1.0\text{ }\mu\text{g kg}^{-1}\text{ min}^{-1}$) and diltiazem ($200\text{--}400\text{ }\mu\text{g kg}^{-1}\text{ min}^{-1}$) given as intravenous infusions on P–R interval, heart rate, and mean arterial blood pressure (MABP) in anaesthetized guinea-pigs. Hearts were allowed to beat spontaneously. Control values are given in the results section. Bars represent means $\pm$ s.e. mean of changes from baseline (horizontal line at 100%) of single determinations from each of six hearts (three for CVT-510, three for diltiazem).

Effect of CVT-510 on atrial and ventricular action potential durations

The A₁ receptor-mediated cellular electrophysiological and contractile effects of adenosine and A₁ receptor agonists in atrial and ventricular myocytes (Belardinelli *et al.*, 1995) can fully account for the effects of CVT-510 presented in this report.

It is well established that adenosine activates the inward-rectifying potassium current, $I_{K_{Ado}}$, in guinea-pig atrial but not ventricular myocytes (Belardinelli *et al.*, 1995; Song & Belardinelli, 1996). This differential effect of adenosine on atrial and ventricular myocytes could be explained by the lower density of inward-rectifying potassium channels (i.e. $I_{K_{(ACh,Ado)}}$) in guinea-pig ventricular versus atrial myocytes (Koumi & Wasserstrom, 1994). Regardless, the shortening of the atrial but not the ventricular action potential by CVT-510 is in keeping with the activation by adenosine of $I_{K_{Ado}}$ found in atrial but not ventricular myocytes. On the other hand, in both atrial and ventricular myocytes, adenosine causes little or no decrease in basal L-type calcium current (Belardinelli *et al.*, 1995; Song & Belardinelli, 1996). Thus, the finding that CVT-510 did not decrease LVP and ventricular dP/dt^{-1} is consistent with reports that adenosine does not affect resting membrane potential, action potential duration and amplitude, or twitch amplitude in ventricular myocytes of various mammalian species, including humans (Belardinelli *et al.*, 1995; Song & Belardinelli, 1996). Similar to adenosine and adenosine receptor agonists, diltiazem reduces action potential duration and contractile force in guinea-pig atrial myocytes (Nabata, 1977). However, unlike adenosine and adenosine receptor agonists, diltiazem shortens the action potential duration and decreases contractile force in guinea-pig ventricular myocytes (Nakajima *et al.*, 1975). In keeping with this latter study (Nakajima *et al.*, 1975) are our findings that diltiazem significantly reduced LVP and dP/dt^{-1} in guinea-pig isolated perfused hearts.

The shortening of atrial action potential and refractory period by adenosine facilitates the induction of atrial flutter or fibrillation (Kabell *et al.*, 1994; DiMarco *et al.*, 1990). In fact, adenosine has been reported to induce transient atrial fibrillation in 2–5% of patients (Kabell *et al.*, 1994; DiMarco

et al., 1990). By the same mechanism, that is shortening of the atrial action potential (Figure 6), CVT-510 could also facilitate the induction of atrial flutter or fibrillation. However, CVT-510 is 3 fold more potent in prolonging the S–H interval ($EC_{50} = 41$ nM) than in shortening the atrial action potential ($EC_{50} = 126$ nM). Thus, it is possible that a significant negative dromotropic effect of CVT-510 can be achieved at a concentration that does not significantly shorten the atrial action potential and refractory period. The differential potencies of adenosine and other A₁ receptor agonists to lengthen the S–H interval and to shorten the atrial action potential are summarized in Table 2. For example, the potency of adenosine to prolong the S–H interval is 1.1 fold greater than its potency to shorten the atrial action potential (Raatikainen *et al.*, 1996). The basis for this differential potency of adenosine and A₁ receptor agonists to slow AV nodal conduction and shorten the atrial potential can be explained by the greater A₁ receptor reserve of the AV node than of atrial myocardium. This conclusion is based on the observation that inactivation of 40% of A₁ receptors with the irreversible antagonist FSCPX (Srinivas *et al.*, 1997) caused a significant attenuation of the maximal shortening of atrial potential but did not reduce the maximal S–H interval prolongation caused by CVT-510 (unpublished observation from our laboratory). This is indicative of a larger A₁ receptor reserve in the AV node than in the atrium.

Therapeutic implications

Paroxysmal supraventricular tachycardias (PSVT) affect 570,000 people with 89,000 new cases diagnosed each year (Orejarena *et al.*, 1998). At present, the most commonly used agent for rapid conversion of SVTs to sinus rhythm is adenosine (Langan, 1997). The limitations of adenosine for the long-term treatment of SVTs are owed to its ultra-short half-life in the circulation (~10 s) and to its vasodilatory effects. In acute cases where adenosine fails to restore normal sinus rhythm or cases in which long term management is required, calcium channel blockers such as verapamil or

Table 2 Potency of adenosine and A₁ receptor agonists to prolong S–H interval and to shorten atrial action potential

	S–H	MAPD	MAPD/S–H
Adenosine	6.5 μ M*	5.8 μ M*	0.89
CCPA	14 nM†	44 nM [#]	3.14
R-PIA	34 nM ⁺	145 nM [#]	2.30
CVT510	41 nM (4)	126 nM (6)	3.07

Potencies are mean EC_{50} values to prolong the stimulus-to-His (S–H) bundle and to shorten the atrial monophasic action potential (MAPD) measured at 90% repolarization in guinea-pig isolated perfused hearts. Numbers in parentheses indicate number of experiments. Superscripts correspond to the following references: *Raatikainen *et al.*, 1996; †Belardinelli *et al.*, 1998; [#]Morey *et al.*, 1998; ⁺Clemo & Belardinelli, 1986.

diltiazem are indicated (Langan, 1997). However, the utility of calcium channel blockers for the treatment of SVTs can be limited by their vasodilatory and negative inotropic actions. As shown in this study, diltiazem at concentrations that cause similar negative dromotropic effects as the A₁ receptor agonist CVT-510, significantly depresses ventricular contractility, increases coronary conductance, and causes hypotension.

In summary, the data reported here suggest that A₁ receptor-selective agonists such as CVT-510 may circumvent the problems associated with both adenosine and calcium channel blockers in the acute and long-term management of SVTs involving the AV node as part of the re-entrant circuit.

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Neuroregulation by vasoactive intestinal peptide (VIP) of mucus secretion in ferret trachea: activation of BK_{Ca} channels and inhibition of neurotransmitter release

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1 The aims of this study were to determine: (1) whether vasoactive intestinal peptide (VIP) regulates cholinergic and 'sensory-efferent' (tachykinergic) ³⁵SO₄ labelled mucus output in ferret trachea *in vitro*, using a VIP antibody, (2) the class of potassium (K⁺) channel involved in VIP-regulation of cholinergic neural secretion using glibenclamide (an ATP-sensitive K⁺ (K_{ATP}) channel inhibitor), iberiotoxin (a large conductance calcium activated K⁺ (BK_{Ca}) channel blocker), and apamin (a small conductance K_{Ca} (SK_{Ca}) channel blocker), and (3) the effect of VIP on cholinergic neurotransmission using [³H]-choline overflow as a marker for acetylcholine (ACh) release.

2 Exogenous VIP (1 and 10 μM) alone increased ³⁵SO₄ output by up to 53% above baseline, but suppressed (by up to 80% at 1 μM) cholinergic and tachykinergic neural secretion without altering secretion induced by ACh or substance P (1 μM each). Endogenous VIP accounted for the minor increase in non-adrenergic, non-cholinergic (NANC), non-tachykinergic neural secretion, which was compatible with the secretory response of exogenous VIP.

3 Iberiotoxin (3 μM), but not apamin (1 μM) or glibenclamide (0.1 μM), reversed the inhibition by VIP (10 nM) of cholinergic neural secretion.

4 Both endogenous VIP (by use of the VIP antibody; 1 : 500 dilution) and exogenous VIP (0.1 μM), the latter by 34%, inhibited ACh release from cholinergic nerve terminals and this suppression was completely reversed by iberiotoxin (0.1 μM).

5 We conclude that, in ferret trachea *in vitro*, endogenous VIP has dual activity whereby its small direct stimulatory action on mucus secretion is secondary to its marked regulation of cholinergic and tachykinergic neurogenic mucus secretion. Regulation is *via* inhibition of neurotransmitter release, consequent upon opening of BK_{Ca} channels. In the context of neurogenic mucus secretion, we propose that VIP joins NO as a neurotransmitter of i-NANC nerves in ferret trachea.

Keywords: Acetylcholine release; airways; cholinergic nerve; ferret trachea; iberiotoxin; mucus secretion; potassium channel; vasoactive intestinal peptide

Abbreviations: ACh, acetylcholine; ATP, adenosine triphosphate; BK_{Ca} (channel), large conductance, calcium-activated potassium (channel); CGRP, calcitonin gene-related peptide; DMSO, dimethyl sulphoxide; i-NANC, inhibitory non-adrenergic, non-cholinergic; K⁺ (channel), potassium (channel); K_{ATP} (channel), adenosine triphosphate sensitive potassium channel; NANC, non-adrenergic, non-cholinergic; NKA, neurokinin A; NO, nitric oxide; NOS, nitric oxide synthase; NPY, neuropeptide Y; PACAP, pituitary adenylate cyclase-activating peptide; PHM, peptide histidine methionine; SK_{Ca} (channel), small conductance calcium-activated potassium channel; SP, substance P; VIP, vasoactive intestinal peptide; VIP-Ab, vasoactive intestinal peptide antibody

Introduction

Airway neural pathways are classified currently as adrenergic, cholinergic and non-adrenergic non-cholinergic (NANC): they are anatomically interlinked and interact functionally. The excitatory and inhibitory neural mechanisms regulating airway smooth muscle function are relatively well defined (Barnes, 1996; Widdicombe, 1996). In contrast, for neurogenic airway mucus secretion, although excitatory pathways are comparatively well characterized (Rogers 1997), inhibitory pathways are poorly defined. In mammalian airways, the predominant neural control for mucus secretion is cholinergic with a minor adrenergic component. There is also a 'sensory-efferent' neural pathway controlling mucus secretion. The nerves comprising the latter pathway are C-fibres sensitive to capsaicin and whose

neurotransmitters are calcitonin gene related peptide (CGRP) and the tachykinins substance P (SP) and neurokinin A (NKA), collectively termed sensory neuropeptides (Ramnarine & Rogers, 1994). In ferret trachea *in vitro*, sensory-efferent pathways account for up to 40% of the total neurogenic secretory response, with the functional response (i.e. mucus secretion) mediated *via* tachykinin NK₁ receptors on the secretory cells (Ramnarine *et al.*, 1994). Nitric oxide (NO) and vasoactive intestinal peptide (VIP) are neurotransmitters of inhibitory NANC (i-NANC) neural pathways regulating the magnitude of neurogenic bronchoconstriction, although their relative involvement varies with species (Lammers *et al.*, 1992). We found that endogenous NO regulated the magnitude of neurogenic mucus secretion in ferret trachea *in vitro* (Ramnarine *et al.*, 1996). The role of VIP as a regulator of neurogenic mucus secretion is unexplored.

Immunohistochemical studies localize VIP-containing airway nerves to intrinsic ganglia, smooth muscle, submucosal

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glands and blood vessels in human (Dey *et al.*, 1981; Laitinen *et al.*, 1985), dog and cat (Dey *et al.*, 1988). VIP coexists with SP and galanin in cat airways (Dey & Zhu, 1994; Dey *et al.*, 1988), nitric oxide synthase (NOS) and acetylcholine (ACh) in human airways (Fischer & Hoffman, 1996; Laitinen *et al.*, 1985), neuropeptide Y (NPY) in guinea-pig trachea (Bowden & Gibbins, 1992), and with SP and NOS in ferret trachea (Dey *et al.*, 1996). By autoradiography, VIP receptors are localized to submucosal glands, epithelium and smooth muscle in airways of pig (Lazarus *et al.*, 1986), rat (Leroux *et al.*, 1984) and human (Fischer *et al.*, 1992). These anatomical studies imply that VIP has a role in modulating airway mucus secretion and may interact with other neurotransmitters.

In the present study, we explored the role of VIP on neural and non-neural control of mucus secretion in ferret trachea *in vitro* in Ussing chambers using $^{35}\text{SO}_4$ as a mucus marker. Firstly, we determined the effect of VIP itself on non-neural mucus secretion. Secondly, we investigated the effect of VIP on cholinergic, tachykinergic, and non-NANC non-tachykinergic neural mucus secretion, using exogenous VIP, a selective VIP antibody (VIP-Ab) (Shimura *et al.*, 1988), the peptidase α -chymotrypsin, or a putative VIP₁ receptor antagonist [D-p-Cl-Phe⁶,Leu¹⁷]-VIP (Usdin *et al.*, 1994) to investigate the role of endogenous VIP. Thirdly, we determined the class of potassium (K^+) channel involved in VIP inhibition using the ATP sensitive K^+ (K_{ATP}) channel inhibitor glibenclamide (Niki *et al.*, 1989; Schmid-Antomarchi *et al.*, 1990), the blocker of calcium activated K^+ (K_{Ca}) channels of small conductance (SK_{Ca} channels) apamin (Banks *et al.*, 1979), and the large conductance K_{Ca} (BK_{Ca}) channel blocker iberiotoxin (Garcia *et al.*, 1991). Finally, to more directly confirm a neuromodulatory role for VIP on cholinergic neurotransmission, we determined the effect of VIP, α -chymotrypsin and VIP-Ab on ACh release from ferret trachea *in vitro* using [^3H]-choline as a marker for ACh (Alberts *et al.*, 1982; Wessler *et al.*, 1991).

Methods

Male ferrets (Regal Rabbits, Great Bookham, Surrey, U.K.) weighing 1.0–2.0 kg were used throughout and were kept four to five in a room. They were allowed 1 week to recuperate after delivery and had free access to food and water. They were terminally anaesthetized with pentobarbitone sodium (Sagatal: 60 mg kg⁻¹, i.p.), bled by incising the left ventricle, and the tracheae were removed and bathed in aerated (95% O_2 + 5% CO_2) Krebs-Henseleit solution of the following composition (mM): NaCl 118, KCl 5.9, MgSO_4 1.2, CaCl_2 2.5, NaHCO_3 25.5 and glucose 5.05 until required (~10 min) for study of either mucus secretion or acetylcholine release.

Tracheal preparation for measurement of mucus secretion

Our methodology for measurement of mucus secretion has been described in detail previously (Meini *et al.*, 1993; Ramnarine *et al.*, 1994). Tracheae were cut longitudinally through the dorsal membrane, opened flat and cut transversely to give four segments. Each segment was pinned and clamped across the aperture separating the two halves of perspex Ussing-type chambers so that the tissue divided the chambers into a 'luminal' (i.e. mucus-producing) and 'submucosal' side. The chambers were rectangular in cross-section to ensure optimal use of the tissue with an exposed surface area for each segment of 1.12 cm². Each side of the tissue was bathed with 5 ml warmed (37°C) Krebs-Henseleit solution which was

oxygenated (95% O_2 :5% CO_2) and circulated using gas-lift pumps.

Radiolabelling of newly synthesized mucus

At time 0 h, $\text{Na}_2^{35}\text{SO}_4$ (0.1 mCi) was added to the submucosal half-chambers, in order to label newly-synthesized intracellular mucus, where it remained throughout the experiment. At unit time intervals, the fluid in the luminal side of the chamber (containing secretions) was collected and replaced with fresh Krebs-Henseleit solution. Baseline stability of spontaneous output of $^{35}\text{SO}_4$ -labelled macromolecules was reached after taking four 30 min collections followed by two 15 min collections (i.e. over 2.5 h following addition of radiolabel). After stabilization, drugs or control solutions were added and the tissues were electrically stimulated (see *Protocols for secretory studies* below).

Electrical stimulation

The tracheal segments could be subjected to an electrical current to stimulate excitable tissues (e.g. nerves). Two pins piercing the tissue on either side were connected *via* outlet wires through the chambers to a Grass stimulator (model S88; Grass Instruments, Quincy, U.S.A.) (Borson *et al.*, 1984). Tissues were stimulated at 2.5 or 10 Hz, 50 V, 0.5 ms for the first 5 min of a 15 min incubation period.

Measurement of $^{35}\text{SO}_4$ output

Luminal fluid, approximately 4 ml and comprising secretions in Krebs-Henseleit solution, was drained into tubes containing 5 g guanidine hydrochloride to dissolve the mucus. The final concentration of guanidine hydrochloride in the fluid was 6 M. Following this, each sample was exhaustively dialyzed against distilled water containing excess Na_2SO_4 and sodium azide (10 mg l⁻¹) using cellulose tubing (Medicell International Ltd., London, U.K.) which allowed molecules of 12–14 kDa or less to pass through. The sodium azide was present to inhibit bacterial growth. The samples were recovered after at least six changes of distilled water when the radioactive count of the dialysis water was the same after dialysis as before dialysis (~20 disintegrations per min [d.p.m.]). The recovered samples were weighed and the remaining radioactivity in 1 ml duplicates of each sample mixed with 2 ml scintillant (Ultima Gold XR, Canberra Packard Ltd., Pangbourne, Berkshire, U.K.) was determined by scintillation spectrometry (model 1900CA Spectrophotometer, Canberra Packard Ltd.). The total radioactivity of each sample was estimated by multiplying the radioactivity present in a 1 ml aliquot of sample by the total weight of the sample (assuming a 1 ml sample weighs 1 g).

Protocols for secretory studies

To investigate the effect of exogenous VIP on non-neural mucus secretion, concentration-response and time course studies of VIP were carried out. The first collection at 2.25 h was the baseline control after which VIP (1 nM–10 μM) or vehicle (distilled water) were added to the submucosal chamber where they remained for the duration of the experiment.

To validate the effectiveness of VIP-Ab in neutralizing the secretory response induced by exogenous VIP, VIP-Ab (final dilution 1:500; Shimura *et al.*, 1988) or control serum was added to the submucosal chamber 30 min prior to the administration of VIP (10 nM–10 μM).

To examine the effects of endogenous VIP in regulating cholinergic neural secretion, VIP-Ab (1 : 500) or control serum, the putative VIP₁ receptor antagonist, [D-p-Cl-Phe⁶,Leu¹⁷]-VIP (0.5 or 5 μ M) (Usdin *et al.*, 1994), or α -chymotrypsin (2 u ml⁻¹) were added to the submucosal chamber 30 min before and during electrical stimulation (2.5 or 10 Hz, 50 V, 0.5 ms for 5 min) in the presence of phentolamine, propranolol (10 μ M for each), and the tachykinin NK₁ receptor antagonist CP-99,994 (3 μ M; McLean *et al.*, 1993) to remove α -adrenergic, β -adrenergic and tachykinergic neural influences respectively.

To examine the effects of exogenous VIP on cholinergic neural secretion, VIP or control vehicle was added to the submucosal chamber 30 min before and during 2.5 or 10 Hz stimulation in the presence of phentolamine, propranolol and CP-99,994 (as above). In addition, in the studies investigating the effect of VIP on ACh release, epithelially-scraped tissues were used in the presence of indomethacin (see below). To assess the influence of this treatment on secretion, the effect of 10 Hz cholinergic stimulation (in the presence of adrenoceptor and NK₁ receptor blockade; as above) was compared between tissues not scraped and without indomethacin, and tissues scraped in the presence of indomethacin (10 μ M).

To determine the effects of endogenous VIP on tachykinergic neural secretion, VIP-Ab (1 : 500) or control serum was added to the submucosal chamber 30 min prior to and during 2.5 or 5 Hz stimulation in the presence of phentolamine, propranolol and atropine (10 μ M each) to eliminate adrenergic and cholinergic neural influences.

To study the effects of exogenous VIP in modulating tachykinergic neural secretion, VIP (1 μ M) was added to the submucosal chamber 30 min before and during 10 Hz stimulation in the presence of phentolamine, propranolol and atropine (10 μ M each).

To assess the contribution of endogenous VIP to NANC non-tachykinergic neural secretion, VIP-Ab was added to the submucosal side for 30 min before and during stimulation at 2.5 or 10 Hz in the presence of atropine, phentolamine, propranolol (10 μ M each) and CP-99,994 (3 μ M).

To test whether or not the post-junctional secretory responses induced by ACh or SP were affected by VIP or VIP-Ab, ACh (1 μ M) or SP (1 μ M) was added to the luminal chamber in the presence of VIP (1 μ M), VIP-Ab or control serum for 30 min.

To investigate the involvement of, and determine the class of, K⁺ channels in VIP-regulation of cholinergic neural secretion (i.e. electrical stimulation in the presence of phentolamine, propranolol and CP-99,994; as above), vehicle solution, glibenclamide (0.1 μ M), apamin (1 μ M) or iberiotoxin (3 μ M) were added to the luminal half-chamber 15 min before VIP (10 nM), which in turn was added 30 min before 10 Hz stimulation.

Tracheal preparation for measurement of ACh release

Release of ACh from cholinergic nerves was measured as described previously (Ward *et al.*, 1993; Patel *et al.*, 1995). Tracheae were bathed in Krebs-Henseleit solution, opened longitudinally by cutting anteriorly through the cartilage, and the epithelium was carefully peeled off. Strips of smooth muscle were dissected from the tracheae and as much cartilage as possible was removed. Indomethacin (10 μ M) was present in the Krebs-Henseleit solution throughout to prevent the formation of endogenous prostanoids which have been demonstrated previously to affect cholinergic neurotransmission and ACh release (Walters *et al.*, 1984; Deckers *et al.*, 1989;

Wessler *et al.*, 1990; Belvisi *et al.*, 1996). The tracheal segments (~1 × 5 mm) were suspended top and bottom with silver wire (Goodfellow Metals, Cambridge, Cambridgeshire, U.K.), mounted in perfusion chambers, and superfused with Krebs-

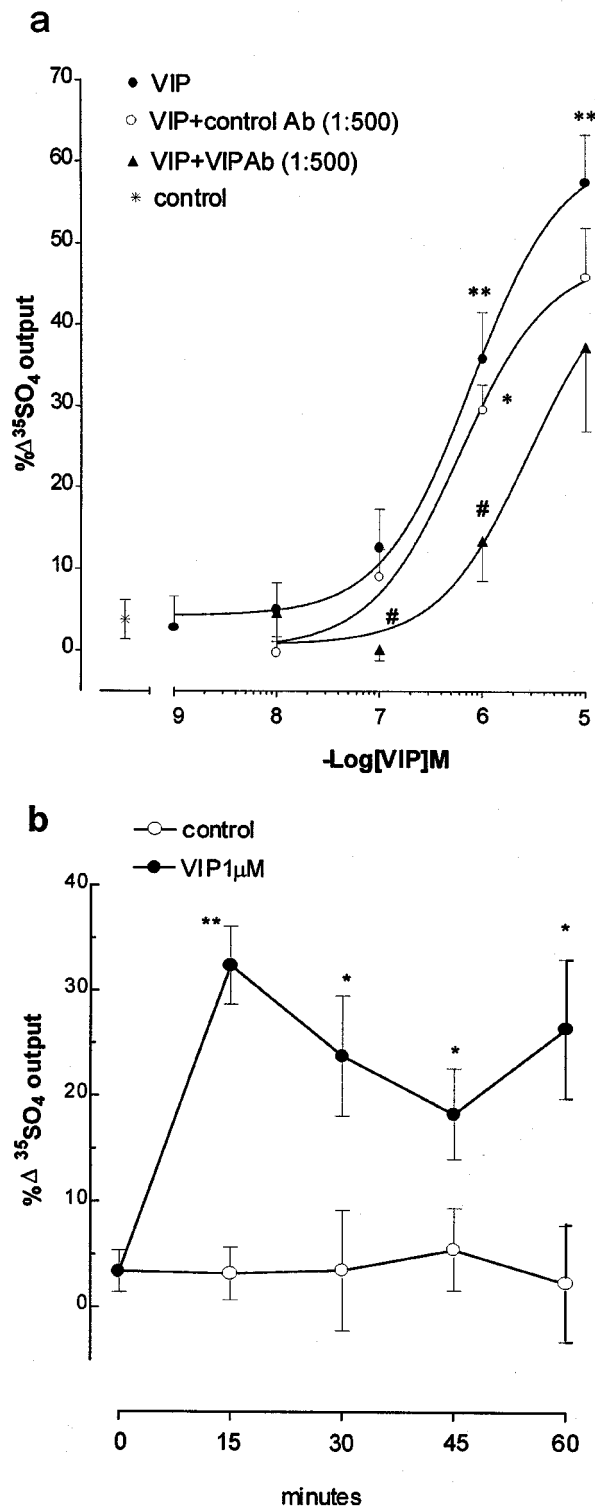


Figure 1 Effect of exogenous vasoactive intestinal peptide (VIP) on mucus secretion in ferret trachea *in vitro*. (a) Concentration-response and inhibition by VIP antibody (VIP-Ab), (b) time course. Data are mean per cent change in output of macromolecules labelled *in situ* with ³⁵SO₄ (a marker for mucus) for 5–8 animals per group; vertical bars are one s.e.mean. **P* < 0.05, ***P* < 0.01 compared with baseline control (a), or with corresponding vehicle control time point (b); #*P* < 0.05 compared with VIP group (a).

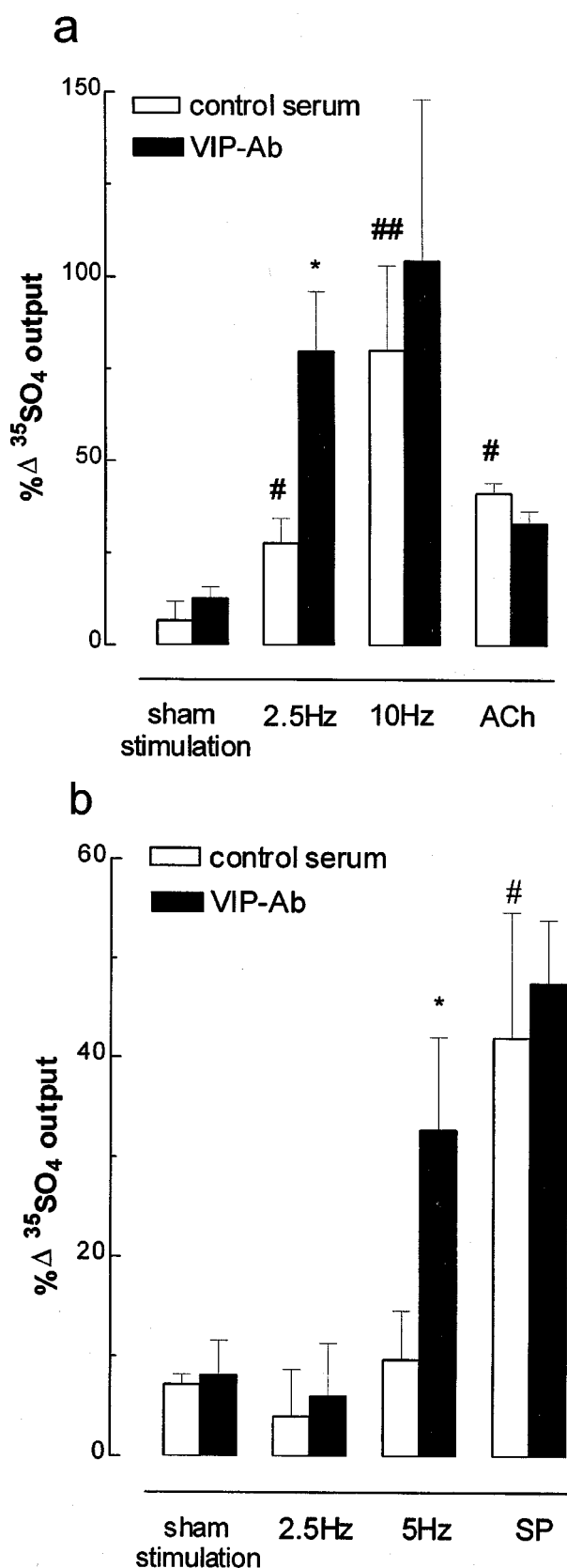


Figure 2 Effect of endogenous vasoactive intestinal peptide (VIP) on neurogenic mucus secretion in ferret trachea *in vitro*. (a) Effect on cholinergic secretion. A VIP antibody (VIP-Ab, 1:500 dilution) was used to exclude VIP, and phentolamine, propranolol (10 μ M each) and the tachykinin NK₁ receptor antagonist CP-99,994 (3 μ M) were used to exclude adrenergic and tachykinergic neural influences at stimulation parameters of 2.5 or 10 Hz, 50 V, 0.5 msec for 5 min. ACh, acetylcholine (1 μ M). (b) Effect on tachykinergic neural secretion. VIP antibody (1:500 dilution) was used to exclude VIP, and phentolamine, propranolol and atropine (10 μ M each) were used

Henseleit solution, bubbled with 95% O₂, 5% CO₂ at 37°C at 1 ml min⁻¹ via a perfusion pump (model 503S; Smith and Nephew Watson-Marlow, Falmouth, Cornwall, U.K.).

Loading with [³H]-choline

The tracheal strips were allowed to equilibrate during a 30-min period of continuous superfusion with Krebs-Henseleit solution, and electrical stimulation (4 Hz, 40 V, 0.5 ms) was applied for the last 10 min of this period (model S88 stimulator; Grass Instrument Co.) via the silver wires. Tissues were then immersed in 1.5 ml oxygenated Krebs-Henseleit solution containing [methyl-³H]-choline chloride (67 nmol liter⁻¹; specific radioactivity 2.78 TBq mmol⁻¹) and electrically stimulated for another 45 min to allow uptake of [³H]-choline into the tissues. At the end of this period, superfusion was continued with Krebs-Henseleit solution containing hemicholinium-3 (10 μ M) to prevent further uptake of [³H]-choline into the tissues. Tissues were then washed for 2.5 h to reach a stable baseline of [³H] release before beginning collections for the experiment (see *Protocols* below). The single term '[³H]' is used herein to acknowledge that the precise identity of the molecule to which the released [³H] is bound is unknown (choline, ACh or phosphorylcholine; see Wessler *et al.*, 1990, 1991). Radioactivity in 1 ml aliquoted sample mixed with 4 ml scintillant (Pico-Fluor 40, Canberra Packard Ltd., Pangbourne, Berkshire, U.K.) was determined by scintillation spectrometry (Model 1900CA Spectrophotometer, Canberra Packard Ltd.).

Protocols for [³H] release studies

Each tracheal tissue segment was stimulated twice (once at 19 min and once at 56 min after equilibration) at 2.5 or 10 Hz, 50 V, 0.5 ms for 1 min, to provoke [³H] overflow. VIP (10 nM and 0.1 μ M), VIP-Ab (1:500), α -chymotrypsin (2 u ml⁻¹) or control solutions were administered 30 min before the second stimulation. In certain experiments, ibuprofen (0.1 μ M) was added 5 min before the administration of VIP. Samples were collected every minute for 3 min before stimulation, for the 1 min of stimulation, every minute for 3 min after stimulation, and at 5 min intervals outside these times.

Drugs and chemicals. The following drugs were used: VIP (porcine; synthetic), substance P, [D-p-Cl-Phe⁶,Leu¹⁷]-VIP (human, porcine, rat; synthetic), α -chymotrypsin, acetylcholine, dimethylsulphoxide (DMSO), indomethacin, tetrodotoxin (TTX) and hemicholinium-3 (Sigma Chemical Company Ltd., Poole, Dorset, U.K.); atropine sulphate (Phoenix Pharmaceuticals Ltd., Pharma Hameln, G.m.b.H., Germany); phentolamine mesylate (Ciba Laboratories, Horsham, West Sussex, U.K.); propranolol hydrochloride (Imperial Chemical Industries Ltd., Macclesfield, Cheshire, U.K.); Na₂³⁵SO₄ and [methyl-³H]-choline chloride (Amersham International plc, Amersham, Berkshire, U.K.); and pentobarbitone sodium B.P. (Sagatal; RMB Animal Health Ltd., Dagenham, Essex,

to exclude adrenergic and cholinergic influences at stimulation parameters of 2.5 or 5 Hz, 50 V, 0.5 msec for 5 min. SP, substance P (1 μ M). Data are mean per cent change in output of macromolecules labelled *in situ* with ³⁵SO₄ (a marker for mucus) for 5–7 animals per group; vertical bars are one s.e.mean. **P* < 0.05 compared with serum control group; #*P* < 0.05, ##*P* < 0.01 compared with sham stimulation group.

U.K.). CP-99,994 (2*S*,3*S*)-3-(2-methoxybenzyl)-amino-2-phenyl-piperidine was a kind gift from Pfizer, Groton, U.S.A. (courtesy Dr R. Michael Snider). VIP-Ab was a rabbit anti-VIP (human, porcine, rat) anti-serum, without cross-reactivity to VIP 1-12 (human, porcine, rat), peptide histidine methionine (PHM)-27 (human), substance P, endothelin-1 (human, porcine, canine, rat, mouse, bovine), secretin (porcine), glucagon (human), galanin (rat), somatostatin, pituitary adenylate cyclase-activating peptide (PACAP)-38 (human, bovine, rat) or PACAP-38 (16-38) (human, bovine, rat), and with little cross-reactivity to VIP 10-28 (human, porcine, rat) (4.4%) or PACAP-27 (human, bovine, rat) (0.02%) (Pennisula Laboratories, Inc., Belmont, U.S.A.).

Data analysis

In Results, data for mucus secretion are the arithmetic mean and one standard error of the mean (s.e.mean). Because baseline d.p.m. displayed variability between tracheal segments, responses obtained from individual segments were calculated as percentage changes in radiolabel output for the difference between response to drug or electrical stimulation and the proceeding collection. Data for ACh release are rate changes for the second stimulation compared with the first stimulation, with the rate coefficient the fractional release of initial [^3H] content over time. The concentration of VIP causing a 50% increase (EC_{50}) in secretion or inhibition (IC_{50})

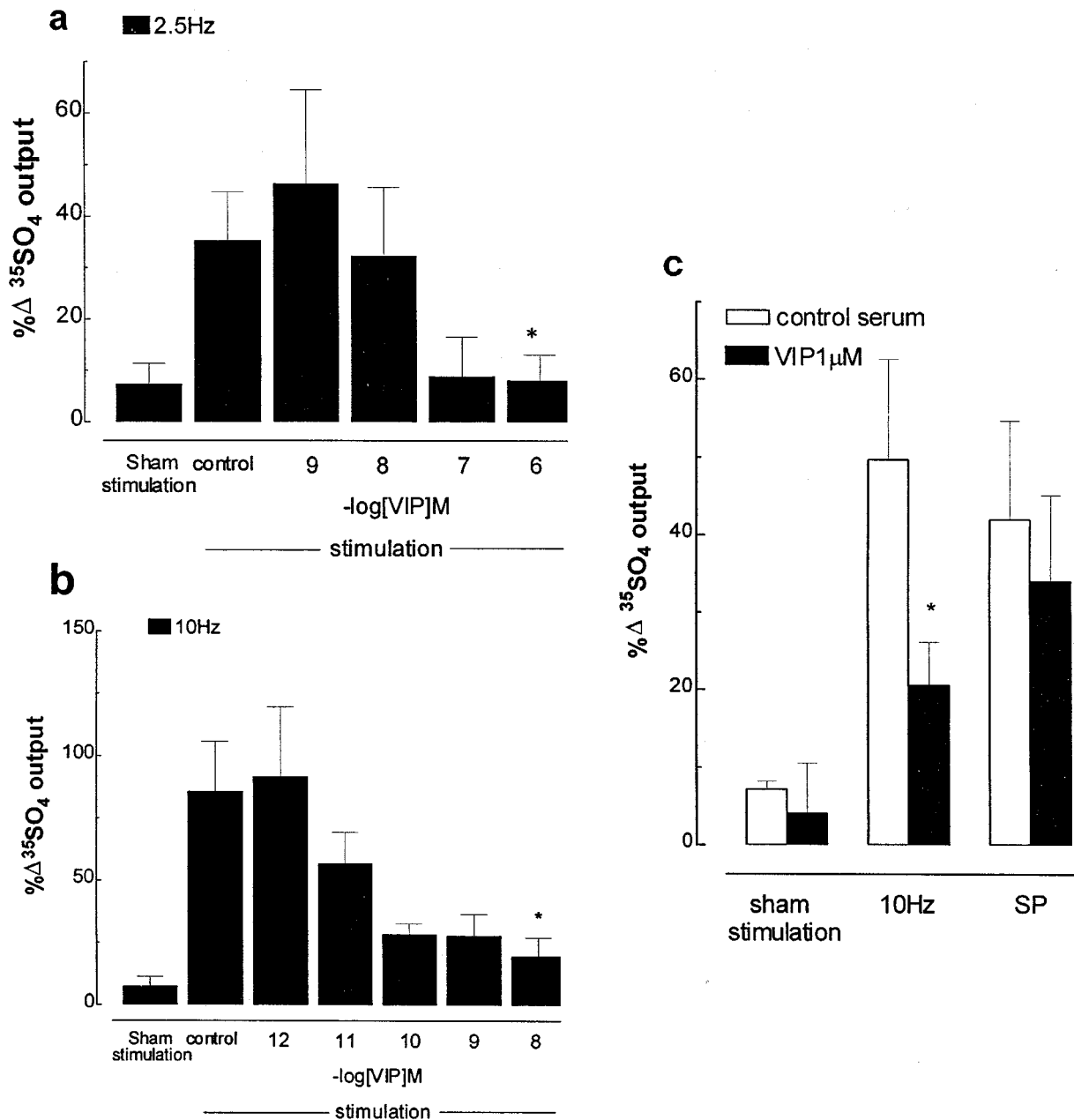


Figure 3 Effect of exogenous vasoactive intestinal peptide (VIP) on neurogenic mucus secretion in ferret trachea *in vitro*. (a and b) Effect on cholinergic neural secretion. Phentolamine, propranolol (10 μM each) and the tachykinin NK_1 receptor antagonist CP-99,994 (3 μM) were used to exclude adrenergic and tachykinergic influences at stimulation parameters of 2.5 (a) or 10 Hz (b), 50 V, 0.5 msec for 5 min. (c) Effect on tachykinergic neural secretion. Phentolamine, propranolol and atropine (10 μM each) were used to exclude adrenergic and cholinergic influences at stimulation parameters of 10 Hz, 50 V, 0.5 msec for 5 min. SP, substance P (1 μM). Data are mean per cent change in output of macromolecules labelled *in situ* with $^{35}\text{SO}_4$ (a marker for mucus) for 5–7 animals per group; vertical bars are one s.e.mean. * $P < 0.05$ compared with stimulation control group (a and b), or with serum control group (c).

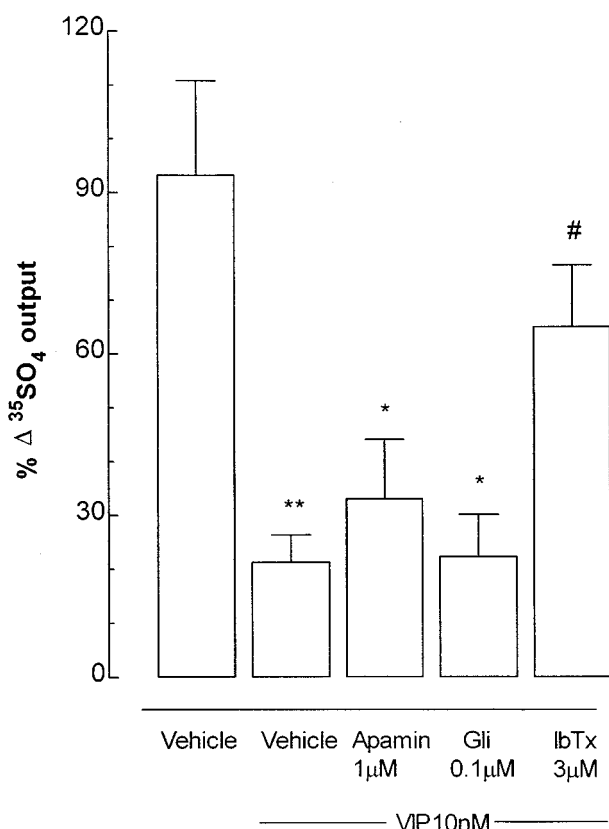


Figure 4 Inhibition by exogenous vasoactive intestinal peptide (VIP) of cholinergic neural mucus secretion in ferret trachea *in vitro*, and the effect of potassium (K^+) channel blockers. Phentolamine, propranolol ($10 \mu M$ each) and the tachykinin NK_1 receptor antagonist CP-99,994 ($3 \mu M$) were used to exclude adrenergic and tachykinergic influences at stimulation parameters of 10 Hz, 50 V, 0.5 msec for 5 min. Gli: glibenclamide, ATP-sensitive K^+ (K_{ATP}) channel inhibitor; apamin, small conductance calcium-activated K^+ (SK_{Ca}) channel blocker; IbTx: iberiotoxin, large conductance calcium-activated K^+ (BK_{Ca}) channel blocker. Data are mean per cent change in output of macromolecules labelled *in situ* with $^{35}SO_4$ (a marker for mucus) for 5–6 animals per group; vertical bars are one s.e.mean. * $P < 0.05$, ** $P < 0.01$ compared with stimulation control group; # $P < 0.05$ compared with VIP + vehicle group.

of neurogenic secretion was calculated by non-linear regression using GraphPad Prism software (Microsoft, San Diego, U.S.A.). Significance of changes in $^{35}SO_4$ output and [^3H]-choline overflow, pre- and post-drug or electrical stimulation, were assessed using the Mann-Whitney U -test between two groups, or the Kruskal-Wallis test followed by Dunns multiple comparison test for multiple groups. The null hypothesis was rejected at $P < 0.05$ (two-tail).

Results

Median baseline radioactivity in the secretion studies was of the order of 740 d.p.m. (range 387–1272 d.p.m., depending upon the specific experiment). There were no significant differences between treatment groups.

Effect of exogenous VIP on mucus secretion

VIP (1 nM – $10 \mu M$) caused an increase in $^{35}SO_4$ output in ferret tracheal segments during the 15 min incubation period after addition. The increase was concentration-dependent with an

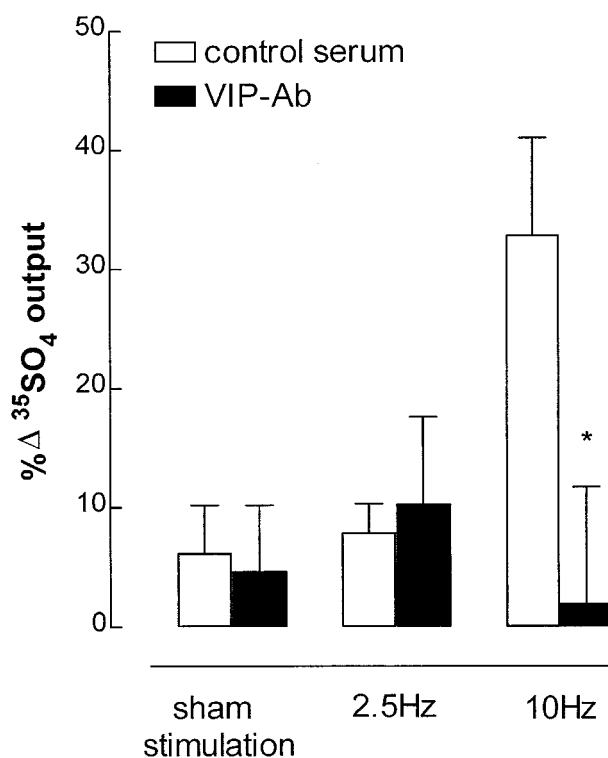


Figure 5 Endogenous vasoactive intestinal peptide (VIP) as mediator of NANC, non-tachykinergic neural mucus secretion in ferret trachea *in vitro*. A VIP antibody (VIP-Ab, 1:500 dilution) was used to exclude VIP, and phentolamine, propranolol, atropine ($10 \mu M$) and the tachykinin NK_1 receptor antagonist CP-99,994 ($3 \mu M$) were used to exclude adrenergic, cholinergic and tachykinergic influences at stimulation parameters of 2.5 or 10 Hz, 50 V, 0.5 msec for 5 min. Data are mean per cent change in output of macromolecules labelled *in situ* with $^{35}SO_4$ (a marker for mucus) for six animals per group; vertical bars are one s.e.mean. * $P < 0.05$ compared with serum control group.

approximate EC_{50} of $0.9 \mu M$ and a maximal increase of 53% above control at $10 \mu M$ (Figure 1a). In the continued presence of VIP ($1 \mu M$) in the submucosal side, $^{35}SO_4$ output peaked during the first 15 min incubation and, although showing a reduced response, was increased significantly above controls for at least 60 min (Figure 1b).

Effects of VIP-Ab on VIP-induced secretion, and cholinergic and tachykinergic neural mucus secretion

Preincubation for 30 min with VIP-Ab caused a rightward shift in the VIP concentration-response curve with significant inhibitions of 90 and 70% respectively at 0.1 and $1 \mu M$ VIP (Figure 1a). Control serum had no significant inhibitory effect on VIP-induced $^{35}SO_4$ output (Figure 1a).

In the presence of phentolamine, propranolol ($10 \mu M$ each) and CP-99,994 ($3 \mu M$), both 2.5 and 10 Hz (50 V, 0.5 ms for 5 min) stimulation significantly induced cholinergic $^{35}SO_4$ output by 21 and 73% respectively above pre-stimulation controls (Figure 2a). VIP-Ab significantly potentiated cholinergic $^{35}SO_4$ output by 219% at 2.5 Hz, but had no significant effect at 10 Hz (an increase of 25% above the stimulated value). VIP-Ab did not affect $^{35}SO_4$ output elicited by exogenous ACh ($1 \mu M$), of an equivalent magnitude to that elicited by 2.5 Hz electrical stimulation (Figure 2a). The putative VIP_1 receptor antagonist, [D-p-Cl-Phe⁶,Leu¹⁷]-VIP (0.5 or $5 \mu M$), did not alter the magnitude of cholinergic $^{35}SO_4$

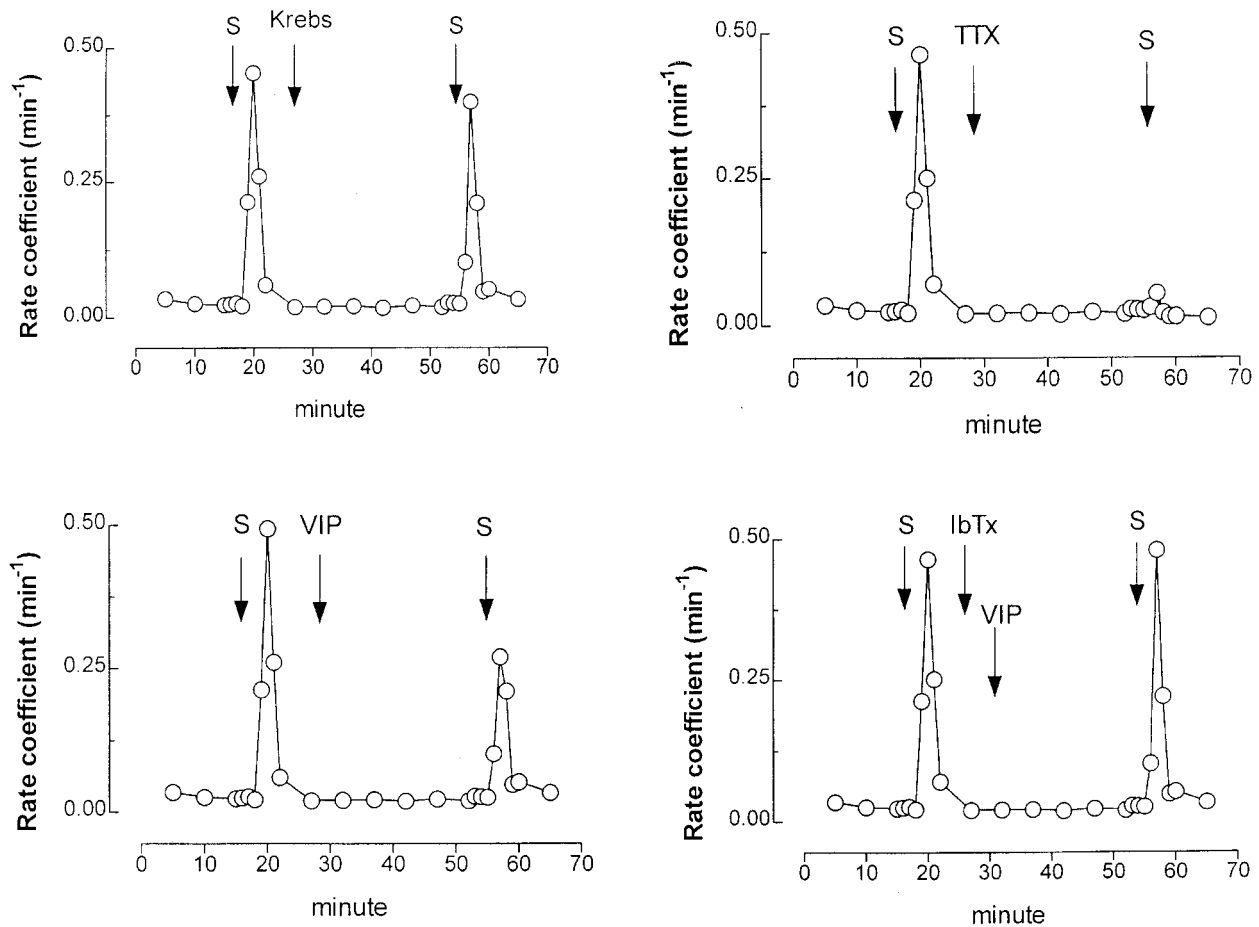


Figure 6 Effect of 10 Hz stimulation (50 V, 0.5 msec, 5 min) on acetylcholine (ACh) release by ferret tracheal strips *in vitro*. Tissues were pre-incubated with [^{3} H]-choline chloride (a marker for ACh) and stimulated electrically (S). Each panel gives the rate of [^{3} H] overflow for an individual tracheal strip. VIP, vasoactive intestinal peptide (0.1 μ M); TTX, tetrodotoxin (0.1 μ M); IbTx, iberiotoxin (0.1 μ M).

output at 2.5 or 10 Hz stimulation, nor did it affect exogenous VIP-elicited $^{35}\text{SO}_4$ output. α -Chymotrypsin (2 u ml $^{-1}$) itself caused a significant increase in $^{35}\text{SO}_4$ output of 40% above control ($n=6$, $P<0.05$). Neither the VIP receptor antagonist nor α -chymotrypsin were utilized further to evaluate the role of endogenous VIP in neurogenic secretion.

In the presence of atropine, phentolamine and propanolol (10 μ M each), VIP-Ab significantly enhanced tachykinergic $^{35}\text{SO}_4$ output by 237% above serum controls at 5 Hz stimulation (Figure 2b). VIP-Ab did not affect $^{35}\text{SO}_4$ output induced by SP (1 μ M) (Figure 2b).

Effect of exogenous VIP on cholinergic and tachykinergic neural mucus secretion

In the presence of phentolamine, propanolol (10 μ M each) and CP-99,994 (3 μ M), exogenous VIP inhibited cholinergic $^{35}\text{SO}_4$ output: at 2.5 Hz stimulation with complete inhibition at 1 μ M (Figure 3a), and at 10 Hz with a maximal inhibition of 80% at 10 nM (Figure 3b). In contrast, exogenous VIP (1 μ M) had an additive effect with ACh in increasing $^{35}\text{SO}_4$ output (41 $\pm$ 3% increase with ACh alone vs 78 $\pm$ 9% increase with ACh + VIP, $P<0.05$). Epithelial scraping and indomethacin had no significant effect on cholinergic $^{35}\text{SO}_4$ output compared with non-scraped tissues without indomethacin: respectively 13 $\pm$ 10% vs 8 $\pm$ 9% increase in output in sham stimulated

tissues, 227 $\pm$ 30% vs 245 $\pm$ 25% in stimulated tissues, 62 $\pm$ 18% vs 96 $\pm$ 20% in stimulated tissues in the presence of VIP (10 nM) ($n=4$ for each group).

In the presence of atropine, phentolamine and propanolol (10 μ M each), 10 Hz stimulation caused an increase in tachykinergic $^{35}\text{SO}_4$ output of 42% above pre-stimulation values, which was inhibited by exogenous VIP (1 μ M) by 59% (Figure 3c). In contrast, SP (1 μ M)-induced increase in $^{35}\text{SO}_4$ output was not significantly changed by pre-treatment with VIP (1 μ M) (Figure 3c).

Effect of K^+ channel blockers on cholinergic neural secretion

In the presence of phentolamine, propanolol (10 μ M each) and CP-99,994 (3 μ M), exogenous VIP (1 μ M) significantly diminished 10 Hz cholinergic secretion by 79% compared with stimulation control, and this effect was reversed (by 60%) by pre-incubation with iberiotoxin (3 μ M), but not by apamin (1 μ M) or glibenclamide (0.1 μ M) (Figure 4).

Effect of VIP-Ab on NANC, non-tachykinergic neural mucus secretion

In the presence of atropine, phentolamine, propanolol (10 μ M each) and CP-99,994 (3 μ M), 10 Hz stimulation significantly

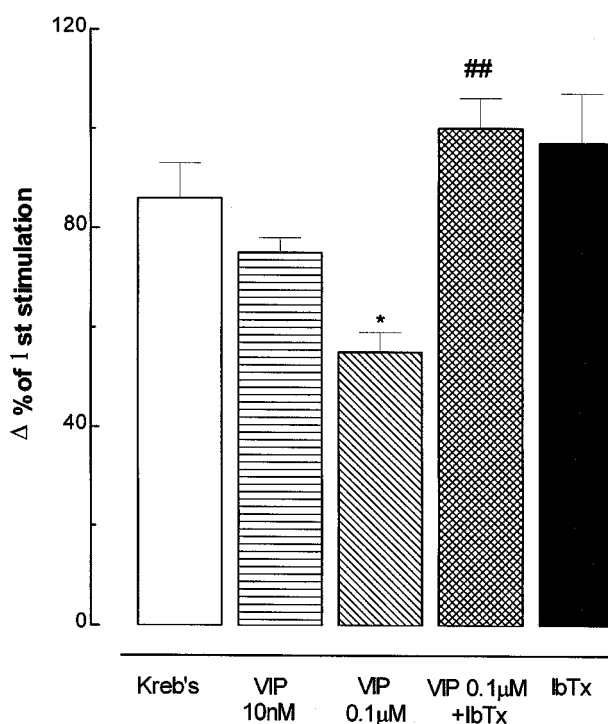


Figure 7 Involvement of large-conductance K^+ (BK_{Ca}) channels in vasoactive intestinal peptide (VIP)-inhibition of acetylcholine (ACh) release in ferret tracheal strips *in vitro*. IbTx, iberiotoxin ($0.1 \mu M$); BK_{Ca} channel blocker. Tissues were pre-incubated with [^3H]-choline chloride (a marker for ACh) and stimulated electrically at 10 Hz, 50 V, 5 msec for 5 min. Data are mean per cent change in rate of [^3H] overflow at a second stimulation compared with the first stimulation for 5–6 animals per group; vertical bars are one s.e.mean. * $P < 0.05$ compared with Krebs's solution alone; ## $P < 0.01$ compared with VIP + IbTx group.

increased $^{35}SO_4$ output by 25% above pre-stimulation control, whereas stimulation at 2.5 Hz did not significantly increase $^{35}SO_4$ output (Figure 5). The 10 Hz stimulation-induced $^{35}SO_4$ output was eliminated by VIP-Ab (Figure 5).

Effects of VIP and iberiotoxin on cholinergic neural [^3H] output

Exogenous VIP ($0.1 \mu M$) significantly reduced [^3H] overflow at 10 Hz stimulation by 34% compared with control (Figures 6 and 7). This effect was completely reversed by pre-administration of iberiotoxin ($0.1 \mu M$) (Figures 6 and 7). Iberiotoxin alone had no significant effect on stimulated [^3H] output (Figure 7). Tetrodotoxin ($3 \mu M$) markedly inhibited (by ~90%) the [^3H] overflow evoked by 10 Hz stimulation (Figure 6): mean 10% ($\pm 12\%$) change in [^3H] overflow compared with first stimulation ($n = 5$, $P < 0.01$).

Effects of VIP-Ab and α -chymotrypsin on cholinergic neural [^3H] output

Pre-treatment with VIP-Ab ($1:500$) or α -chymotrypsin (2 u ml^{-1}) potentiated neural [^3H] output at 2.5 Hz by 44 and 40%, respectively (Figures 8 and 9). Tetrodotoxin ($3 \mu M$) markedly inhibited (by ~95%) the [^3H] overflow evoked by 2.5 Hz stimulation (Figure 8): mean 5% ($\pm 10\%$) change in [^3H] overflow compared with first stimulation ($n = 5$, $P < 0.01$).

Discussion

In the present study in ferret trachea *in vitro*, exogenous VIP increased $^{35}SO_4$ -labelled mucus output whereas endogenous VIP regulated the magnitude of neurogenic secretion. The increase in $^{35}SO_4$ output is consistent with an increase in mucus secretion by submucosal glands, because there are few surface epithelial goblet cells but large numbers of submucosal glands in ferret trachea (Robinson *et al.*, 1986; Meini *et al.*, 1993). By autoradiography, under basal conditions there is selective uptake and retention of $^{35}SO_4$ by tracheal submucosal glands, rather than epithelium, in cat (Davies *et al.*, 1990) and ferret (Gashi *et al.*, 1987), with some labelling of cartilage. Stimulation of ferret trachea *in vitro* increased radioactive counts in the incubation medium, with concomitant loss of autoradiographic grains from the glands (Gashi, 1987): neither cartilage nor epithelium showed loss of grains. In cats, tracheal washings labelled with $^{35}SO_4$ have a molecular weight and buoyant density typical of a mucin molecule (Davies *et al.*, 1990). Thus, the increased output of $^{35}SO_4$ observed herein is indicative of an increase in tracheal submucosal gland mucus secretion.

We demonstrated herein that exogenous VIP caused a small increase in secretion in a concentration-dependent fashion with an increase of 32% above control at $1 \mu M$. This observation is consistent with previous reports showing that VIP induces release of a variety of mucus markers with similar potency (maximal/near maximal effects at $\sim 1 \mu M$) in a number of different *in vitro* airway preparations: ferret trachea (Peatfield *et al.*, 1983; Gashi *et al.*, 1986), cat isolated tracheal submucosal glands (Shimura *et al.*, 1988), rat trachea (Wagner *et al.*, 1998) and human nasal mucosal explants (Baraniuk *et al.*, 1990; Mullol *et al.*, 1992). VIP also stimulated rhinorrhea *in vivo* in human volunteers (Chatelain *et al.*, 1995). With secretory activity apparent at $\sim 1 \mu M$, it has to be concluded that VIP is not a potent secretagogue. However, it would appear from the present study that the activity of VIP is related primarily to neuroregulation of airway secretion rather than to induction of secretion. The VIP-induced increase in mucus secretion found herein was inhibited by a VIP-Ab. VIP-Ab has previously been shown to inhibit the potentiation by VIP of methacholine-elicited [^3H]-glycoconjugate release by cat isolated submucosal glands (Shimura *et al.*, 1988), and to inhibit smooth muscle relaxation induced by i-NANC electrical stimulation in cat and guinea-pig trachea, a response mediated by VIP (Li & Rand, 1991). Taken together, airway responses to exogenous and endogenous VIP are effectively suppressed by VIP-Ab, making it a useful tool for investigating the biological role of endogenous VIP. α -Chymotrypsin is a peptidase which has also been used to investigate the effect of endogenous VIP. For example, α -chymotrypsin greatly reduced the relaxant response to exogenous VIP and to NANC electrical stimulation in guinea-pig tracheal smooth muscle (Ellis & Farmer, 1989a; Tucker *et al.*, 1990; Li & Rand, 1991), which gave support to the notion that VIP is a neurotransmitter of the i-NANC nervous system in guinea-pig airways. However, in the present study, we found that α -chymotrypsin itself caused an increase in $^{35}SO_4$ output which precluded its use in characterizing the role of exogenous VIP on secretion, and of endogenous VIP on neurogenic secretion. In the present study, a putative VIP₁ antagonist, [D-p-Cl-Phe⁶,Leu¹⁷]-VIP, did not alter the magnitude of cholinergic neural secretion nor did it diminish exogenous VIP-induced mucus secretion. This antagonist has variable effects depending upon species and preparation (Ellis & Farmer, 1989b; Aizawa *et al.*, 1994; Shvilkin *et al.*, 1994), but is not suitable for

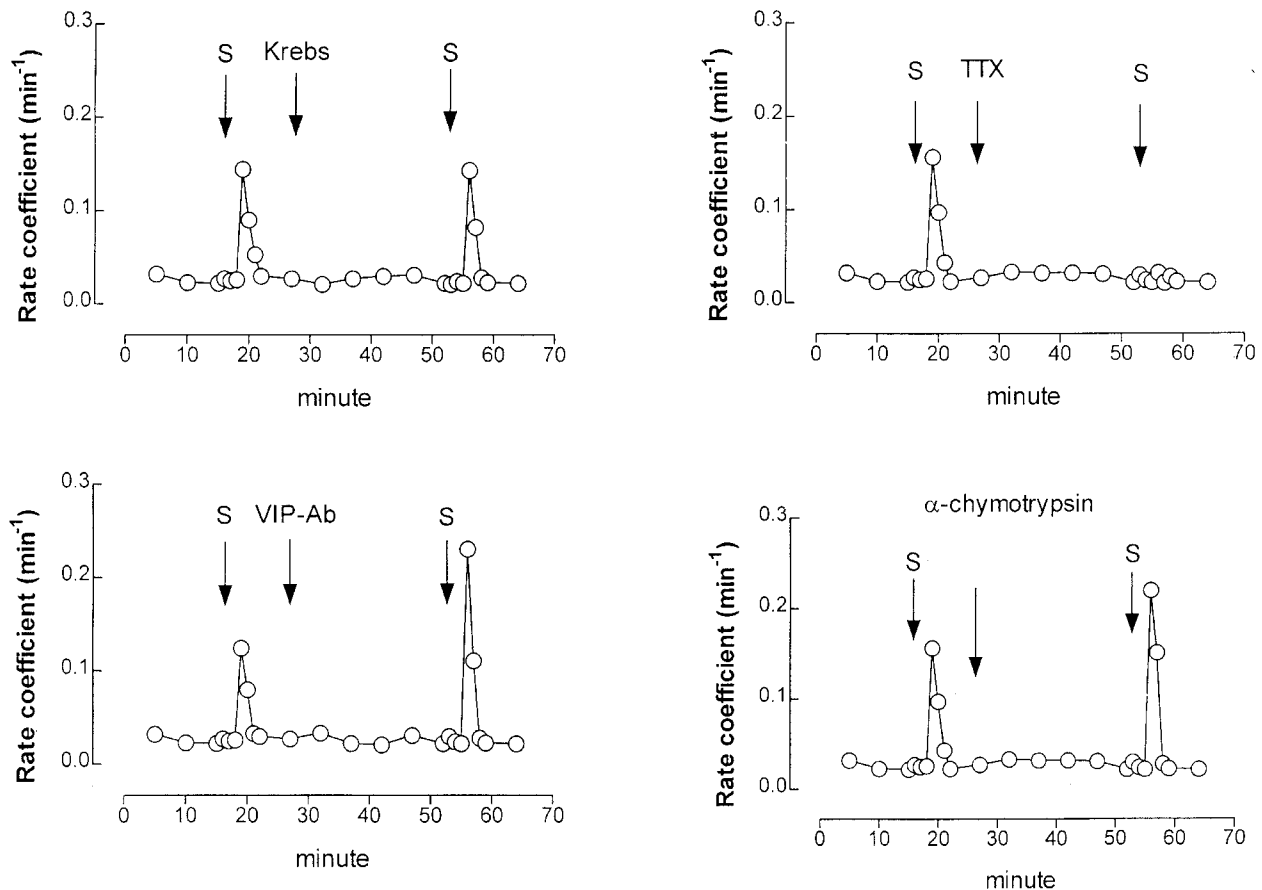


Figure 8 Effect of 2.5 Hz stimulation (50 V, 0.5 msec, 5 min) on acetylcholine (ACh) release in ferret tracheal strips *in vitro*. Tissues were pre-incubated with [^{3} H]-choline chloride (a marker for ACh) and stimulated electrically (S). Each panel gives the rate of [^{3} H] overflow for an individual tracheal strip. VIP-Ab, antibody to vasocative intestinal peptide (1:500 dilution); TTX, tetrodotoxin (0.1 μ M); α -chymotrypsin (2 u ml $^{-1}$).

investigating the role of endogenous VIP in neurogenic mucus secretion in ferret trachea.

We found herein that exogenous VIP markedly inhibited cholinergic neural secretion at both 2.5 and 10 Hz, and that VIP-Ab potentiated cholinergic neural secretion (at 2.5 Hz). In contrast, exogenous VIP (1 μ M) and ACh combined had an additive effect on secretion, whilst VIP-Ab had no significant effect on ACh-induced secretion. These results suggest that VIP regulates prejunctional cholinergic neuro-transmission to mucus secretion in ferret trachea rather than affecting the post-junctional activity of ACh. There is evidence to suggest that VIP regulates cholinergic neurotransmission in guinea-pig and ferret tracheal smooth muscle prejunctionally, post-junctionally or ganglionically. For example, VIP inhibited both ganglionic and post-ganglionic cholinergic contractile responses in an isolated innervated guinea-pig tracheal preparation in response to vagal stimulation or electrical field stimulation (EFS) (Martin *et al.*, 1990). Similarly, VIP inhibited guinea-pig tracheal smooth muscle contraction evoked by EFS, and also decreased ACh-induced contraction (Shigyo *et al.*, 1997). Depending upon concentration, VIP may augment (at 1 pM–1 nM) or suppress (at 10 nM–0.2 μ M) cholinergic neurotransmission in ferret tracheal smooth muscle (Sekizawa *et al.*, 1988). VIP also has variable effects on secretion, depending upon stimulus. VIP inhibited maintained methacholine-stimulated mucous gland cell liquid secretion *in vitro* in ferret trachea, but potentiated maintained phenylephr-

ine-stimulated serous gland cell liquid secretion (Webber & Widdicombe, 1987). In contrast, VIP augmented methacholine-induced [^{3} H]-glycoconjugate secretion from isolated cat tracheal submucosal glands (Shimura *et al.*, 1988). Thus, the conflicting data of the latter two studies, combined with our present data of no inhibitory or potentiating effect for VIP on ACh-induced secretion, indicate that the effect of VIP on cholinomimetic-induced secretion is complex and varies with animal species, study design, and degree of isolation of the glands. It is possible that isolation of the cat gland from the epithelium removes an inhibitory influence (Sasaki *et al.*, 1989). However, in the present study, epithelial scraping did not alter the secretory response to cholinergic nerve stimulation.

In both our present study and the latter study using the isolated cat gland, cyclooxygenase inhibition did not affect stimulated mucus output, which suggests that endogenous prostaglandins do not regulate secretion. Interestingly, depending upon the prostaglandin, exogenous prostaglandins can increase, decrease or have no effect on different parameters of secretion in ferret trachea *in vitro* (Deffebach *et al.*, 1990). Thus, the data of the latter study combined with the lack of effect of cyclooxygenase discussed above indicate either that prostaglandins are not generated endogenously under the above experimental conditions, or that the opposing effects of different generated prostaglandins leads to an overall lack of secretory response.

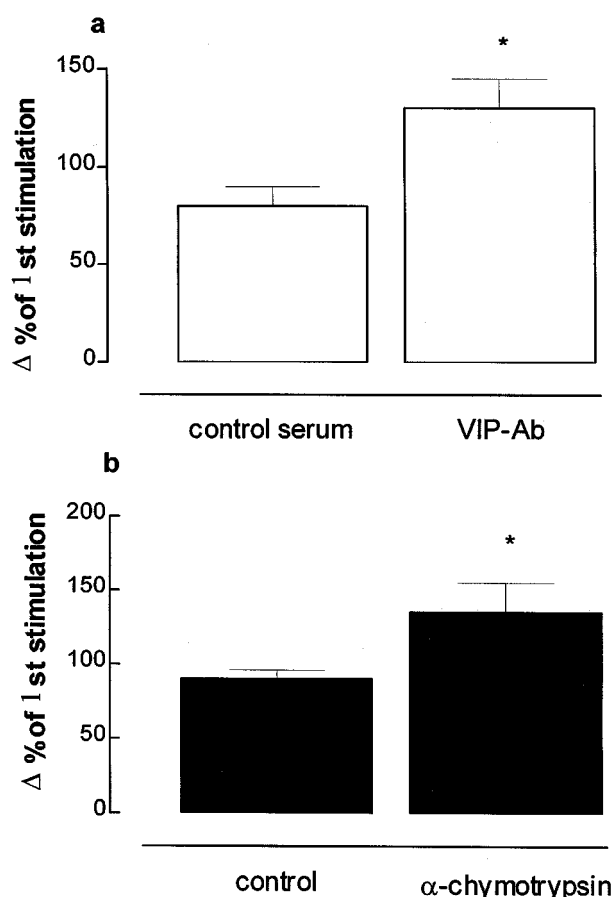


Figure 9 Effect of endogenous vasoactive intestinal peptide (VIP) on acetylcholine (ACh) release in ferret tracheal strips *in vitro*. A VIP antibody (VIP-Ab, 1:500 dilution; a) or α -chymotrypsin (2 μM ; b) were used to exclude VIP. Tissues were pre-incubated with $[^3\text{H}]$ -choline chloride (a marker for ACh) and stimulated electrically at 2.5 Hz, 50 V, 5 msec for 5 min. Data are mean per cent change in rate of $[^3\text{H}]$ overflow at a second stimulation compared with the first stimulation for five animals per group; vertical bars are one s.e.mean. * $P < 0.05$ compared with control group.

We found herein that exogenous VIP significantly suppressed tachykininergic neural secretion at 10 Hz stimulation and VIP-Ab markedly potentiated tachykininergic neural secretion at 5 Hz stimulation. Neither VIP nor VIP-Ab altered the secretory response to exogenous SP. These results suggest that VIP modulates the release of tachykinins *via* a prejunctional mechanism. A similar conclusion has been reached for VIP-inhibition of tachykininergic contractility of guinea-pig smooth muscle (Stretton *et al.*, 1991). VIP also inhibited SP-induced ACh release in rabbit tracheal smooth muscle strips (Giuseppe *et al.*, 1995).

In the presence of atropine, propranolol, phentolamine and CP-99,994 (i.e. NANC, non-tachykininergic), 10 Hz stimulation evoked a significant increase in mucus secretion of 25% above control, which was abolished by VIP-Ab indicating that VIP was the mediator of the response. It is interesting that the magnitude of the response was equivalent to that induced by exogenous VIP (1 μM). The concentration of VIP in the tissues, after release from nerves, is unknown, although local concentrations may be high. However, whatever the concentration near the secretory cells, the small increases in secretion elicited by either exogenous or endogenous VIP do not indicate that VIP is an important airway secretagogue. Rather, neuronal VIP elicits two responses: one is a small stimulatory

effect upon mucus secretion, but this is offset by its effect in curbing prejunctional cholinergic and tachykininergic neural secretion.

Incubation with $[^3\text{H}]$ -choline and subsequent overflow of $[^3\text{H}]$ can substitute for bioassay methods in the measurement of ACh release from guinea-pig ileum (Alberts *et al.*, 1982) and has been applied to a variety of airway smooth muscle preparations (Wessler *et al.*, 1990, 1991; Ward *et al.*, 1993; Patel *et al.*, 1995). In the present study, tetrodotoxin virtually abolished $[^3\text{H}]$ overflow evoked by electrical stimulation of ferret tracheal smooth muscle strips which supports the concept that $[^3\text{H}]$ overflow in our ACh release preparation is of neural origin. We found that exogenous VIP significantly inhibited $[^3\text{H}]$ overflow, whereas VIP-Ab and α -chymotrypsin augmented $[^3\text{H}]$ overflow. These data support the contention that endogenous VIP regulates the magnitude of neurogenic tracheal mucus secretion in ferret *via* inhibition of ACh release.

In this study, we found that the BK_{Ca} channel inhibitor, iberiotoxin, but not the SK_{Ca} channel inhibitor, apamin, or the K_{ATP} channel inhibitor, glibenclamide, reversed the VIP-inhibition of cholinergic neural secretion. Similarly, iberiotoxin reversed the suppression of ACh release by exogenous VIP. Reversal in both instances was unlikely to be due to any possible potentiating effect of iberiotoxin on neurotransmission because we have shown previously that iberiotoxin has no significant effect on neurogenic mucus output (Ramnarine *et al.*, 1998), and herein did not affect stimulated $[^3\text{H}]$ overflow. These data suggest that VIP-inhibition of cholinergic neurotransmission is mediated *via* opening of BK_{Ca} channels. We have found previously that selective opioid OP_1 and OP_3 receptor agonists inhibit neurogenic mucus secretion in ferret trachea at a prejunctional site *via* opening of BK_{Ca} channels (Ramnarine *et al.*, 1998).

VIP inhibited glycoconjugate secretion and lysozyme release from normal human tracheal explants. In contrast, in explants with 'bronchitic' changes, VIP did not inhibit baseline or methacholine-stimulated glycoconjugate release, or mucous or serous cell discharge (Cole *et al.*, 1981). In severe asthma, there is evidence of an imbalance in NANC nerves within the airways; specifically a deficiency of VIP- and an excess of SP-immunoreactive nerves (Ollerenshaw *et al.*, 1989, 1991), but not in symptomatic mild to moderate asthmatics (Howarth *et al.*, 1995). In studies where plasma levels of VIP and SP were correlated with airway function, it was concluded that an over-excitation of excitatory-NANC and a deficiency in i-NANC activity were closely related to asthma attacks and bronchial hyperresponsiveness (Liu *et al.*, 1993a,b). Rapid degradation of VIP by a variety of enzymes in asthmatic airways, for example mast cell tryptase (Caughey *et al.*, 1988), and increased plasma levels and affinity of catalytic anti-VIP antibodies in mild to moderate asthmatics (Paul *et al.*, 1995) might contribute to the deficiency in VIP. This might be one explanation for the ineffectiveness of inhaled VIP as a bronchodilator in asthmatics (Barnes & Dixon, 1984).

In conclusion, in ferret trachea *in vitro*, endogenous VIP regulates cholinergic and tachykininergic neural secretion at a prejunctional site *via* opening of BK_{Ca} channels. In the context of mucus secretion, we propose that VIP joins NO as a neurotransmitter of i-NANC neural pathways in ferret trachea.

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Effect of acute and long-term treatment with 17- β -estradiol on the vasomotor responses in the rat aorta

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1 This study sought to evaluate whether the effects of acute and long-term treatment with 17- β -estradiol on the vasomotor responses in rat aortic rings are mediated through the same mechanism.

2 Ovariectomized rats were treated daily with either 17- β -estradiol-3-benzoate (100 μ g kg⁻¹) or vehicle for 1 week.

3 The effect of long-term 17- β -estradiol treatment on the responses to cumulative doses of phenylephrine, 5-HT, calcium, potassium and 17- β -estradiol was determined in aortic rings. In the same rings, the effect of acute exposure to 17- β -estradiol (5 and 10 μ M) on the dose response curves for phenylephrine, 5-HT, calcium, potassium and acetylcholine were estimated. The measurements were made in rings with and without intact endothelium. The tone-related basal release of nitric oxide (NO) was measured in rings with intact endothelium.

4 Long-term 17- β -estradiol treatment reduced the maximum developed contraction to all contracting agents studied. This effect was abolished in endothelium denuded vessels. Acute 17- β -estradiol treatment also reduced maximal contraction. This effect, however, was independent of the endothelium.

5 Long-term 17- β -estradiol treatment significantly increased the ability of the rings to dilate in response to acetylcholine whereas acute exposure to 17- β -estradiol had no effect. The tone-related release of NO was significantly increased after long-term exposure to 17- β -estradiol.

6 In conclusion, this study indicate that the acute and long-term effects of 17- β -estradiol in the rat aorta are mediated through different mechanisms. The long-term effect is mediated through the endothelium most likely by increasing NO release. In contrast, the acute effect of 17- β -estradiol seems to be through an effect on the vascular smooth muscle cells.

Keywords: 17- β -Estradiol; acute; aorta; calcium; long-term; nitric oxide; rat; vasomotor response

Abbreviations: EC₅₀-value, concentration of agonist causing a half-maximal response; L-NAME, N^ω-nitro-L-arginine methyl ester; NO, nitric oxide

Introduction

Cardiovascular disease is more prevalent in men and post-menopausal women than in pre-menopausal women or post-menopausal women treated with oestrogen replacement therapy (Sullivan *et al.*, 1988; Barret-Connor & Bush, 1991; Stampfer *et al.*, 1991). Observations which suggest a cardioprotective effect of oestrogen. Although randomized clinical trials are yet to come, the protective action of oestrogen has been demonstrated in numerous animal studies (Kushwaha & Hazzard 1981; Adams *et al.*, 1990; Holm *et al.*, 1995; Sulistiyani *et al.*, 1995).

The cardioprotective effect of oestrogen has been explained by its ability to lower plasma cholesterol (Walsh *et al.*, 1991). However, oestrogen also has a direct effect on the arterial wall (Bush *et al.*, 1987; Holm *et al.*, 1997). Long-term treatment of cholesterol-fed monkeys with 17- β -estradiol has been shown to augment the endothelium dependent vasodilatory response to acetylcholine, an effect which also has been illustrated in humans (Williams *et al.*, 1990; Herrington *et al.*, 1994; Collins *et al.*, 1995). This effect has also been demonstrated after acute administration of 17- β -estradiol (<20 min) (Williams *et al.*, 1992). These findings are of particular interest as several studies have demonstrated a relationship between endothelium-dependent vasodilatation and the development of

atherosclerosis in both animals and humans (Ludmer *et al.*, 1986; Gilligan *et al.*, 1994; Reis *et al.*, 1994; Collins *et al.*, 1995). Considering these observations, it has been hypothesized that an impaired endothelium-dependent vasodilatation is a predictor for the development of atherosclerosis (Hayashi *et al.*, 1995). Accordingly, drugs which are able to prevent this reduction could potentially prevent the development of atherosclerosis (Hayashi *et al.*, 1997).

The function of the endothelium is easily studied in an isolated vessel preparation and this method has been used extensively to study the effects of acute and long-term treatment of 17- β -estradiol on the vasomotor responses of different vessels (Vargas *et al.*, 1989; Miller & Vanhoutte 1990; Jiang *et al.*, 1992; Paredes-Carbajal *et al.*, 1995). However, the effects of the two treatments have never been directly compared. This in spite of the fact that the effect seen after acute exposure to 17- β -estradiol *in vitro* have been used to explain the cardioprotective role of long-term 17- β -estradiol treatment (Collins *et al.*, 1993; Han *et al.*, 1995; Kitazawa *et al.*, 1997). One obvious difference between the two types of 17- β -estradiol treatments are the concentration needed to initiate the effects seen. While the plasma concentration needed to mediate the long-term effects of 17- β -estradiol is within the nanomolar range, it requires concentrations that are approximately 1000 times higher to initiate the acute effect seen *in vitro*. Some authors, however, have suggested that the dose question

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regarding the concentration is more of a technical nature as the actual concentration achieved in the tissue *in vivo* might be higher than the circulating plasma level (Collins *et al.*, 1993; Kitazawa *et al.*, 1997). This effect is explained partially by the lipophilic nature of 17- β -estradiol and the ability of vascular smooth muscle cells to synthesize 17- β -estradiol (Bayard *et al.*, 1995). A similar phenomenon is known to occur with the calcium channel blocker nifedipine (Opie & Singh, 1987) where a higher concentration *in vitro* than *in vivo* is necessary to elicit vascular effects. Furthermore, one of the limitations of the previous studies has been that only very few agents which affect the vasomotor responses have been studied. This questions whether the effect seen is specific for the individual agent or it is of a more general nature.

The current study therefore, examines whether the effects of acute and long-term 17- β -estradiol treatment on the vasomotor responses of the rat aorta by using a variety of agents with different mechanisms of action. Additionally, the interaction between acute and long-term 17- β -estradiol treatment is examined.

Methods

Animals

Sixty-two sexually mature, 8-weeks-old, female Sprague-Dawley rats (200–220 g) from Møllegaards Breeding Centre (Ll. Skedsved, Denmark) were individually housed under controlled conditions with food and water *ad libitum*. In all rats, bilateral ovariectomy was performed through an incision on the back under halothane anaesthesia. After surgery, buprenorphine (0.015 mg rat⁻¹, s.c.) was administered to reduce postsurgical pain. A 6–7 day recovery period was allowed prior to the initiation of treatment. The animals were divided into two experimental groups treated daily with either 17- β -estradiol-3-benzoate (100 μ g kg⁻¹, s.c.) or vehicle for seven days. This treatment was defined as long-term treatment. All the experiments were performed in accordance with the Danish legislation on use of animals in research.

Aortic ring preparation

On the day of experiment, the rats were euthanized by cervical dislocation. The thoracic aorta was immediately isolated and cut into four rings (3 mm long). Each ring was suspended in an organ bath (5 ml) between two parallel stainless steel hooks. One hook was fixed, while the other was connected to a force transducer for the measurement of isometric tension. The organ baths contained a Krebs solution at 37°C, bubbled with 95% O₂ and 5% CO₂. The composition of the Krebs solution was as follows (mM): NaCl 121.5; KCl 4.7; CaCl₂ 2.5; MgSO₄ 1.2; KH₂PO₄ 1.2, NaCHO₃ 25.0 and glucose 11.1. In Krebs solutions with high concentrations of potassium, the potassium was exchanged with sodium on a molar basis. The rings were initially stretched until a basal tension of 2.0 g and allowed to equilibrate for at least 45 min. At a basal tension of 2.0 g the rings were found to develop maximal active tension to stimulation with a contracting agent.

Bioassay

Each experiment began with the repeated contraction of the rings with 80 nM phenylephrine until the responses were

reproducible. The responses were defined as reproducible when the fluctuation around the mean value of three consecutive responses was less than $\pm 10\%$. Rings from the two long-term treatment groups were always studied in parallel. In the first part of the study, a set of rings with functionally intact endothelium were studied. The presence of functional endothelium was verified at the beginning of each experiment by the ability of acetylcholine (10 μ M) to relax a precontracted vessel (Furchgott & Zawadzki, 1980). Vessels that were able to relax less than 20% were excluded from the study. In the second part of the study rings denuded from the endothelium were investigated. The rings were excluded from this part of the study if they were able to relax to acetylcholine. Except for the acetylcholine dose-response curve and the basal release of nitric oxide (NO) which for obvious reasons was not determined in the endothelium denuded arteries the following dose response curves were determined in both parts of the study. Each ring was exposed to only one contracting or dilating agent.

The influence of 17- β -estradiol on the dose response curves for phenylephrine, 5-HT and potassium

The effect of long-term 17- β -estradiol treatment on cumulative dose response curves for either phenylephrine, 5-HT or potassium was determined in the concentration range of 10⁻⁹–10⁻⁵ M, 10⁻⁸–10⁻³ M and 5–126 mM, respectively. Following this determination, the rings were washed in Krebs solution and allowed to relax. The effect of acute 17- β -estradiol treatment on the dose response curves was then evaluated by adding 5 μ M of 17- β -estradiol to the bathing solution 30 min prior to obtaining a second series of dose response curves. This procedure was repeated with 10 μ M 17- β -estradiol. Pilot studies had demonstrated that the dose response curves were reproducible over the time of the experiments.

The influence of 17- β -estradiol on the dose-response curve for calcium

The effect of 17- β -estradiol on the dose response curve for calcium was determined after removal of the extracellular calcium. Twenty minutes prior to the determination, the extracellular calcium was removed by adding a calcium-free Krebs solution containing 100 μ M EDTA to the organ bath. Complete removal of the extracellular calcium was tested by demonstrating that no contraction occurred in response to a calcium-free Krebs solution containing potassium (100 mM). A cumulative dose response curve for calcium between 1 μ M and 7.5 mM was determined. Although maximum contraction was not reached at 7.5 mM calcium, it was not possible to increase the concentration of calcium further since the calcium-sensitivity of the tissue was found to decrease after exposure to higher concentrations of calcium. The influence of acute exposure to 17- β -estradiol was investigated as described above.

The influence of 17- β -estradiol on the dose response curve for acetylcholine

A cumulative dose response curve for acetylcholine (10⁻⁸–10⁻⁵ M) was determined on rings precontracted with phenylephrine (10 μ M) in order to investigate the influence of long-term 17- β -estradiol treatment on endothelium dependent vasodilatation. The influence of acute 17- β -estradiol treatment was investigated as described above. A time control was included in these experiments.

Basal release of NO

The effect of long term 17- β -estradiol treatment on the basal release of NO was quantified by measuring the contraction developed to 100 μ M N $^{\omega}$ -nitro-L-arginine methyl ester (L-NAME) on precontracted rings. The rings were precontracted with phenylephrine to 30% of the contraction developed to 126 mM potassium.

The direct vasodilating effect of 17- β -estradiol

To determine if the vasodilating effect of 17- β -estradiol was affected by long-term 17- β -estradiol treatment, a cumulative dose response curve for 17- β -estradiol (10^{-8} – 10^{-3} M) was obtained in precontracted rings. The rings were precontracted with a dose of phenylephrine that was equivalent to 50% of the contraction developed to potassium (126 mM)

Evaluation of oestrogen exposure in vivo

To evaluate the *in vivo* exposure to 17- β -estradiol, blood samples from 17- β -estradiol-3-benzoate on vehicle treated animals ($n=6$) were taken and the plasma level of 17- β -estradiol was determined by Medi-Lab (Copenhagen, Denmark). Furthermore, the uteri from these animals were dissected free and weighed.

Data analysis

For each ring, the response to the highest concentration of the contracting agent was defined as the maximum contraction (100%), this also applies to the dose response curves for calcium. The EC₅₀-values were calculated by Excel (Microsoft®) by fitting an exponential curve to the data. The effects of the various agents on the vascular reactivity were compared by a two way analysis of variance. A *P*-value below 0.05 was taken as significant.

Drugs

Acetylcholine, L-phenylephrine, 5-HT, kreatininsulphate, N $^{\omega}$ -nitro-L-arginine methyl ester and water-soluble 17- β -estradiol (from Sigma Chemicals) was dissolved in isotonic glucose solution (278 mM). 17- β -estradiol-3-benzoate (Sigma) was suspended in saline and propylene glycol (Fluka) 1:1. All other chemicals used were obtained from Merck.

Results

Acute and long-term effects of 17- β -estradiol treatment on contraction in rings with intact endothelium

Long-term treatment with 17- β -estradiol significantly ($P=0.002$, $n=24$) decreased the maximal developed tension to all the contracting agents studied by 13–17%, as compared to the control (Figure 1). The sensitivity (calculated as the concentration of agonist causing a half-maximal response (EC₅₀-value)) to neither phenylephrine, 5-HT nor calcium was affected (Table 1, Figure 1). The sensitivity to potassium, however, was decreased by 20% as compared to the control group ($P=0.01$, $n=6$) (Table 1).

As in the case of long-term exposure to 17- β -estradiol, acute exposure to 17- β -estradiol also decreased the maximal developed tension to all contracting agents investigated compared to the control situation (Figure 2), although it

only reached statistical significance after exposure to 10 μ M. It was, however, not possible to estimate the maximal contraction developed to calcium after acute exposure to 17- β -estradiol (see Methods and Figure 2c). The sensitivity to both phenylephrine and 5-HT was reduced after acute exposure to 17- β -estradiol although it only reached statistical significance after exposure to 10 μ M in the case of phenylephrine. Acute exposure to 17- β -estradiol caused an almost parallel right-ward shift of the dose response curve for calcium (Figure 2c). Under the assumption that the same maximal contraction could be reached as in the control rings, the sensitivity to calcium in the presence of 17- β -estradiol was calculated. Under these circumstances the sensitivity to calcium was statistically significant and reduced both in the presence of 5 and 10 μ M 17- β -estradiol. The sensitivity to potassium was not affected by acute exposure to 17- β -estradiol.

There was no interaction between the effects of long-term and acute exposure to 17- β -estradiol on either the maximum contraction or the sensitivity i.e. the effect of acute exposure to 17- β -estradiol was not dependent on the long-term treatment.

Acute and long-term effects of 17- β -estradiol treatment on dilation

Long-term treatment with 17- β -estradiol significantly increased the maximum relaxation to acetylcholine by 10% ($P=0.038$; $n=9$) without affecting the sensitivity (Figure 3). Acute treatment with 17- β -estradiol, however, had no effect on either the maximum contraction or the sensitivity to acetylcholine (data not shown).

17- β -estradiol dose dependently dilated the rings with a maximum relaxation of 65–68% and an EC₅₀-value of 16–22 μ M. This effect was not affected by long-term 17- β -estradiol treatment (Figure 4).

Tone related NO-release

Long-term 17- β -estradiol treatment increased the basal release of NO measured as contraction to L-NAME from 40% in the control rings to 57% in the rings treated with 17- β -estradiol ($P=0.034$; $n=7$).

Effect of endothelium denudation on contraction after acute and long-term 17- β -estradiol treatment

In a separate set of rings, the endothelium was denuded. In these rings an effect of the long-term 17- β -estradiol treatment was observed on the maximal contraction to any of the contracting agent studied. ($P=0.28$, $n=26-32$, Figure 1). The long-term 17- β -estradiol treatment did not affect the EC₅₀-value to any of the contracting agents. (Table 2). However, independent of the long-term treatment with 17- β -estradiol the rings denuded of endothelium were 2–6 times more sensitive to phenylephrine and 5-HT than the rings with functional endothelium (compare Tables 1 and 2). The acute 17- β -estradiol treatment was independent of the presence of the endothelium (Figure 5). The same was true for the direct vasodilatory effect of 17- β -estradiol (Figure 4).

Level of in vivo exposure to 17- β -estradiol

The plasma levels of 17- β -estradiol in the samples taken from treated animals were ≈ 100 pg ml⁻¹ (0.4 nM) whereas the concentration in the vehicle treated animals were below the

detection level of 20 pg ml⁻¹. The normal plasma level of 17- β -estradiol in rats that have been reported in the literature varies from 20–360 pg ml⁻¹ for non-pregnant rats (Wellman *et al.*,

1996; Huang *et al.*, 1997). The physiological effect of the treatment was detected by an increase in the uterus weight from 133 $\pm$ 5 mg to 549 $\pm$ 26 mg ($P < 0.05$, $n = 6$).

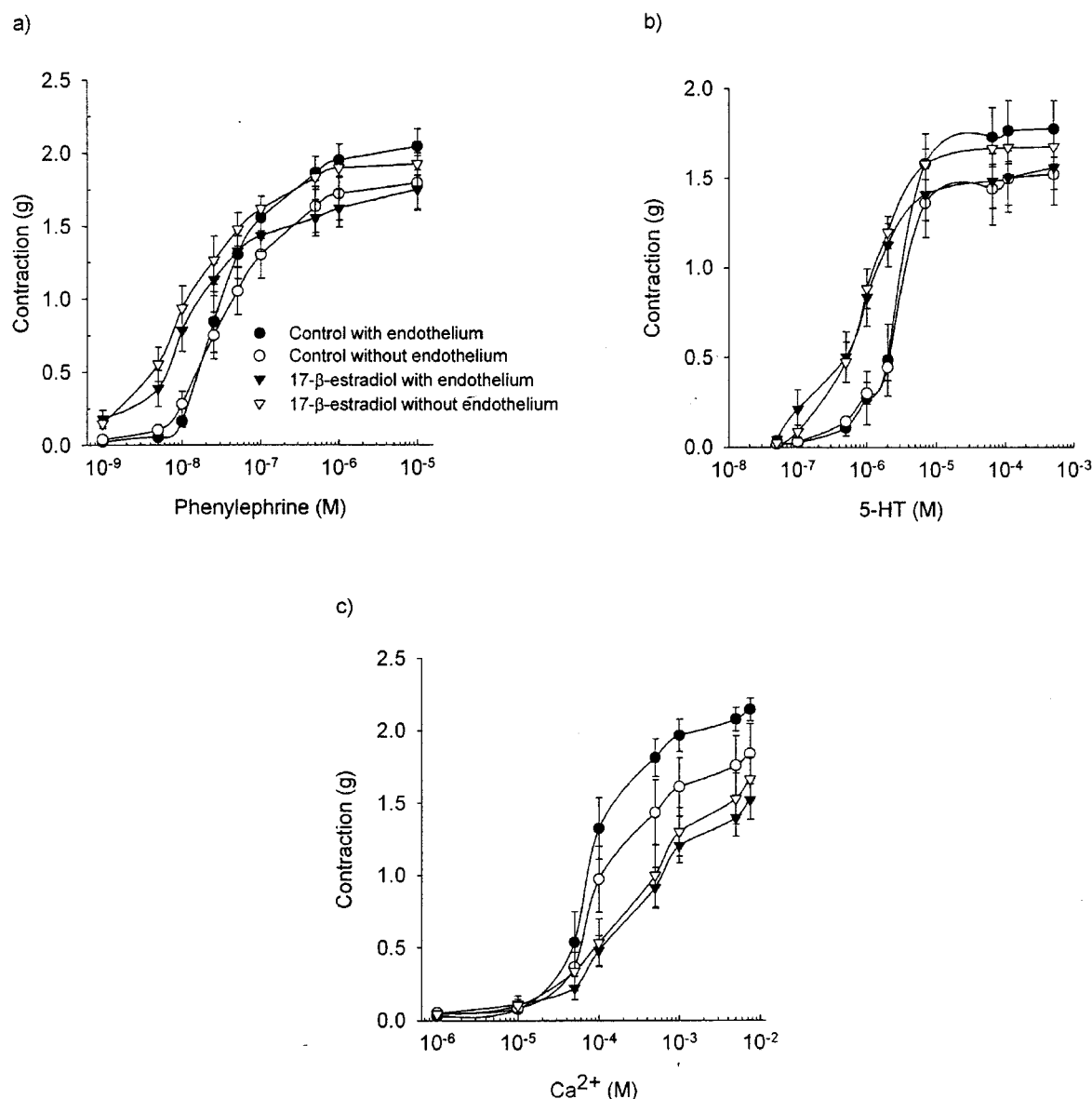


Figure 1 The effect of long-term exposure to 17- β -estradiol-3-benzoate (100 (μ g day⁻¹ kg⁻¹)) on the dose response curve for (a) phenylephrine (b) 5-HT and (c) calcium in endothelium intact and endothelium denuded rings.

Table 1 -Log (EC₅₀) to different contracting agents in rings with intact endothelium

Contracting agent	In vivo treatment	Control	Acute treatment	
			17- β -estradiol (5 μ M)	17- β -estradiol (10 μ M)
Phenylephrine	Vehicle	7.24 $\pm$ 0.08 (6)	7.09 $\pm$ 0.10 (6)	6.77 $\pm$ 0.25# (6)
	Estradiol	7.27 $\pm$ 0.07 (6)	7.09 $\pm$ 0.06 (6)	7.03 $\pm$ 0.10# (6)
5-HT	Vehicle	5.38 $\pm$ 0.08 (6)	5.09 $\pm$ 0.15# (6)	5.11 $\pm$ 0.14# (6)
	Estradiol	5.42 $\pm$ 0.05 (6)	5.15 $\pm$ 0.07# (6)	4.97 $\pm$ 0.08# (6)
Ca ²⁺	Vehicle	3.99 $\pm$ 0.09 (6)	3.21 $\pm$ 0.11# (6)	2.94 $\pm$ 0.11# (6)
	Estradiol	3.88 $\pm$ 0.09 (6)	3.03 $\pm$ 0.19# (6)	2.56 $\pm$ 0.25# (6)
Potassium	Vehicle	1.54 $\pm$ 0.02 (6)	1.52 $\pm$ 0.03 (6)	1.49 $\pm$ 0.04 (6)
	Estradiol	1.46 $\pm$ 0.01* (6)	1.48 $\pm$ 0.03 (6)	1.37 $\pm$ 0.05 (6)

(Mean $\pm$ s.e.mean) (n). *Indicates that there is a significant difference between the *in vivo* treatments. #Indicates that there is a significant difference compared to the control for the acute treatment.

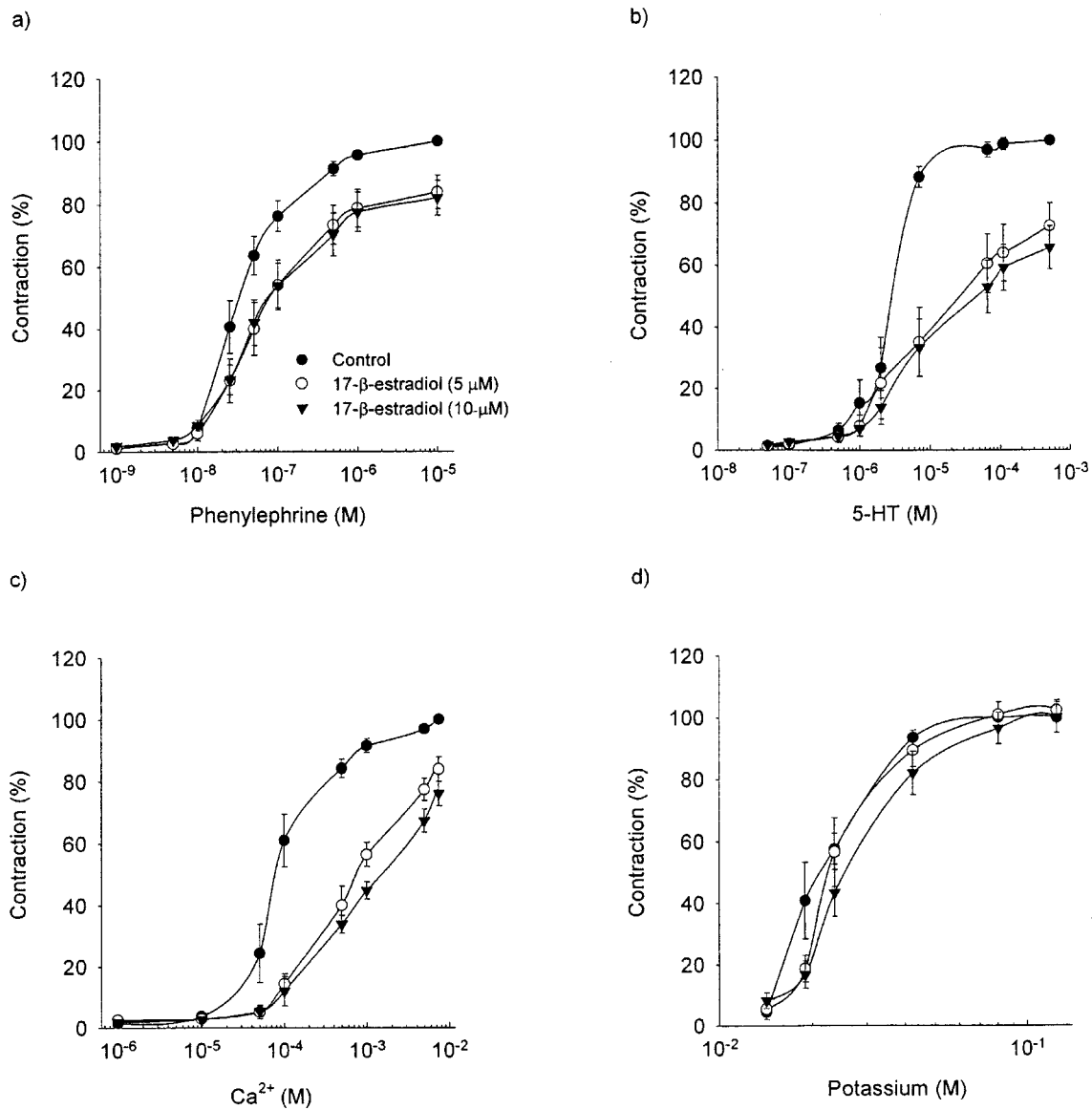


Figure 2 The effect of acute exposure to 17- β -estradiol on the dose response curve for (a) phenylephrine (b) 5-HT (c) calcium and (d) potassium in endothelium intact rings exposed to vehicle for seven days.

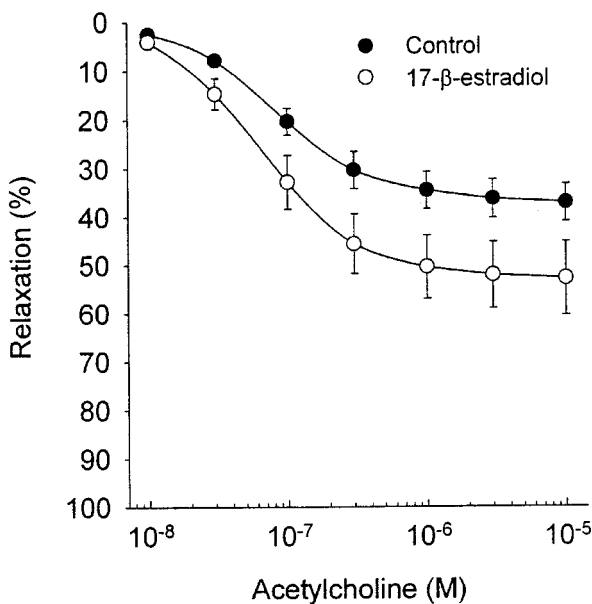


Figure 3 The long-term effect of 17- β -estradiol-3-benzoate (100 (μ g day) kg^{-1}) or vehicle on the dose response curve for acetylcholine.

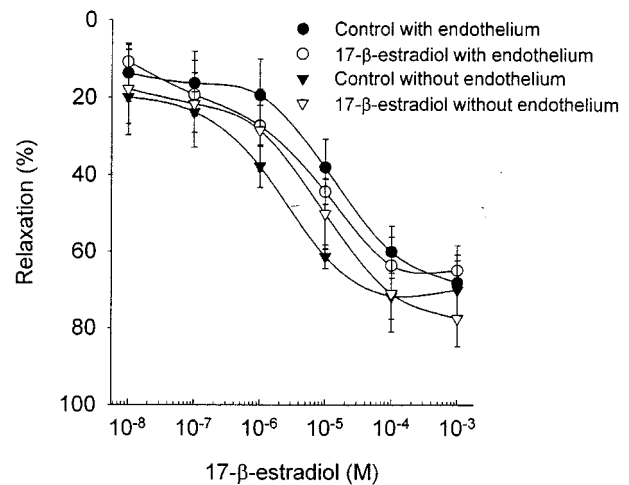


Figure 4 The effect of long-term exposure to 17- β -estradiol-3-benzoate (100 (μ g day) kg^{-1}) on the dose response curve 17- β -estradiol in endothelium intact and endothelium denuded rings.

Table 2 $-\text{Log}(\text{EC}_{50})$ to different contracting agents in rings with denuded endothelium

Contracting agent	In vivo treatment	Control	Acute treatment	
			17- β -estradiol (5 μM)	17- β -estradiol (10 μM)
Phenylephrine	Vehicle	7.98 $\pm$ 0.21 (9)	7.34 $\pm$ 0.05# (9)	7.06 $\pm$ 0.14# (9)
	Estradiol	8.09 $\pm$ 0.33 (8)	7.13 $\pm$ 0.15# (8)	7.24 $\pm$ 0.14# (8)
5-HT	Vehicle	6.00 $\pm$ 0.14 (9)	5.67 $\pm$ 0.03# (9)	5.18 $\pm$ 0.12# (9)
	Estradiol	5.80 $\pm$ 0.09 (7)	5.46 $\pm$ 0.11# (6)	5.50 $\pm$ 0.09# (7)
Ca^{2+}	Vehicle	3.79 $\pm$ 0.04 (9)	3.31 $\pm$ 0.24# (9)	3.05 $\pm$ 0.11# (9)
	Estradiol	3.58 $\pm$ 0.14 (7)	3.08 $\pm$ 0.20# (6)	2.78 $\pm$ 0.22# (7)

(Mean $\pm$ s.e.mean) (n). #Indicates that there is a significant difference compared to the control for the acute treatment.

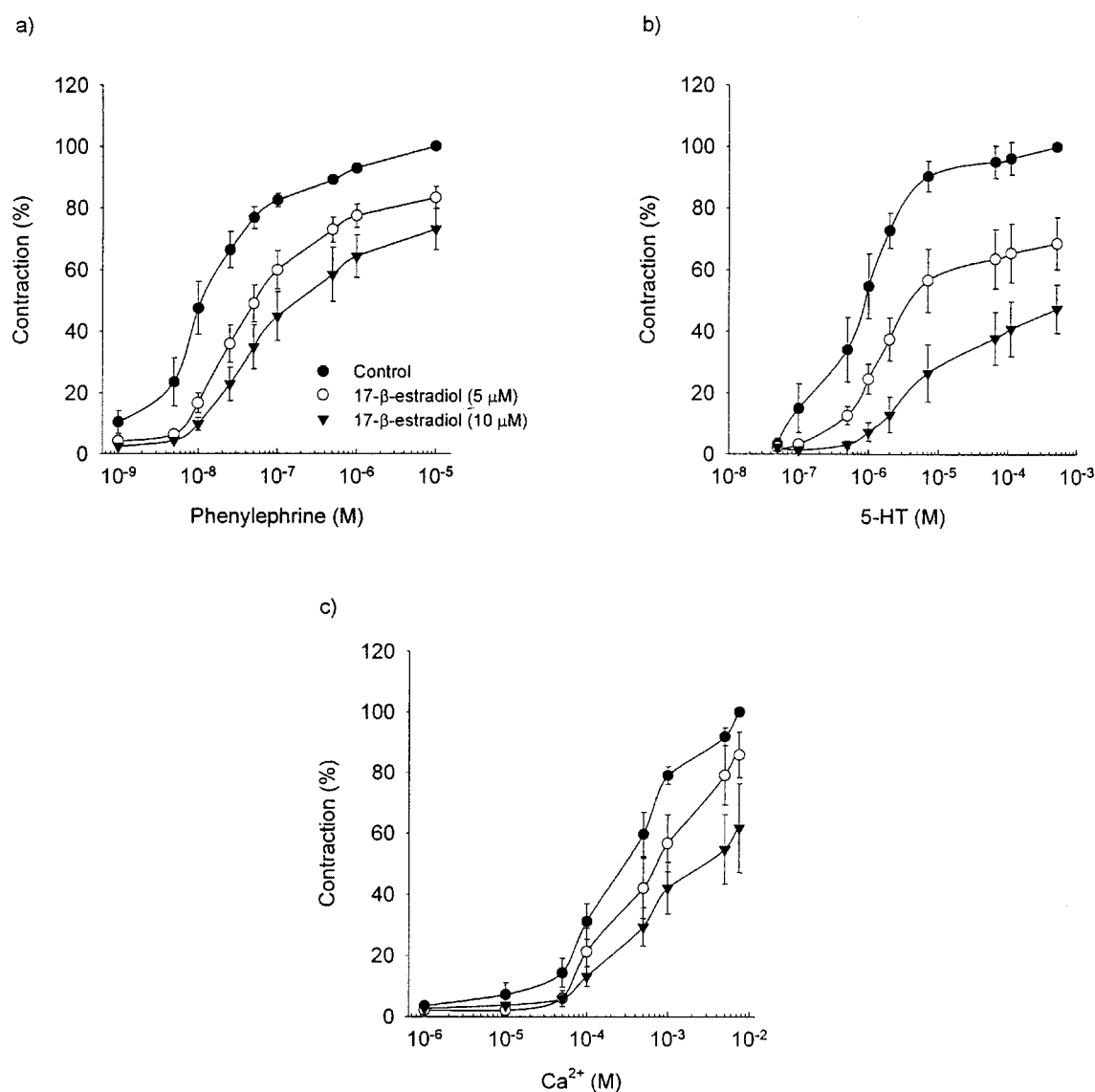


Figure 5 The effect of acute exposure to 17- β -estradiol on the dose response curve for (a) phenylephrine, (b) 5-HT and (c) calcium in endothelium denuded rings exposed to vehicle for seven days.

Discussion

To the best of our knowledge, this is the first study to demonstrate that the effects of acute and long-term 17- β -estradiol treatment on vasomotor responses may not be mediated through the same mechanisms. While the acute effect was independent of the presence of a functional endothelium, the effect of long-term treatment was

abolished after the endothelium was removed. In addition, this study demonstrates that the acute effect of 17- β -estradiol treatment is unaffected by long-term treatment.

Long-term effects of 17- β -estradiol

The maximum developed contraction to phenylephrine was significantly decreased after long-term 17- β -estradiol treat-

ment in this study, an effect which has previously been demonstrated both *in vivo* and *in vitro* (Ezimokhai *et al.*, 1994; Williams *et al.*, 1994; Paredes-Carbajal *et al.*, 1995). It has been proposed that long-term 17- β -estradiol treatment affects the α -adrenergic receptor density and/or affinity (Bento & Moraes, 1992; Collucci *et al.*, 1982; Gisclard *et al.*, 1988; Larson *et al.*, 1984). However, the effect observed in this study seems more general in nature since the maximum developed contraction to two different receptor ligands (α_1 -adrenoceptor and 5-HT) and the contraction mediated by passive depolarization of the vascular smooth muscle was affected. This is supported by the work of Honda *et al.* (1996) and Vedernikov *et al.* (1997) who also found that the maximum developed contraction to potassium was affected by long-term 17- β -estradiol treatment. Thus, the long-term effect of 17- β -estradiol cannot be explained solely by an effect on receptor density and/or receptor affinity.

Another mechanism suggested to explain the long-term effect of 17- β -estradiol on the vasomotor responses is *via* processes in the endothelium. The endothelium produces and releases various vasoactive substances that modulate the response of the underlying vascular smooth muscle cells (Furchgott & Zawadzki, 1980; Furchgott & Vanhoutte, 1989; Vita *et al.*, 1990). One of the mediators has been identified as NO that is released under agonist stimulation (Palmer *et al.*, 1987). There is, however, also evidence for a continuous release of NO both *in vivo* and *in vitro* (Griffith *et al.*, 1984; Rees *et al.*, 1989). Indeed, we found a dose dependent relaxation to acetylcholine in rings with intact endothelium and evidence for a basal release of NO. Both these effects were increased after long-term 17- β -estradiol treatment. It therefore seems possible that one of the mechanisms by which long-term 17- β -estradiol treatment can depress the contraction is by increasing the release of NO from the endothelial cells.

To investigate the role of the endothelium in the long-term effect of 17- β -estradiol, we included a set of experiments in which the endothelium was removed. Removal of the endothelium abolished the effect of the long-term 17- β -estradiol treatment, indicating that the site of action of long-term 17- β -estradiol treatment is associated with the endothelium, a finding which has also been previously reported by others (Maddox *et al.*, 1987; Paredes-Carbajal *et al.*, 1995; Wellman *et al.*, 1996; Meyer *et al.*, 1997). These findings are supported by reports studying various effects of 17- β -estradiol on the endothelial NO synthases all showing that a treatment period of approximately 24 h is necessary for the effect to become apparent (Weiner *et al.*, 1994; Hayashi *et al.*, 1995; Hishikawa *et al.*, 1995; Magness *et al.*, 1997; Wingrove & Stevenson, 1997).

Our findings therefore, suggest that long-term 17- β -estradiol treatment affects the vasomotor responses through increasing the release of endothelium dependent NO. This process, in turn, decreases the ability of the aorta to contract to different contracting agents and increases the dilation to acetylcholine. The endothelium, however, also produces various other vasoactive substances which is affected by estrogen treatment e.g. prostaglandins and endothelin-1 (Myers *et al.*, 1996; Wingrove & Stevenson, 1997). However, it was not the scope of these paper to investigate a particular mechanism by which long-term 17- β -estradiol affects vascular reactivity but only to elucidate whether it is reasonable to assume that the mechanism by which acute and long-term treatment with 17- β -estradiol is identical.

Acute effects of 17- β -estradiol

In this study we demonstrated a direct dilatory effect of 17- β -estradiol, an effect which was observed within 5 min after the application of the compound to the vessel. Based on this finding and previous reports (Jiang *et al.*, 1991; Mügge *et al.*, 1993; Chester *et al.*, 1995) it seems unlikely that this effect is mediated through the classical nuclear/cytostolic oestrogen receptors, although both the classical oestrogen receptor and specific binding site for steroidal sex hormones in cell membranes have been described (Pietras & Sezego, 1979; Karas *et al.*, 1994; Losordo *et al.*, 1994). Also, to support this hypothesis various studies have shown that the acute effects of 17- β -estradiol was unaffected by inhibiting the protein synthesis (Wellman *et al.*, 1996; Kitazawa *et al.*, 1997). Furthermore, the effects have been demonstrated to be readily reversible, which is incompatible with an effect on gene activation (Kitazawa *et al.*, 1997) and the binding of various oestrogenic compounds to the nuclear oestrogen receptor do not reflect their ability to inhibit evoked contraction (Kitazawa *et al.*, 1997). On the other hand, the effects of the acute 17- β -estradiol exposure do not seem to be due to an unspecific action of 17- β -estradiol on the cell membrane since it does not affect cell membrane resting potential and 17- β -estradiol and 17- α -estradiol have the same physio-chemical properties but not the same inhibitory effect on contraction (Vargas *et al.*, 1989; Thomas *et al.*, 1995).

It has been hypothesized by several authors that 17- β -estradiol is a calcium-channel antagonist (Stice *et al.*, 1987; Jiang *et al.*, 1991; 1992; Collins *et al.*, 1993; Salas *et al.*, 1994). Our acute 17- β -estradiol treatment caused an almost parallel right-ward shift in the dose response curve for calcium under depolarization condition, supporting this hypothesis. Inhibition of calcium-influx could explain the endothelium independent inhibition of the contraction seen after acute 17- β -estradiol treatment in this study. Indeed, this is supported by the observations of Vargas *et al.* (1989) who found that the inhibitory effect of 17- β -estradiol on the dose response curve for phenylephrine was completely abolished under calcium-free conditions. Also, it has been reported that the inhibitory effect of 17- β -estradiol on the contractile response to U46619 was weaker under calcium-free conditions (Han *et al.*, 1995). Work by Han *et al.* (1995) has shown that 17- β -estradiol relaxes arteries by inhibiting calcium influx without affecting the calcium sensitivity of the contractile proteins or the resting tone of the arteries whereas patch-clamp studies have further revealed that 17- β -estradiol inhibits the voltage operated calcium-current (L-type) in vascular smooth muscle cells. Findings which are in harmony with the parallel shift of the calcium dose response curve under depolarization condition (Jiang *et al.*, 1992; Zhang *et al.*, 1994). All findings explain the parallel right-ward shift of the calcium-dose response curve after acute 17- β -estradiol treatment and the endothelium independent inhibition of the contraction to various agents seen after acute 17- β -estradiol treatment.

Even though the hypothesis of 17- β -estradiol being a calcium-antagonist seems to explain most of the effects of acute 17- β -estradiol treatment, it does not explain the direct vasodilatory effect of 17- β -estradiol. Indeed, the vasodilatory effect of 17- β -estradiol has been demonstrated to be independent of the presence of extracellular calcium (Babai *et al.*, 1995). However, neither does this effect seem to be mediated through an increase in NO release, since it was found to be endothelium independent. In contrast to the effect of long-term 17- β -estradiol treatment where most of the observed

effects seems to be explained by an increase in NO synthesis and release, this mechanism does not explain any of the effects seen after acute 17- β -estradiol treatment, since we did not observe any effect on the NO system after acute 17- β -estradiol treatment. Taken together, these results demonstrate that the effect of 17- β -estradiol on NO production is only seen after long-term treatment.

Another possible mechanism of action for 17- β -estradiol could be through inhibition of tyrosine kinases in the vascular smooth muscle cell. Tyrosine kinases play an important role in the development of contraction mediated by different agonists, an effect which is accomplished within minutes (Laniyonu *et al.*, 1994; Jinsi & Deth 1995; Toma *et al.*, 1995; Yousif *et al.*, 1997). Indeed, many of the so called phyto-oestrogens, compounds with oestrogenic activity isolated from plant material, are known tyrosine kinase inhibitors (e.g. genistein and tyrophostine). Also, both 17- β -estradiol and tyrosine kinase inhibitors has been described to modulate arterial contractility *in vivo* as well as *in vitro* (Williams *et al.*, 1994; Paredes-Carbajal *et al.*, 1995; Jinsi & Deth 1995; Sauro *et al.*,

1996) an effect which has been shown to be independent of extracellular calcium (Abebe & Agrawal, 1995). The direct vasodilating effect of 17- β -estradiol could therefore be mediated *via* an inhibition of the tyrosine kinases in the vascular smooth muscle cell, however, this theory remains speculative at the moment.

In conclusion, this study demonstrates that the acute and long-term effect of 17- β -estradiol in the rat aorta is probably mediated through different mechanisms. Furthermore, this study suggests that the long-term effect is mediated through the endothelium most likely by increasing NO release. The acute effect seems to be mediated through an effect on the calcium homeostasis in the vascular smooth muscle cells independent of the endothelium. Certainly other mechanisms can be involved in both effects.

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Differences in the actions of some blockers of the calcium-activated potassium permeability in mammalian red cells

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1 The actions of some inhibitors of the Ca^{2+} -activated K^+ permeability in mammalian red cells have been compared.

2 Block of the permeability was assessed from the reduction in the net loss of K^+ that followed the application of the Ca^{2+} ionophore A23187 ($2\ \mu\text{M}$) to rabbit red cells suspended at a haematocrit of 1% in a low potassium solution ($[\text{K}]_0$ 0.12–0.17 mM) at 37°C . Net movement of K^+ was measured using a K^+ -sensitive electrode placed in the suspension.

3 The concentrations ($\mu\text{M} \pm \text{s.d.}$) of the compounds tested causing 50% inhibition of K^+ loss were: quinine, 37 ± 3 ; cetiedil, 26 ± 1 ; the cetiedil congeners UCL 1269, UCL 1274 and UCL 1495, ~ 150 , 8.2 ± 0.1 , 0.92 ± 0.03 respectively; clotrimazole, 1.2 ± 0.1 ; nitrendipine, 3.6 ± 0.5 and charybdotoxin, 0.015 ± 0.002 .

4 The characteristics of the block suggested that compounds could be placed in two groups. For one set (quinine, cetiedil, and the UCL congeners), the concentration-inhibition curves were steeper (Hill coefficient, n_H , ≥ 2.7) than for the other (clotrimazole, nitrendipine, charybdotoxin) for which $n_H \approx 1$.

5 Compounds in the first set alone became less active on raising the concentration of K^+ in the external solution to 5.4 mM.

6 The rate of K^+ loss induced by A23187 slowed in the presence of high concentrations of cetiedil and its analogues, suggesting a use-dependent component to the inhibitory action. This was not seen with clotrimazole.

7 The blocking action of the cetiedil analogue UCL 1274 could not be overcome by an increase in external Ca^{2+} and its potency was unaltered when K^+ loss was induced by the application of Pb^{2+} ($10\ \mu\text{M}$) rather than by A23187.

8 These results, taken with the findings of others, suggest that agents that block the red cell Ca^{2+} -activated K^+ permeability can be placed in two groups with different mechanisms of action. The differences can be explained by supposing that clotrimazole and charybdotoxin act at the outer face of the channel whereas cetiedil and its congeners may block within it, either at or near the K^+ binding site that determines the flow of K^+ .

Keywords: Cetiedil; cetiedil analogues; clotrimazole; charybdotoxin; nitrendipine; red cells; calcium-activated potassium permeability; Gárdos channel; use dependence

Introduction

An increase in the cytosolic concentration of Ca^{2+} causes the erythrocytes of most mammalian species to become much more permeable to K^+ , as first shown by Gárdos (1958; see Sarkadi & Gárdos, 1985 for a review). This action is mediated by the opening of Ca^{2+} -activated K^+ channels ('Gárdos channels') that are voltage-insensitive and have a single channel conductance of $\sim 15\ \text{pS}$ (Grygorczyk & Schwarz, 1983; Christophersen, 1991; Leinders *et al.*, 1992a,b; Dunn, 1998). Since this lies between that of the small conductance (SK_{Ca}) and large conductance (BK_{Ca}) varieties of Ca^{2+} -activated K^+ channel, they are regarded as belonging to an intermediate conductance (IK_{Ca}) subtype (for reviews see Cook & Quast, 1990; Haylett & Jenkinson, 1990). The recent cloning and sequencing by Ishii *et al.* (1997) of a human intermediate conductance Ca^{2+} -activated K^+ channel with the properties of the Gárdos channel has shown it to be structurally related to, but distinct from, the SK_{Ca} subtype.

This erythrocyte Ca^{2+} -activated K^+ permeability ($\text{P}_{\text{K}(\text{Ca})}$) has been known for some years to be blocked by quinine and quinidine (Armando-Hardy *et al.*, 1975; Reichstein & Rothstein, 1981), carbocyanine dyes (Simons, 1979), cetiedil (Berkowitz & Orringer, 1982, 1984; Christophersen & Vestergaard-Bogind, 1985; Roxburgh *et al.*, 1996) and charybdotoxin (Wolff *et al.*, 1988; Brugnara *et al.*, 1993a) though not by apamin (Burgess *et al.*, 1981; for additional references see Schwarz & Passow, 1983; Sarkadi & Gárdos, 1985). More recently, nifedipine (Kaji, 1990), nitrendipine (Ellory *et al.*, 1992) and clotrimazole (Alvarez *et al.*, 1992; Brugnara *et al.*, 1993b) have also been shown to be potent inhibitors. The present paper is concerned with the characteristics of the blockade caused by these diverse substances which are also of interest because of their potential value in the treatment of sickle cell disease (Benjamin *et al.*, 1986; for reviews see Joiner, 1993; Bunn, 1997). Our findings suggest that quinine, cetiedil and three close analogues of cetiedil (UCL 1269, UCL 1274 and UCL 1495) act in a different way to clotrimazole, nitrendipine and charybdotoxin. Some of the results have been published in a preliminary form (Benton *et al.*, 1994).

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Methods

Preparation of red blood cells

Blood (2–5 ml) was withdrawn from the ear vein of adult New Zealand White rabbits and mixed with heparin (20 units ml^{-1} whole blood). It was stored at 4°C for up to 2 h until used. The blood was centrifuged (3 min at $1600 \times g$) and the supernatant and buffy coat aspirated and discarded. The packed cells were then resuspended in five volumes of a solution containing (in mM): NaCl 145, KCl 0.1, MgSO_4 1, EDTA 1, TRIS 10, inosine 10. The pH was adjusted to 7.4 by adding 1 M NaOH. This suspension was centrifuged and the pellet re-suspended twice. After the final centrifugation, the cells were stored as a pellet in this solution at 4°C for up to 3 days.

Construction and use of K^+ sensitive electrodes

The application of the Ca^{2+} -ionophore A23187 to a suspension of red cells at 37°C causes a net K^+ loss which is attributable to the opening of Ca^{2+} -activated K^+ channels. The amount of K^+ lost can be measured by means of a K^+ -sensitive electrode placed in the suspension. This provides a convenient indirect measure of the increase in K^+ permeability, and of the effect of drugs thereon (see e.g. Burgess *et al.*, 1981; Cook & Haylett, 1985). The K^+ -sensitive electrodes were prepared using a minor modification of the method described by Hill *et al.* (1978). Valinomycin (4 mg), sodium tetraphenylborate (1 mg) and high molecular weight polyvinyl chloride (PVC, 25 mg) were dissolved in 1 ml tetrahydrofuran and 75 μl dibutyl sebacate. This solution was stored at -20°C . Membranes were formed by applying 5 μl of the solution to filter paper discs inserted into the end of a short length (~ 2 cm) of PVC tubing (2.0 mm, i.d.). After the solvent had evaporated a further 5 μl was applied and the membranes were allowed to dry for at least 2 h. They could then be stored in a dessicator at 4°C for several weeks. When required for use the PVC tube carrying the membrane was filled with a solution containing NaCl (145 mM) and KCl (5.4 mM) prior to connection to a second closely fitting tube housing an Ag/AgCl bead. The reference electrode consisted of a PVC tube containing a similar bead and filled with the salt solution used to suspend the cells. The potential difference between the electrodes was measured by means of a high impedance amplifier (WPI F-223 A) and displayed on a potentiometric pen recorder (Kipp & Zonen BD 100). The performance of the electrode was checked before each experiment by applying standard concentrations of K^+ covering the range to be used. The recording chamber comprised a water jacketed glass bath containing 2 ml of the red cell suspension. The suspension was stirred by a small magnetic 'flea' driven by a rotating magnet placed below the chamber.

Measurement of K^+ loss induced by the calcium ionophore A23187

Most of the experiments were performed using a low K^+ bathing solution, for two main reasons. First, as is shown, cetidil and its congeners become more effective when the external K^+ is reduced so that it is then possible to use lower concentrations of the blocking agents, some of which were rather inactive and available in limited quantity. Second, small increases in the K^+ content of the external solution resulting from loss of K^+ from the cells are more easily detected by means of a K^+ -sensitive electrode when $[\text{K}]_0$ is initially low.

The standard low K^+ bathing solution contained (mM): NaCl 145, KCl 0.1, MgSO_4 1, CaCl_2 1, inosine 10, TRIS 10. The pH was adjusted to 7.4 by addition of 1 M NaOH. 2 ml of this solution were placed in the recording chamber and 20 μl of packed red cells were added to give a final haematocrit of just under 1%. Because of the K^+ content of the fluid adhering to the cells, their addition caused the concentration of K^+ to rise to 0.12–0.17 mM, as indicated by the signal from the K^+ sensitive electrode.

When the reading from the electrode had become steady, K^+ loss from the cells *via* the Ca^{2+} -activated K^+ permeability was initiated by the addition of a supramaximal concentration of A23187 (2 μM , added as 4 μl of a 1 mM solution in DMSO) to the cell suspension. This caused a rapid release of K^+ . Three minutes after the application of A23187, digitonin (100 μM) was added to produce cell lysis and so allow the total available K^+ to be estimated (Cook & Haylett, 1985). The magnitude of the K^+ loss initiated by A23187 could then be calculated as the increase in $[\text{K}]_0$ 3 min after addition of A23187, expressed as a percentage of the total increase after addition of digitonin. This is equivalent to the quantity of K^+ released by A23187 as a percentage of total K^+ content of the cells.

The inhibitory effects of $\text{P}_{\text{K}(\text{Ca})}$ -blocking drugs were tested by adding a small volume (usually $< 5 \mu\text{l}$) of a concentrated stock solution to the cell suspension for a preincubation period (usually 3 min) before applying A23187 to initiate K^+ loss *via* Ca^{2+} -activated K^+ -channels. The loss of K^+ in the presence of the drug was then compared with that in its absence, so that the inhibition caused by the drug could be expressed as a percentage. Much longer preincubation periods (up to 2 h) were explored in some experiments. In these instances, packed red cells (20 μl) were added to a glass vial containing 2 ml of the standard low K^+ solution containing the drug. The vial was gently shaken in a water bath at 37°C for the time required. Its contents were then transferred to the recording chamber prior to the application of A23187.

Because the K^+ content of the incubation fluid was continuously monitored, the rate at which the cells lost K^+ when treated with A23187 could also be determined. This was done by expressing the amount (ΔQ) of K^+ lost during successive 20 s periods as a fraction of the K^+ content (Q) of the cells midway in that period. Dividing this fraction by the time (Δt , normally 20 s) over which the loss occurred provided an estimate of the rate coefficient (k) for K^+ loss:

$$k = \frac{\Delta Q}{Q \cdot \Delta t}$$

Changes in this coefficient provided a second index of the action of drugs that block $\text{P}_{\text{K}(\text{Ca})}$. Both indices were used in the present work though most of the results presented are based on measurements of the amount of K^+ lost after a standard 3 min application of A23187.

Activation of $\text{P}_{\text{K}(\text{Ca})}$ by Pb^{2+}

The cells were prepared and handled as in the experiments with A23187. Lead II nitrate was added to the bath to give nominal Pb^{2+} concentration of 10 μM . The concentration of free Pb^{2+} is likely to have been lower because lead ions are extensively bound by red blood cell proteins (see e.g. Simons, 1993). Since the action of lead was slower than that of A23187, and had a greater latency, the K^+ loss was measured after 5 min. It was noticed that lengthy exposure to lead had a deleterious effect on the K^+ sensitive electrodes. This could be avoided by washing the bath and the electrodes with a solution containing 10 μM EDTA.

Preparation of buffered calcium solutions

Buffered calcium solutions were prepared in a solution containing (in mM) NaCl 145, KCl 0.1, MgSO_4 1, inosine 10, HEPES 10, EGTA 5, pH 7.4. The desired Ca^{2+} concentration was attained by adding the appropriate volume of 1 M CaCl_2 and re-adjusting pH to 7.4 with 1 M NaOH. Free Ca^{2+} concentrations were calculated using the program REACT (written by G.L. Smith and kindly provided by him) based on the affinity constants listed by Smith & Miller (1985).

Data analysis

Values are given as the mean $\pm$ s.e.mean except when the Hill equation was fitted to concentration-response data. This was done using a weighted least-squares minimization program (CVFIT) written by Professor D. Colquhoun (Department of Pharmacology, University College London). It provided estimates of the molar concentration (the IC_{50}) of the compounds that caused 50% inhibition of A23187-induced K^+ loss, and of the Hill coefficient (n_H), $\pm$ an approximate standard deviation (see Colquhoun *et al.*, 1974, for details). When comparing the effects of changes in external K^+ on the potency of the compounds, the same program was used to estimate the factors (equivalent to the equi-effective molar ratios) by which the concentration of the compound had to be altered to achieve the same inhibition under the new experimental conditions.

Drugs and reagents

Charybdotoxin, nitrendipine, high molecular weight polyvinyl chloride (PVC) and cetiedil (the ($\pm$)-2-hexahydro-1*H*-azepin-1-yl) ethyl ester of 2-cyclohexyl-2-(3-thienyl) ethanoic acid) were generous gifts from Dr P.N. Strong, Bayer, ICI, and Innothéra (Arceuil, France), respectively. The compounds UCL 1274, UCL 1269 and UCL 1495 were synthesized in the Department of Chemistry at University College London by standard methods to be described elsewhere. UCL 1269 ('hydroxy cetiedil') is the ($\pm$)-2-(hexahydro-1*H*-azepin-1-yl) ethyl ester of 2-cyclohexyl-2-(3-thienyl)-2-hydroxy ethanoic acid, UCL 1274 is the 2-(hexahydro-1*H*-azepin-1-yl) ethyl ester of 2,2,2-triphenylethanoic acid and UCL 1495 is the 2-[N-(5-ethyl-2-methyl-piperidino)] ethyl ester of 2,2,2-triphenylethanoic acid. Dibutyl sebacate, dimethyl sulphoxide (DMSO), inosine, valinomycin, clotrimazole, A23187 (calcimycin), EGTA (1,2-di(2-aminoethoxy)ethane-tetra-acetic acid), quinine and heparin were purchased from Sigma, TRIS (tris (hydroxymethyl)aminomethane) from Calbiochem and sodium tetraphenyl borate from Koch-Light. Standard salts were of Analar quality and were obtained from BDH, who also provided EDTA (ethylene diamine tetra-acetic acid), digitonin and tetrahydrofuran.

Results

Preliminary experiments showed that the blocking action of cetiedil and several of its congeners was strongly dependent on the time for which the red cells had been exposed to the compound. To study this, rabbit red cells were preincubated for different periods with a concentration of the compound that produced 50–80% inhibition of the $\text{P}_{(\text{K}(\text{Ca}))}$ -mediated increase in K^+ loss initiated by the Ca^{2+} ionophore A23187. It can be seen in Figure 1 that the action of cetiedil and hydroxy cetiedil (UCL 1269) developed only slowly so that a relatively

long preincubation was necessary in order to obtain a true measure of their effectiveness. In contrast, the actions of nitrendipine (Figure 1, lower right) and clotrimazole were complete within 60 s, the shortest preincubation period practicable with the technique used.

Though the factors that underlie the slow onset of action of the cetiedil series have not been studied in any detail, the onset was noted to be approximately exponential in time course, with a rate constant that increased with the activity of the compound. Table 1 lists the rate constants for cetiedil, UCL 1269 and UCL 1274 together with the concentrations causing half maximal inhibition (IC_{50}). Because the potency of these substances is strongly correlated with their lipophilicity (Benton *et al.*, 1994) a measure of lipophilicity (Σf) is also given. This was estimated from the hydrophobic fragmental constants (f) introduced by Nys & Rekker (1973) and refined by Rekker & de Kort (1979). It is clear that the most potent (and lipophilic) of the three compounds, UCL 1274, acts rapidly, at a rate such that a 3-min incubation period was adequate. This has also been seen in subsequent work with more active analogues of cetiedil and UCL 1274, including UCL 1495 (D.C.H. Benton, M. Malik, C.R. Ganellin and C.J. Roxburgh, unpublished observations).

The concentration-dependence of the blocking action

The next aim was to study the concentration dependence of the blocking action. Figure 2 presents inhibition-concentration curves for six inhibitors of the Ca^{2+} -activated K^+ -permeability of red cells. The curves are clearly steeper for four of the compounds (quinine, cetiedil, UCL 1274 and UCL 1495) than for clotrimazole and nitrendipine. Table 2 lists the parameters obtained on fitting the Hill equation to these data and to the results of similar experiments with charybdotoxin. The values for the Hill coefficient, n_H , suggest that the compounds can be placed in two categories. For one set (quinine, cetiedil, UCL 1274 and UCL 1495) the value of n_H is considerably greater than for the second (nitrendipine, clotrimazole and charybdotoxin).

Two limitations of these findings deserve mention. First, a satisfactory concentration-response relationship could not be obtained for the cetiedil analogue UCL 1269, probably because of its relative inactivity and slow onset of action. The prolonged incubation with high concentrations needed to establish the upper region of the curve is likely to have adversely affected the cells. Second, and more important, a puzzling and as yet unexplained feature of the present observations was that although individual experiments, and

Table 1 Values of the rate constants (k) obtained on fitting the expression $y = y_{\text{max}} (1 - e^{-kt})$ to the results of Figure 1

Compound	k (min^{-1} , $\pm$ approx s.d.)	IC_{50} (μM , $\pm$ approx s.d.)	Σf
UCL 1269	0.056 ± 0.012	~ 150	4.76
Cetiedil	0.154 ± 0.024	26 ± 1	5.71
UCL 1274	1.31 ± 0.17	8.2 ± 0.1	6.50

The concentration of each compound causing half maximal inhibition is also listed, together with an estimate of the compound's lipophilicity (the Σf value of Rekker & de Kort (1979); see text). Note that, because of the indirectness of the responses measured, k should be regarded only as a fitting constant and not as an estimate of the onset rate constant for the combination of the compounds with their site of action.

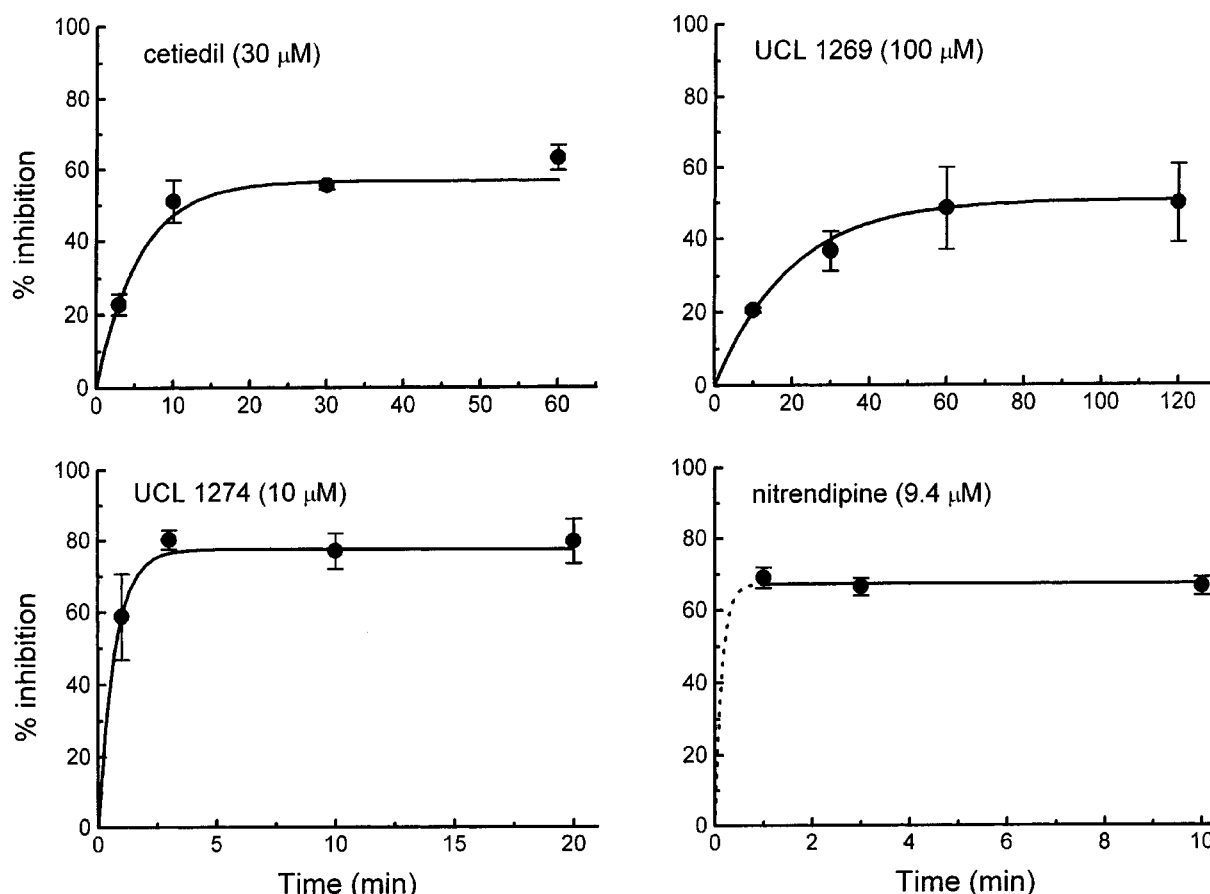


Figure 1 The dependence on preincubation time of the $\text{P}_{\text{K}(\text{Ca})}$ blocking action of nitrendipine, cetiedil and two analogues of cetiedil, UCL 1269 and UCL 1274. Each compound was incubated with a suspension of rabbit red blood cells for the periods indicated (abscissa) prior to adding the Ca^{2+} -ionophore A23187 to the suspension in order to initiate K^+ loss through Ca^{2+} -activated K^+ channels. The amount of K^+ lost was measured using a K^+ -sensitive electrode placed in the suspension (see also Figure 4a). The total K^+ loss 3 min after applying A23187 in the presence of the test compound was expressed as a percentage of the loss in its absence (ordinate). Each point is the mean of 3–6 observations and the error bars indicate the s.e.mean. For cetiedil, UCL 1269 and UCL 1274 the curves have been drawn by fitting the expression $y = y_{\text{max}} (1 - e^{-kt})$, where y is percentage inhibition, k is a rate constant and t is time (see also Table 1). The onset of the action of nitrendipine was too rapid to be resolved by present technique and the broken line has been constructed using a value of k of 7 min^{-1} , to indicate a lower limit.

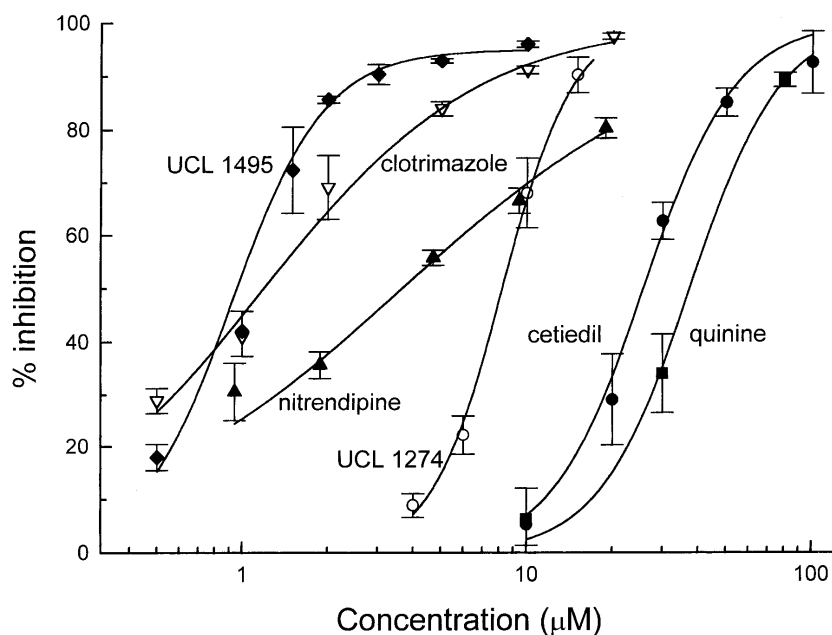


Figure 2 The concentration dependence of the inhibitory actions of clotrimazole, nitrendipine, quinine, cetiedil, UCL 1274 and UCL 1495 on A23187-induced K^+ loss from rabbit red cells bathed in a low K^+ solution. The preincubation times were 60 min for cetiedil, UCL 1274 and UCL 1495, and 3 min for quinine, clotrimazole and nitrendipine. Each point is the mean of 3–8 observations and the error bars show the s.e.means. The lines have been drawn according to the Hill equation, using the parameter values listed in Table 2.

sets of experiments on successive days, gave reproducible results, the IC_{50} values for the compounds were found to vary, by as much as 3 fold on occasion, when determined at longer intervals. The variation was seen even with cells from the same animal. This could reflect differences in the state of the cells which may in turn influence the complex equilibria known to exist between closed and open states of the Gárdos channel (see e.g. Leinders *et al.*, 1992a). Further work would be needed

Table 2 Hill slopes (n_H) and IC_{50} values for fits of the Hill equation to the data shown in Figure 2

Compound	n_H ($\pm$ approx s.d.)	IC_{50} ($\mu\text{M} \pm$ approx s.d.)
Quinine	2.77 ± 0.36	37 ± 3
Cetiedil	2.73 ± 0.32	26 ± 1
UCL 1274	3.55 ± 0.33	8.2 ± 0.1
UCL 1495	2.71 ± 0.14	0.92 ± 0.03
Nitrendipine	0.83 ± 0.11	3.6 ± 0.5
Clotrimazole	1.16 ± 0.04	1.2 ± 0.1
Charybdotoxin	0.72 ± 0.09	0.015 ± 0.002

Results with charybdotoxin have also been included. Except for UCL 1495, the maximum inhibitions did not differ significantly from 100% and the fits were constrained to this value. The maximum for UCL 1495 was constrained to 95%.

to identify the underlying cause. In view of this uncertainty, and bearing in mind that the measures of channel block are rather indirect, the IC_{50} values listed in Tables 1 and 2 clearly can not be regarded as equivalent to the dissociation equilibrium constants for the combination of the blocking compounds with their sites of action.

The influence of external K^+

A study of the K^+ -dependence of the blocking action confirmed a second difference between the two groups of compounds. The work of Armando-Hardy *et al.* (1975) and of Reichstein & Rothstein (1981) has shown that the inhibitory action of quinine on the Ca^{2+} -activated K^+ permeability of red cells is reduced when the concentration of K^+ in the external solution is increased. In contrast, the blocking action of nitrendipine is little affected by large changes in external K^+ (Ellory *et al.*, 1992). Figure 3 shows the consequences of raising $[\text{K}]_0$ from between 0.12 and 0.17 mM to 5.4 mM on the blocking action of cetiedil, UCL 1495, charybdotoxin, nitrendipine and clotrimazole. It is evident that cetiedil and UCL 1495 resemble quinine in becoming less effective when $[\text{K}]_0$ is increased. In contrast, the inhibitory actions of nitrendipine, charybdotoxin and clotrimazole are not significantly affected (see also Table 3).

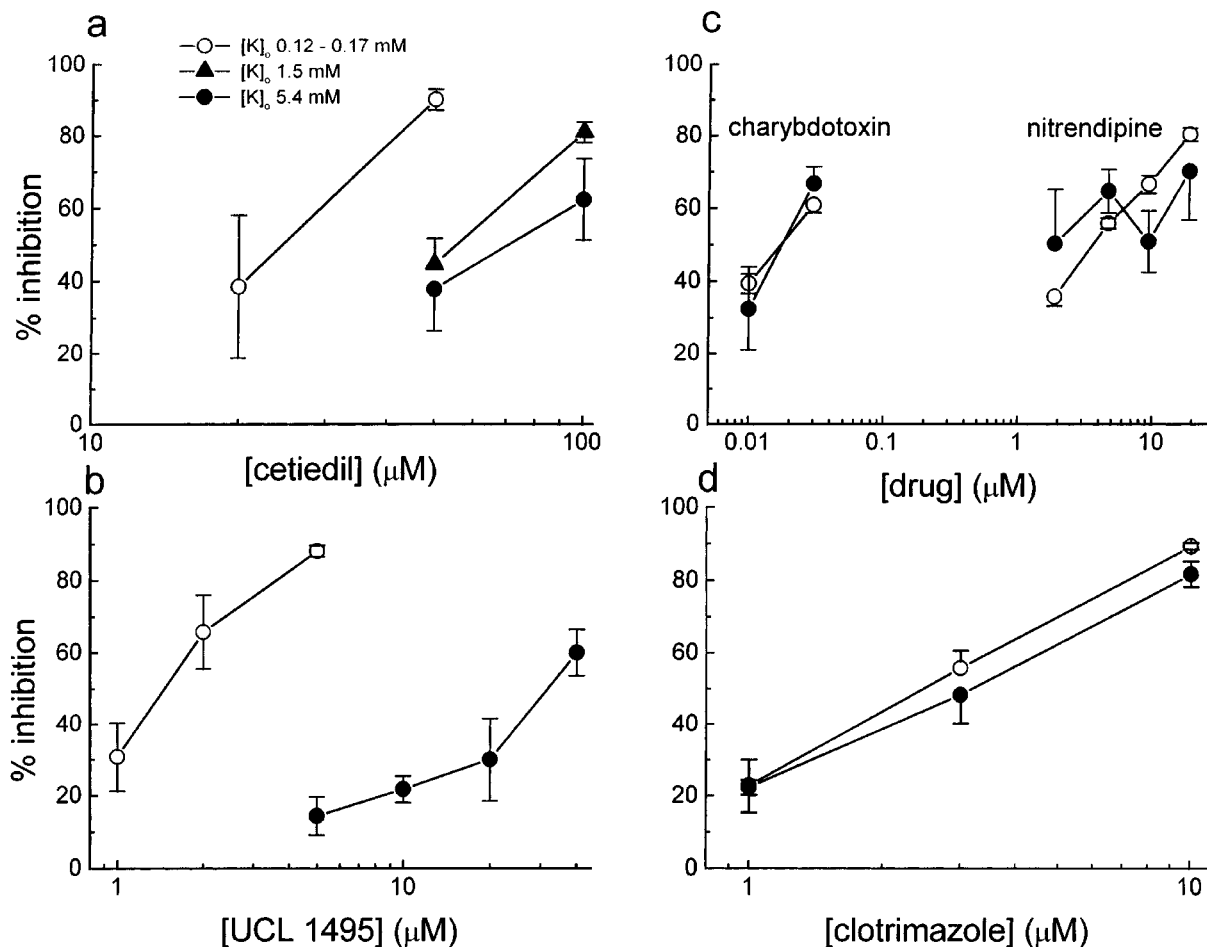


Figure 3 The effect of increasing the external concentration of K^+ on the blocking action of (a) cetiedil, (b) UCL 1495, (c) charybdotoxin and nitrendipine, (d) clotrimazole. Each point is the mean of 3–8 observations and the error bars show the s.e.mean. The cells were incubated with the blocking agents for 1 h before the application of A23187 to initiate K^+ loss, except for nitrendipine and clotrimazole which were applied 3 min beforehand.

Table 3 The factor by which the concentration of each of the compounds listed had to be increased in order to produce the same inhibition of $\text{P}_{\text{K}(\text{Ca})}$ when the concentration of K^+ in the external fluid was raised from 0.12–0.17 to 5.4 mM (see also Figure 3)

Compound	Factor ($\pm$ approx s.d.)
Cetiedil	3.12 ± 0.74 (cf. 2.37 ± 0.31 in 1.5 mM K^+)
UCL 1495	22 ± 5
Charybdotoxin	0.82 ± 0.44
Nitrendipine	0.92 ± 0.24
Clotrimazole	1.3 ± 0.21

Differences in the time course of A23187-stimulated K^+ loss in the presence of cetiedil and clotrimazole

Inspection of the time course of the K^+ loss induced by A23187 in the presence of the blocking agents suggested a third difference in the actions of the two groups of compounds identified above. Figure 4a shows sample records and the combined results from several experiments to compare cetiedil and clotrimazole have been plotted in Figure 4b. It can be seen that although clotrimazole at $3 \mu\text{M}$ greatly reduced the rate of loss of K^+ , this loss continued throughout the presence of the drug. Similar results were obtained with nitrendipine and charybdotoxin. In contrast, with a high concentration of cetiedil or its congeners (e.g., UCL 1274, UCL 1495), the rate of loss rapidly fell (see Figure 4a, ii) so that by the end of the 3-min application of A23187 in the presence of cetiedil ($50 \mu\text{M}$), it had almost ceased.

To quantitate this difference, the amount of K^+ leaving the cells in successive 20-s intervals was measured from the results shown in Figure 4b. A rate coefficient, k , for K^+ loss was then calculated as described in the Results section. The values obtained are shown in Figure 5a. The initial rate of loss is reduced almost equally (by $\sim 85\%$) by cetiedil and clotrimazole at concentrations of 50 and $3 \mu\text{M}$ respectively. However, with increasing time, the rate falls steeply in the presence of cetiedil (squares) but not clotrimazole (triangles). Figure 5b presents the ratios of the rate constants at 70 and 10 s. There is a clear difference in the patterns seen with increasing concentrations of clotrimazole and cetiedil.

An incidental observation was that the loss of K^+ caused by A23187 in the absence of blocking agents did not follow simple first order kinetics, as evidenced by the decline in the 'control' rate coefficients (Figure 5a, circles). This was not surprising because of the concomitant changes in membrane potential and concentration gradients that would be expected to occur: after 3 min exposure to A23187, the cells will have lost $\sim 70\%$ of their K^+ content and their volume will have fallen because of the accompanying loss of Cl^- and water. These changes can be expected to be much smaller in the presence of a large concentration of clotrimazole and accordingly the loss of K^+ is then closer to first order, as indicated by the relatively small decline with time in the rate coefficient (Figure 5a, triangles).

The finding that cetiedil and clotrimazole differ in their effects on the time course of A23187-induced K^+ loss has two implications. First, it suggests a difference in the mechanism of the blocking action. The inhibition caused by cetiedil but not clotrimazole appears to be use-dependent in the sense that it becomes greater in the continued presence of A23187. This would be expected were cetiedil to have some preferential affinity for open ion channels (see Discussion). Second, it is clear that the apparent magnitude of the block caused by the $\text{P}_{\text{K}(\text{Ca})}$ blockers will depend on the time at which the

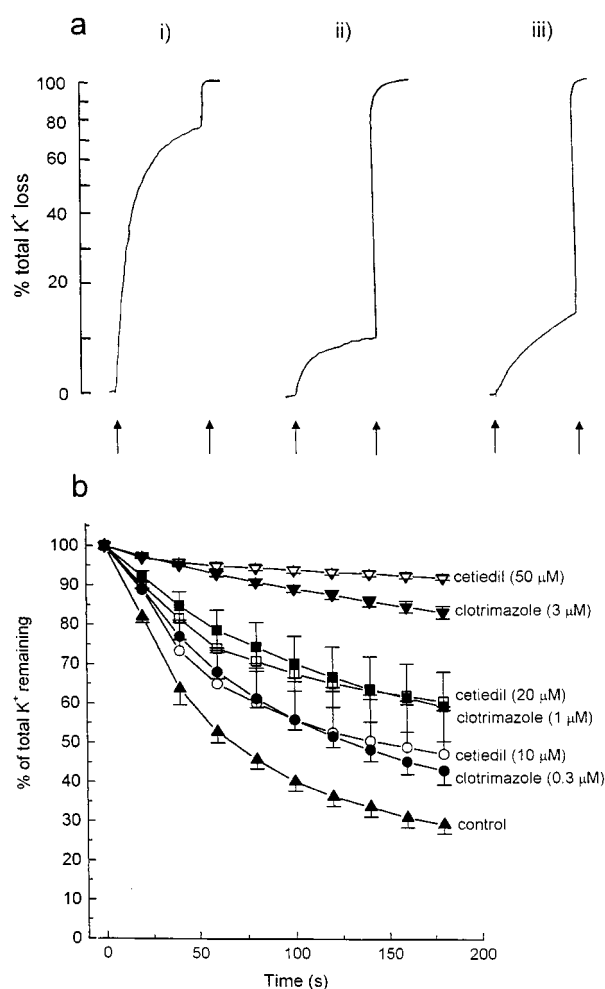


Figure 4 (a) Records showing the loss of K^+ from a suspension of rabbit erythrocytes exposed to A23187 ($2 \mu\text{M}$) either alone (i) or in the presence of either $50 \mu\text{M}$ cetiedil (ii) or $3 \mu\text{M}$ clotrimazole (iii). A23187 was applied at the first of each pair of arrows, followed ~ 3 min later (second arrow) by digitonin ($100 \mu\text{M}$) to cause lysis and so allow the total K^+ content of the cells to be determined. Cetiedil and clotrimazole were added 3 min prior to A23187. Because the absolute value of the initial and final K^+ concentration varied a little, the traces have been scaled to the same amplitude and accordingly the ordinate shows K^+ loss as a percentage of the total K^+ content, as measured following the addition of digitonin. Typically, the concentration of K^+ in the suspension rose from ~ 0.15 (before A23187) to 0.8 mM (after digitonin). The nonlinearity of the ordinate scale reflects mainly the logarithmic relationship between $[\text{K}^+]$ and the potential measured by the K^+ -sensitive electrode used to detect the net movement of K^+ between the erythrocytes and the solution in which they were suspended. (b) Averaged results. The values are the means of six observations for the control and three for the test compounds. Vertical bars indicate the s.e.mean.

measurement is made. Figure 6 shows concentration response curves constructed using the reduction in the rate coefficients measured at 10 and 170 s after A23187 had been added to the cell suspension. At 170 s the IC_{50} 's estimated from Hill plots were 16 ± 2 and $1.4 \pm 0.3 \mu\text{M}$ for cetiedil and clotrimazole respectively. When measured at 10 s the corresponding values were 20 ± 2 for cetiedil and $0.47 \pm 0.06 \mu\text{M}$ for clotrimazole. Thus whereas the IC_{50} for clotrimazole increased if the measurements were taken later in the response, that for cetiedil became a little lower if anything. An increase with time in the potency of cetiedil is consistent with use-dependent inhibition, as already discussed. The apparent decrease in the

effectiveness of clotrimazole is likely to arise because, as already noted, when the K^+ flux in the presence of the blocker is greatly reduced, the loss of K^+ approximates more closely to first order kinetics, in contrast to the loss from the control cells (see also Figure 5a).

It is also worth noting that the slopes of the concentration-inhibition curves constructed in this way were consistently greater for cetiedil than for clotrimazole, in keeping with the results presented in Figure 2 and Table 2. Thus the estimates of the Hill slope, n_H , were 1.0 ± 0.1 and 1.4 ± 0.4 clotrimazole at 10 s and 170 s respectively, as compared with 1.9 ± 0.2 and 2.2 ± 0.2 for cetiedil at the same times.

Additional experiments

A common feature of the work so far described was the use of the Ca^{2+} ionophore A23187 to activate the $\text{P}_{\text{K(Ca)}}$ mechanism.

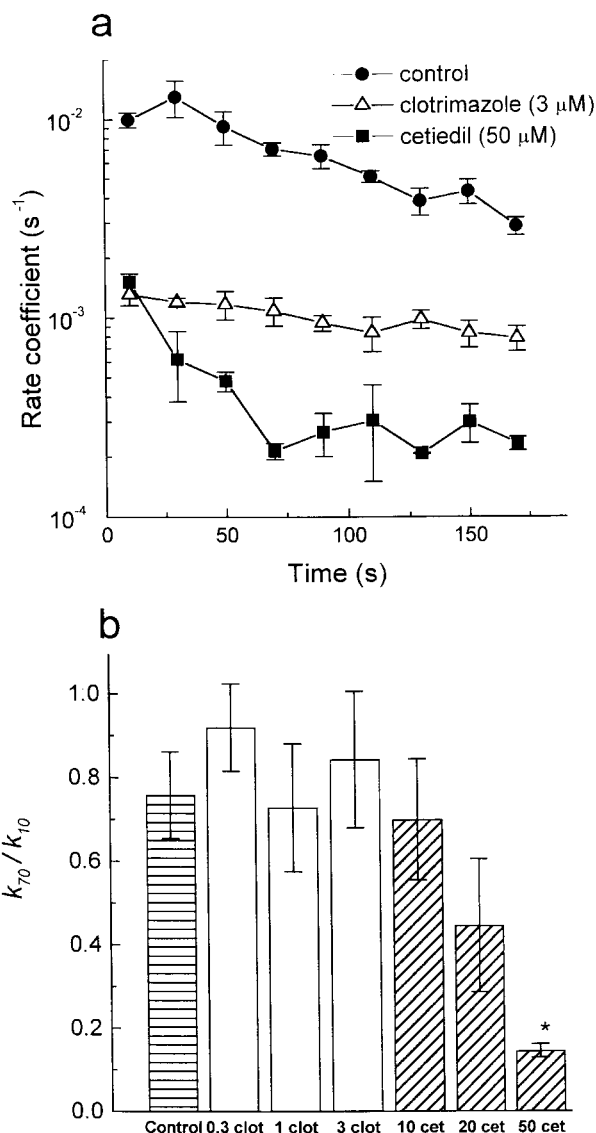


Figure 5 (a) The time course of changes in the rate coefficient (ordinate: note log scale) for A23187-induced K^+ loss from erythrocytes in the absence ($n=6$) or presence of either 50 μM cetiedil ($n=3$) or 3 μM clotrimazole ($n=3$). Abscissa: time after the introduction of A23187. (b) The ratios of the rate coefficients at 70 and 10 s after applying A23187. Results are shown for clotrimazole (clot, μM concentrations) and cetiedil (cet, μM concentrations). This ratio becomes smaller as the concentration of cetiedil is increased. * denotes significance at the 5% level as indicated by an unpaired *t*-test.

A possible if unlikely complication was that the blockers might have interfered with the action of the ionophore. To rule this out, Pb^{2+} rather than A23187 was employed in a few experiments. Pb^{2+} ions are known to enter erythrocytes *via* the anion exchanger (Simons, 1984) and to activate the Ca^{2+} -dependent K^+ channels by a direct effect not involving Ca^{2+} (Shields *et al.*, 1985). In keeping with this, the addition of Pb^{2+} to rabbit erythrocytes suspended in the standard low K^+ solution caused a loss of K^+ comparable to, though a little slower than, that seen with A23187. In six such experiments, the mean K^+ loss in response to a 5-min application of Pb^{2+} at 10 μM (a maximal concentration) was $53 \pm 1\%$, as compared with $58.4 \pm 5\%$ ($n=6$) with the standard application of A23187 (2 μM, also maximal). As figure 7a shows, the cetiedil congener UCL 1274 was as effective in blocking K^+ loss induced by Pb^{2+} as by A23187. The IC_{50} 's observed in this set of experiments were 5.4 ± 0.4 μM (with Pb^{2+}) and 5.3 ± 0.4 μM (A23187).

UCL 1274 was also used in a final set of experiments to determine whether its blocking action could be surmounted by an increase in Ca^{2+} concentration. A direct competitive interaction between the blocker and Ca^{2+} at its recognition site at the inner face of the K^+ channel seemed unlikely for several reasons. For example, an intracellular site of action would not be in keeping with the rapidity of the effect of UCL 1274 (see Figure 1). Also, cetiedil, to which UCL 1274 is chemically closely related, is much less active when applied to the inner as compared with the outer face of the membrane (Dunn, 1998, and unpublished observations). To test the surmountability of the block, the effect of UCL 1274 on A23187-induced K^+ loss was re-examined using external solutions containing different buffered concentrations of Ca^{2+} . The results are shown in Figure 7b and as expected are in keeping with noncompetitive rather than simple reversible competitive block. Fitting the Hill equation to the data provided an estimate of $69 \pm 3\%$ for the maximum K^+ loss in the absence of the blocker and an EC_{50} of 2.2 ± 0.4 μM for the action of Ca^{2+} , which is in keeping with previous values (see e.g. Simons, 1976). The Hill coefficient was 1.9 ± 0.3 which is also consistent with earlier work. In the presence of 6 μM UCL 1274, the maximum K^+ loss was reduced to $39 \pm 2\%$.

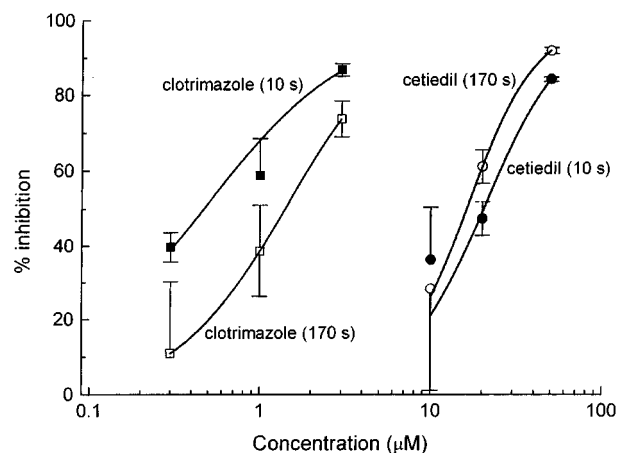


Figure 6 The concentration dependence of the effect of clotrimazole and cetiedil on the rate coefficients for K^+ loss measured at 10 s (filled symbols) and 170 s (open symbols) after the application of A23187. The values plotted are the coefficients in the presence of the blocker expressed as a percentage of the corresponding values in its absence. Vertical bars indicate the s.e.mean ($n=3$). The lines have been drawn according to the Hill equation (see text).

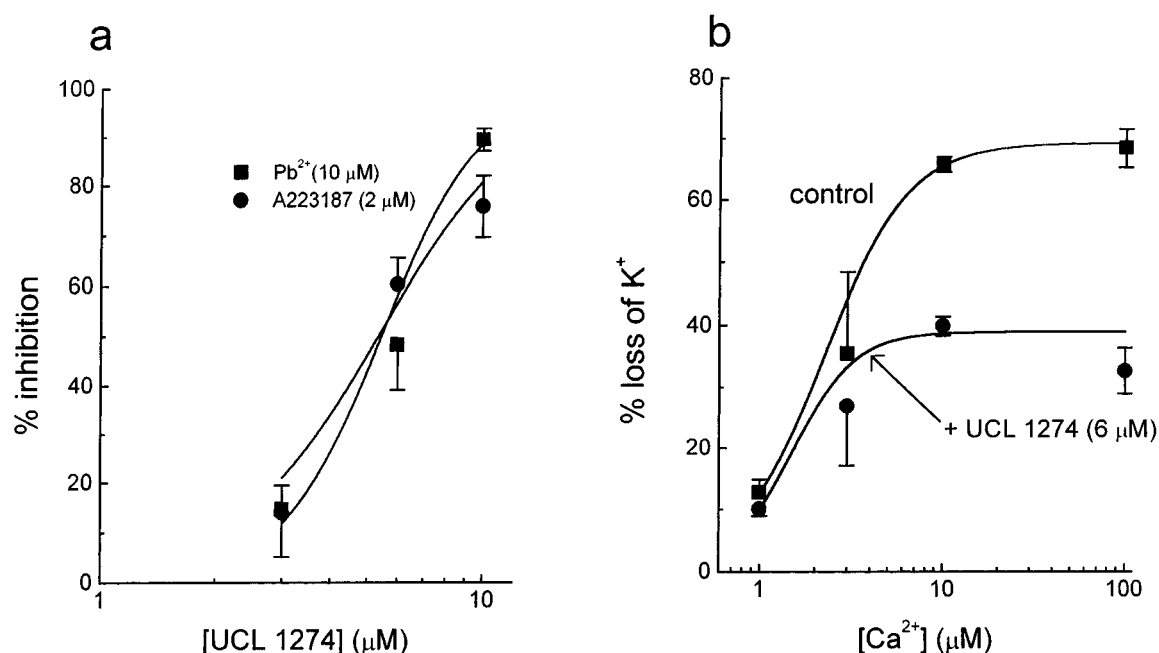


Figure 7 (a) Inhibition by UCL 1274 of K^+ loss from rabbit erythrocytes exposed to either A23187 (2 μM) or Pb^{2+} (10 μM). Each point is the mean of 3–4 observations and the vertical bars indicate the s.e.mean. (b) The relationship between the concentration of external Ca^{2+} and the release of K^+ in response to A23187 (2 μM) applied in the absence or presence of UCL 1274 (6 μM). The erythrocytes were suspended in solutions containing buffered Ca^{2+} at the concentrations shown on the abscissa. The lines have been drawn according to the Hill equation ($E_{\text{max}} = 69 \pm 3$ and $39 \pm 2\%$, and $n_{\text{H}} = 1.9 \pm 0.3$ and 2.6 ± 1.1 , in the absence and presence respectively of UCL 1274).

This corresponds to 44% inhibition which is in keeping with the results presented in Figure 2. Similar findings for the action of the $\text{P}_{\text{K}(\text{Ca})}$ blocker nifedipine on human red cells have been reported by Kaji (1990).

Discussion

The main new finding is of differences in the characteristics of the block of the red cell Ca^{2+} -activated K^+ permeability by clotrimazole and by cetiedil and its congeners, as well as by quinine. Thus the inhibitory effect of cetiedil, but not clotrimazole, is increased by a reduction in external K^+ (Figure 3); the concentration-inhibition curves for the two sets of compounds have very different slopes (Figures 2 and 6) and there appears to be a use-dependent component of the blocking action of cetiedil but not clotrimazole (Figures 4 and 5).

These differences can be discussed in relation to the properties of the Gárdos channels. It is known from single channel recordings (Grygorczyk & Schwarz, 1983, 1985) and from flux measurements (Vestergaard-Bogind *et al.*, 1985) that they have the characteristics of long pores that can contain more than one K^+ ion (probably 2–3) at the same time. Movement of K^+ through such a multi-ion channel involves the successive binding and dissociation of individual K^+ ions and this binding underlies the permeation characteristics of the channel (Hille & Schwartz, 1978; Hille, 1992). The present findings with cetiedil could be accounted for by supposing that it either acts at these sites or influences their properties. Thus, were it to compete with K^+ for them, or alter their affinity for K^+ through an allosteric effect, the observed K^+ dependence of the blocking action could be explained. The steepness ($n_{\text{H}} > 2$; see Table 2) of the concentration-inhibition curves observed with cetiedil and its congeners UCL 1274 and UCL

1495 is also in keeping with this since Hill slopes of greater than unity are expected for the action of blockers that act at the binding sites within multi-ion channels (see e.g. Adelman & French, 1978; Hille, 1992).

Blockade of the Gárdos channel by a carbocyanine dye (3,3'-diethylthiadicarbocyanine; diS-C₂(5)) is similarly reduced by an increase in external K^+ , as shown by Simons (1979) who already suggested that displacement of the dye by K^+ within the channel might be involved. Simons also noted that the dependence of the block on the extent to which the Ca^{2+} -activated K^+ permeability had been activated suggested that diS-C₂(5) binds either only or preferentially to open channels. It was found in the present work that in the presence of a relatively high concentration of cetiedil, the rate at which red blood cells lost K^+ in response to indirect activation of the Gárdos channel by A23187 declined sharply with time though it was clearly already reduced at the earliest point at which measurements could be made (Figure 5). This can be explained by supposing that cetiedil, like the carbocyanine dyes, blocks open channels but also has some affinity for the shut form. A preference for open channels could be relevant to the possibility that potent blockers of the Gárdos channel may prove useful in the treatment of sickle cell disease. The potential value of clotrimazole in this condition is under study (Brugnara *et al.*, 1996; see also Bunn, 1997).

The correlation between the potency of cetiedil and its analogues and their lipophilicity (Table 1; Benton *et al.*, 1994) suggests that lipophilicity is an important factor in determining the concentration reached at the site of action. One possibility is that these compounds must first enter the cell, as suggested by Grygorczyk & Schwarz (1985) for the Gárdos channel blocking action of quinine (though see Reichstein & Rothstein, 1981, for a contrary view). This seems unlikely to hold for cetiedil in view of single channel recordings (Dunn, 1997, 1998) that show that cetiedil is only weakly active when applied to

the cytoplasmic surface of inside-out patches isolated from human red cells. An action within the channel rather than at either its inner or outer surface seems more probable, particularly in view of our findings (i) that quaternization of cetiedil greatly reduces its activity (Benton *et al.*, 1994), and (ii) that the action of cetiedil and of hydroxy cetiedil (UCL 1269), a less lipophilic derivative of cetiedil, increased with the period of preincubation with the cells (Figure 1). The observation (Roxburgh *et al.*, 1996) that the enantiomers of cetiedil are equi-active as blockers of the Gárdos channel also suggests that the lipophilicity of the molecule is more important than its shape as a determinant of activity. However, in recent studies with more potent analogues of cetiedil, we have obtained evidence that structural features can also play a role (unpublished observations by D.C.H. Benton, M. Malik, C.R. Ganellin, S. Athmani, Z. Miscony & D.H. Jenkinson).

To summarize to this point, the evidence suggests that cetiedil and its congeners either act at or affect the K^+ binding sites within the channel that determine its selectivity. Access is strongly influenced by the lipophilicity of the compound and does not occur from the inner surface of the membrane. Interestingly, the same conclusions have been reached for the Ca^{2+} channel blocking action of the dihydropyridines, as discussed by Hockerman *et al.* (1997).

Our findings with clotrimazole, taken in conjunction with the work of others (Brugnara *et al.*, 1993a, 1995) suggest that it inhibits in a quite different way. The Gárdos channel is blocked by charybdotoxin (Wolff *et al.*, 1988; Brugnara *et al.*, 1993a) and the binding of labelled charybdotoxin is inhibited by clotrimazole (Brugnara *et al.*, 1995). This suggests that both substances act at the outer region of the channel, rather than within it. A common mechanism of action of clotrimazole and charybdotoxin (and one distinct from that of cetiedil) is also suggested by the finding that the block is little affected by a reduction in external K^+ (Figure 3). Nor does the block by clotrimazole increase with time in the manner seen with cetiedil (Figures 4 and 5). Finally, the similarity of the Hill coefficients observed for the inhibitory effects of clotrimazole, charybdotoxin and nitrendipine is also consistent with a common mechanism of action, whereas the coefficients for cetiedil,

quinine and the cetiedil analogues UCL 1274 and UCL 1495 were consistently greater (Table 2).

Though the evidence strongly suggests that clotrimazole, like charybdotoxin, acts at the outer face of the channel, this may not be the only site involved. Recent single channel recordings by Dunn (1997, 1998) have shown that clotrimazole is a potent blocker of the Gárdos channel even when applied to the inner face of the red cell membrane. The contribution that this makes to the overall inhibition seen when clotrimazole is applied to intact cells remains to be determined.

In our work the IC_{50} value for the inhibitory action of clotrimazole on Ca^{2+} -activated K^+ loss from rabbit erythrocytes was found to be $1.2 \pm 0.1 \mu\text{M}$. Alvarez *et al.* (1992) and Brugnara *et al.* (1993b) using human cells have reported a much greater potency ($\text{IC}_{50} \approx 50 \text{ nM}$). Two factors seem likely to contribute to the discrepancy. First, the IC_{50} 's listed in Table 2 are based on measurements taken at the end of a 3-min exposure to A23187. This time point was chosen for consistency with our studies with cetiedil whose blocking action increases with time (Figure 4). On recalculating the potency of clotrimazole but now from the reduction in the rate of loss when A23187 was first applied, an IC_{50} of 470 nM was obtained (Figure 6). Second, we have found that rabbit erythrocytes are less sensitive to clotrimazole than human cells, by a factor of ~ 2 (M. Malik & D.H. Jenkinson, unpublished data). The same factors are likely to account for the somewhat lower potencies observed for charybdotoxin and nitrendipine in the present work.

To conclude, we have found differences in the effects of Gárdos channel blockers that are likely to reflect different modes of interaction with the channel. Cetiedil and compounds related to it seem likely to act within the channel, at or near the K^+ binding sites that determine permeation, whereas the evidence so far available suggests that clotrimazole and charybdotoxin become attached to its outer surface.

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[³H]-Mesulergine labels 5-HT₇ sites in rat brain and guinea-pig ileum but not rat jejunum

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1 The primary aim of this investigation was to determine whether binding sites corresponding to the 5-HT₇ receptor could be detected in smooth muscle of the rat jejunum. Binding studies in rat brain (whole brain minus cerebellum) and guinea-pig ileal longitudinal muscle were also undertaken in order to compare the binding characteristics of these tissues. Studies were performed using [³H]-mesulergine, as it has a high affinity for 5-HT₇ receptors.

2 In the rat brain and guinea-pig ileum, pK_D values for [³H]-mesulergine of 8.0 ± 0.04 and 7.9 ± 0.11 (*n* = 3) and B_{max} values of 9.9 ± 0.3 and 21.5 ± 4.9 fmol mg⁻¹ protein were obtained respectively, but no binding was detected in the rat jejunum. [³H]-mesulergine binding in the rat brain and guinea-pig ileum was displaced with the agonists 5-carboxamidotryptamine (5-CT) > 5-hydroxytryptamine (5-HT) > 5-methoxytryptamine (5-MeOT) > sumatriptan and the antagonists risperidone > LSD > metergoline > ritanserin > pindolol.

3 Despite the lack of [³H]-mesulergine binding in the rat jejunum, functional studies undertaken revealed a biphasic contractile response to 5-HT which was partly blocked by ondansetron (1 μM). The residual response was present in over 50% of tissues studied and was found to be inhibited by risperidone > LSD > metergoline > mesulergine = ritanserin > pindolol, but was unaffected by RS 102221 (3 μM), cinanserin (30 nM), yohimbine (0.1 μM) and GR 113808 (1 μM). In addition, the agonist order of potency was 5-CT > 5-HT > 5-MeOT > sumatriptan.

4 In conclusion, binding studies performed with [³H]-mesulergine were able to detect 5-HT₇ sites in rat brain and guinea-pig ileum, but not in rat jejunum, where a functional 5-HT₇-like receptor was present.

Keywords: 5-HT₇ receptor; [³H]-mesulergine; rat jejunum; guinea-pig ileum; rat brain

Introduction

Recent advances in receptor classification using molecular cloning techniques have resulted in the identification of additional 5-HT receptor subtypes such as 5-HT₅, 5-HT₆ and 5-HT₇ to the four major subtypes already present. The 5-HT₇ receptor has been cloned from rat (Lovenberg *et al.*, 1993; Ruat *et al.*, 1993), mouse (Plassat *et al.*, 1993), human (Bard *et al.*, 1993) and guinea-pig (Tsou *et al.*, 1994). Whilst evidence for the endogenous expression and function of the gene products which encode the 5-HT₅ and 5-HT₆ receptors is yet to be obtained, the 5-HT₇ receptor is now recognized as the receptor previously classified as being 5-HT₁-like (Tsou *et al.*, 1994; Eglen *et al.*, 1997).

The 5-HT₇ receptor is positively coupled to adenylate cyclase and although there are no selective 5-HT₇ receptor ligands available at present, the identification of 5-HT₇ sites has been established using the operational criteria of rank order of agonist and antagonist potency at recombinant 5-HT₇ receptors (Eglen *et al.*, 1994; Sleight *et al.*, 1995b). The expressed 5-HT₇ receptor has a high affinity (pK_i 8.0–10.0) for 5-carboxamidotryptamine (5-CT), 5-hydroxytryptamine (5-HT), 5-methoxytryptamine (5-MeOT), clozapine, LSD, and mesulergine, moderate affinity (pK_i 6–7.9) for (±)-2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydronaphthalene (8-OH-DPAT), methysergide, ergotamine and spiperone and low affinity (pK_i < 6.0) for pindolol, cyanopindolol, and buspirone (Hoyer *et al.*, 1994; To *et al.*, 1995). These attributes comprise a unique pharmacological profile for the 5-HT₇ receptor,

which distinguishes it from other closely related receptors such as the 5-HT_{1A} receptor.

The 5-HT₇ receptor has been identified in regions of rat and guinea-pig brain (Tsou *et al.*, 1994; Shenker *et al.*, 1987), in particular the limbic system and thalamocortical regions, where it has been suggested that it may have a role in affective behaviours (To *et al.*, 1995; Gustafson *et al.*, 1996). Functional studies have identified 5-HT₇ receptors in porcine vena cava and myometrium (Sumner *et al.*, 1989; Kitazawa *et al.*, 1998), canine coronary artery (Cushing & Cohen, 1992), marmoset aorta (Dyer *et al.*, 1994) and in guinea-pig ileum (Feniuk *et al.*, 1984; Kalkman *et al.*, 1986; Carter *et al.*, 1995).

Until recently, the pharmacological profile of the smooth muscle 5-HT₇ receptor was thought to be consistent across species, where activation of the receptor has been shown to result in relaxation. However, smooth muscle contraction by a postjunctional 5-HT₇-like receptor in the rat jejunum has also been reported (McLean & Coupar, 1996), raising the possibility of either a different transduction mechanism or further subtypes of the 5-HT₇ receptor. The latter is supported by the finding of cDNA in the rat which differed in the carboxy terminal of the seven transmembrane structure of the expressed 5-HT₇ receptor (Lovenberg *et al.*, 1993; Ruat *et al.*, 1993). The generation of receptors differing in the carboxy regions can result in differential coupling to G-proteins (Lucas & Hen, 1995). In addition there have been isolated reports of an apparent species difference in the 5-HT₇ receptor. For instance, the affinity of clozapine for the rat 5-HT₇ receptor was found to be almost two orders of magnitude greater than the affinity of the compound at the mouse 5-HT₇ receptor (Sleight *et al.*, 1995b).

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Hence, the primary aim of this investigation was to determine whether binding sites corresponding to 5-HT₇ receptors could be detected in smooth muscle homogenates of rat jejunum. In addition, binding studies in rat brain and guinea-pig ileum were undertaken to identify 5-HT₇ sites, in order to compare the binding characteristics in different tissues. 5-HT₇ sites have been previously labelled in the brain using the agonists [³H]-5-CT and [³H]-5-HT (Sleight *et al.*, 1995a; To *et al.*, 1995), but often binding with an agonist radioligand may be complicated by the presence of multiple affinity states for the ligand, whereas this is generally not a problem with the use of an antagonist radioligand (Kenakin, 1984).

Methods

Radioligand binding experiments

Membrane preparations *Rat brain* Hooded Wistar rats of either sex were decapitated followed by rapid removal of the brain. The cerebellum was removed, the remaining mass weighed, and homogenized with 10 volumes of 50 mM Na₂HPO₄ phosphate buffer pH 7.4, using 20 strokes of a glass homogenizer. Following centrifugation at 1000 × *g* for 10 min at −2°C, the supernatant was re-centrifuged at 40,000 × *g* for 20 min at −2°C. The resulting pellet was then washed with a further 10 volumes of buffer before the final centrifugation at 40,000 × *g* at −2°C for 20 min. The final pellet was collected and frozen at −80°C until required for radioligand binding studies.

Guinea-pig ileum Homogenates were prepared according to the method of Kalkman *et al.* (1986), who detected a 5-HT₇-like receptor using [¹²⁵I]-LSD. Briefly, guinea-pigs of either sex were stunned and exsanguinated before removal of the ileum and flushing out of the intra-luminal contents with phosphate buffer. The segments (*ca.* 20 cm) were stretched over a glass rod, to enable separation of the longitudinal and circular muscle layers by gentle rubbing and peeling of the top layer. The wet mass was then collected, weighed, mixed with 10 volumes of buffer, homogenized in an Ultra Turrax set at 0.75 × maximum speed for 10 s, and centrifuged at 700 × *g* for 10 min at −2°C. After centrifugation the buffy coat was skimmed off the surface, and the remaining supernatant and pellet re-homogenized, passed through a double layer of coarse mesh gauze and re-centrifuged at 14,000 × *g* for 30 min. The pellet was collected, then washed with 10 volumes of buffer and re-centrifuged as previously. The final pellet was collected and stored as above.

Rat jejunum Homogenates were prepared according to a similar method employed by Pinkus *et al.* (1990) in an investigation of 5-HT₃ binding sites using [³H]-zacopride. Briefly, hooded Wistar rats of either sex were stunned and exsanguinated before removal of about 20 cm of jejunum, measured from the ligament of Trietz. Phosphate buffer was used to flush out the intra-luminal contents. The intestine was cut vertically and the mucosa scraped off with a glass slide. The remaining tissue was mixed with 50 ml of buffer, homogenized in an Ultra Turrax set at 0.75 × maximum speed for 10 s, and centrifuged at 500 × *g* for 5 min at −2°C. The supernatant was collected and centrifuged at 45,000 × *g* for 20 min. The pellet was then collected, washed with 20 ml of buffer and re-centrifuged as previously. The final pellet was collected and stored as above.

Receptor binding assays

Binding studies were performed using the ligand [³H]-mesulergine. Mesulergine has a high affinity for 5-HT₇ receptors as well as an affinity for 5-HT_{2A} and 5-HT_{2C} receptors, dopamine D₂ receptors and α₁/α₂ adrenoceptors (Closse, 1983; Rinne, 1983; Pazos *et al.*, 1985; Hoyer *et al.*, 1994). In order to overcome the binding of mesulergine to receptors other than 5-HT₇, masking drugs consisting of cinanserin (30 nM), RS 102221 (3 μM), raclopride (1 μM), prazosin (0.1 μM) and yohimbine (0.1 μM) were used, respectively. The concentrations of the masking drugs were chosen so that there was a theoretical occupancy of at least 90% of their respective receptor with little effect (<20% in total) on 5-HT₇ receptor binding.

In addition, phosphate buffer (Na₂HPO₄ 50 mM) was chosen instead of Tris-HCl (Tris 50 mM, NaCl 140 mM and MgCl₂ 5 mM) as it has been reported that the specific binding of [³H]-mesulergine is reduced in the presence of 100 mM or greater of sodium (Closse, 1983). Sodium ascorbate (1 μM) and pargyline (10 μM) were added to the buffer with experiments involving 5-HT.

Saturation curves were constructed in rat brain, jejunum and guinea-pig ileum. In each case, an aliquot of 200 μl containing between 1–1.8, 2–2.5 and 0.8–1.2 mg of protein, respectively, determined by the Bradford method (Bradford, 1976), was used with various concentrations of the radioligand ranging from 0.3–30 nM (final concentration) and buffer containing the masking drugs to give a total volume of 500 μl. Non-specific binding was defined by risperidone (1 μM) and experiments were performed in triplicate.

The assay composition for competition studies in rat brain was as follows: 100 μl of the membrane preparation, 50 μl of the radioligand (10 nM, final concentration), 50 μl of the displacing agent at various concentrations, and buffer containing the masking drugs to give a total volume of 350 μl. Displacing agents were added in 13 different concentrations and the experiment performed in duplicate. In guinea-pig ileum and rat jejunum this was varied slightly to conserve radioligand. The assay medium was as follows: 150 μl of the membrane preparation, 25 μl of the radioligand (10 nM, final concentration), 25 μl of the displacing agent at various concentrations, and buffer containing the masking drugs to give a total volume of 250 μl. Displacing agents were added in at least five different concentrations and the experiment performed in quadruplicate.

In both saturation and competition studies, the drugs and buffer were incubated initially for 10 min at 37°C before addition of the homogenate and incubation at 37°C for a further 60 min. Preliminary studies showed no significant difference in specific binding (*P* > 0.05) between experiments incubated for 30, 60 and 90 min. Termination of the experiment was performed by vacuum filtration through Whatman GF/B filters presoaked in 0.5% polyethylene glycol and risperidone (1 μM). Each filter was placed in a plastic vial with 5 ml of Filtercount (Packard), vortexed, and the radioactivity determined by a scintillation counter (Packard Tricarb 2000, CA, U.S.A.) after at least 5 h had elapsed.

Functional studies

Hooded Wistar rats of either sex were stunned and exsanguinated before removal of about 20 cm of jejunum, measured from the ligament of Trietz (McLean & Coupar, 1996). The intraluminal contents were flushed out using warm modified Krebs-Henseleit solution of the following composi-

tion (mM): NaCl 116, KCl 5.4, MgSO₄ 0.6, CaCl₂ 2.5, NaH₂PO₄ 1.2, NaHCO₃ 25, glucose 11.1. Four segments of jejunum (3–4 cm in length), were set up separately on tissue holders under 1 g weight tension and equilibrated in 20 ml organ baths containing Krebs-Henseleit solution gassed with 95% O₂: 5% CO₂ and maintained at 37°C. Responses were measured using an isotonic transducer (HUGO Basile) connected to a Grass model 79D polygraph recorder. Following equilibration, non-cumulative concentration-response curves were constructed for the agonists to avoid desensitization, as reported by McLean & Coupar (1996). Preliminary experiments showed non-reproducible curves, in agreement with previous findings in the rat jejunum (McLean & Coupar, 1996), therefore only one concentration-response curve was recorded in each tissue segment. Curves were established as described by addition of increasing concentrations of the agonist to the bathing solution at 10 min intervals, and agonists left in contact with the tissues for <30 s. Paired segments were used in order to measure the effect of antagonists, one always serving as control and the others to compare the response of the agonist in the presence of different antagonist concentrations. The effect of the agonists in the absence and presence of antagonist were expressed as a percentage of a maximum contraction obtained with acetylcholine (1 μM) in each segment at the end of the experiment. Antagonists were incubated for 40 min before testing of agonists.

Drugs

The following drugs were used: 5-carboxamidotryptamine, cinanserin, lysergic acid diethylamide (LSD), metergoline, 5-methoxytryptamine, (–) pindolol, raclopride L-tartrate, risperidone, tetrodotoxin citrate (Research Biochemicals International, Natick, MA, U.S.A.), acetylcholine chloride, atropine hydrochloride, 5-hydroxytryptamine creatinine sulphate, pargyline hydrochloride (Sigma, Poole, U.K.), prazosin hydrochloride, yohimbine hydrochloride (ICN Biomedicals, Aurora, OH, U.S.A.), haloperidol (Searle, Melbourne, Australia), [³H]-mesulergine (Amersham, Amersham, U.K.), RS 102221 8-[5-(2,4-dimethoxy-5-(4-trifluoromethylphenylsulphonamido)phenyl-5-oxopentyl)-1,3,8 triazaspiro[4.5]decane-2,4-dione (Tocris Cookson, Bristol, U.K.), ritanserin (Janssen-Cilag, Sydney, Australia), GR 113808 ({1-[2-(methylsulphonylamino)ethyl]-4-piperidinyl}methyl 1-methyl-1H-indole-3-carboxylate, ondansetron hydrochloride, sumatriptan succinate (Glaxo, Melbourne, Australia). Ritanserin, pindolol and cinanserin were dissolved in methanol, ondansetron, risperidone and metergoline were dissolved in ethanol and RS 102221 was dissolved in dimethylsulphoxide (DMSO) before dilution to the required concentrations. The volumes of solvent used constituted less than 0.02% of the final concentration in both functional and binding studies and had no effect in either study. All other drugs were dissolved in distilled water.

Data analysis

Equilibrium dissociation constants (K_D) for saturation studies were obtained using non-linear regression analysis and competition data were analysed according to one or two-site binding models. The equation used for fitting one site competition curves was:

$$y = \min + (\max - \min) / (1 + 10^{\times - \text{Log IC}_{50}}),$$

where min is the apparent minimum, max is the apparent maximum and Log IC₅₀ is the logarithm of the concentration of the competing drug, $\times$, required to inhibit the binding of the

radioligand by 50%. The equation used for fitting two-site competition curves was:

$$y = \min + (\max - \min) [F1 / (1 + 10^{\times - \text{Log IC}_{50(1)}}) + (1 - F1) / (1 + 10^{\times - \text{Log IC}_{50(2)}})],$$

where min is the apparent minimum, max is the apparent maximum, F1 is the fraction of receptors with an affinity described by Log IC₅₀₍₁₎ (the logarithm of the concentration of the competing drug, $\times$, required to inhibit the binding of the radioligand by 50%) and the remaining receptors have an affinity described by Log IC₅₀₍₂₎. K_i values were obtained using the Cheng & Prusoff equation (1973).

Contractions to serotonergic agonists were expressed as a percentage of the maximum contraction obtained to acetylcholine (1 μM). The EC₅₀ values, concentrations producing 50% of the maximum response elicited by the agonists, were estimated from non-linear regression plots of single curves. The data was analysed by fitting a logistic curve of the form:

$$y = \min + \sum_{i=1,2,3 \dots n} (\max_i - \min) / (1 + 10^{(\text{Log EC}_{50} - \times)^{n_H}}),$$

where i is apparent number of curves, max is the apparent maximum, min is the minimum point, Log EC₅₀ is the logarithm of the concentration, $\times$, producing 50% of the maximum response of each individual curve and n_H is the slope factor. Antagonist potencies were expressed as either a pK_B or an apparent pK_B value. Initially, pA_2 values were calculated from experiments in which three concentrations of antagonist were tested, using the method of Arunlakshana & Schild (1959). The slope of the Schild plot was calculated and compared to a slope of unity as expected for a competitive antagonist, using Student's unpaired t -test. When the slope did not differ significantly from unity ($P < 0.05$), an estimate of the pK_B value was made by fitting a regression with the slope constrained to unity. Apparent pK_B values were calculated from the relationship:

$$pK_B = \log(\text{CR} - 1) - \log[B],$$

where CR is the concentration ratio of the agonist in the presence and absence of antagonist (B), (Furchgott, 1972).

Student's t -test was used for comparison of individual means and Dunnett's test was used when multiple means were compared to a common control; the criterion for statistical significance for both tests was set at $P < 0.05$. Arithmetic and geometric means are given with mean $\pm$ s.e.mean or mean with 95% confidence intervals, respectively. Pearson correlation coefficients were calculated using the computer program Graph Pad Prism 2.0 (GraphPad Software, San Diego, CA, U.S.A.). All other calculations and graphics were also performed using Graph Pad Prism 2.0.

Results

Saturation curves in the rat brain were performed in the presence of raclopride (1 μM) to inhibit the binding of [³H]-mesulergine to dopamine D₂ receptors. The additional inhibition of α -adrenoceptors with prazosin (0.1 μM) and yohimbine (0.1 μM) and 5-HT_{2A} receptors using cinanserin (30 nM) had little effect on the specific binding of the radioligand. However, inhibition of 5-HT_{2C} receptors using RS 102221 was found to significantly ($P < 0.05$) decrease the specific binding of [³H]-mesulergine (0.3–30 nM), indicating the possibility of non-homogeneous binding sites recognized by the radioligand (Figure 1).

At a concentration of 0.3 μM (100 $\times$ K_D value at 5-HT_{2C} receptors), RS 102221 did not sufficiently block 5-HT_{2C} receptors when 30 nM of [³H]-mesulergine was used. This is

illustrated in Figure 1, where an increase in specific binding occurred when 30 nM of [³H]-mesulergine was used. To overcome this problem, the concentration of RS 102221 was increased 10 fold to 3.0 μ M. This concentration was found to be sufficient in reducing the counts obtained when using 30 nM of [³H]-mesulergine, and the curve reached a plateau as previously. A pK_D value of 8.0 ± 0.04 and a B_{max} of 9.9 ± 0.3 fmole mg protein⁻¹ ($n=3$) was calculated.

The rank order of potency of the displacing drugs studied in the rat brain was as follows: (agonists) 5-CT > 5-HT $\geq$ 5-

MeOT > sumatriptan and (antagonists) risperidone $\geq$ LSD $\geq$ metergoline > ritanserin > pindolol (Table 1). All agonists best fitted a two-site competition model (Figure 2a, Table 1) and all antagonist ligands best fitted a one-site competition model except for ritanserin which best fitted a two-site model (Figure 2b, Table 1). In a preliminary experiment, haloperidol (0.01 nM to 1 μ M), a compound with weak affinity at 5-HT₇ receptors, did not displace [³H]-mesulergine binding. The correlation between the pK_i values obtained in the rat brain and previously published values from recombinant 5-HT₇

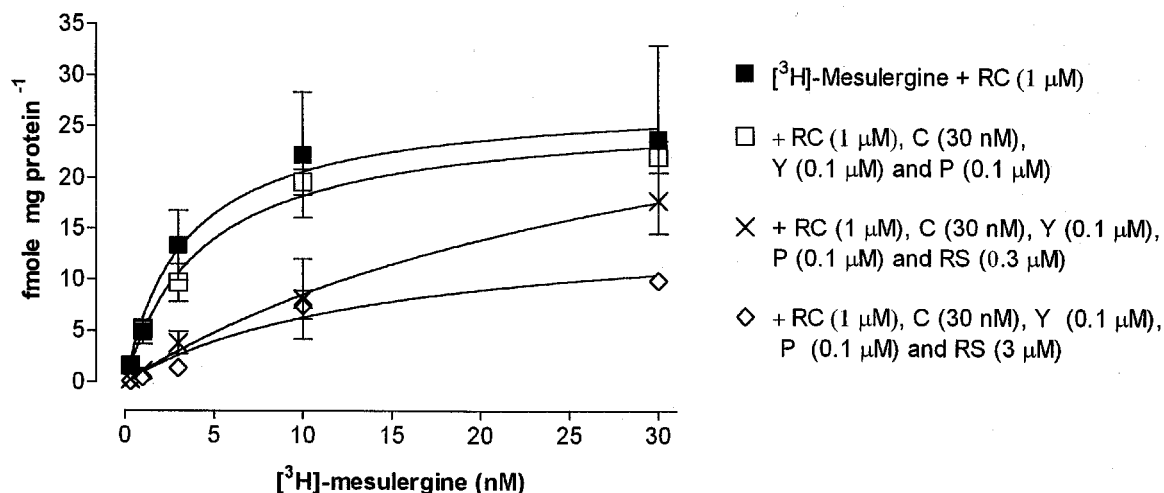


Figure 1 Saturation curve of [³H]-mesulergine binding in the rat brain in the absence and presence of yohimbine (Y: 0.1 μ M), prazosin (P: 0.1 μ M) cinanserin (C: 30 nM) and RS 102221 (RS: 0.3 and 3 μ M). All experiments were performed in the presence of raclopride (RC: 1 μ M). Abscissa: Concentration of [³H]-mesulergine in nM; Ordinate: fmole mg of protein⁻¹. Each point is the mean $\pm$ s.e. mean from three experiments.

Table 1 Comparison of ligand affinities in guinea-pig ileum, rat brain and isolated rat jejunum. All data are expressed as mean values with 95% confidence intervals noted in parentheses. High and low affinity values as well as the per cent fraction are shown for compounds displaying two-site competition

Compound	Rat brain pK_i or pK_D	Guinea-pig ileum pK_i or pK_D	Rat jejunum pK_B or apparent pK_B
<i>Agonists</i>			
5-CT	11.4 (11.0–11.7) 39% 8.0 (7.7–8.3) 61% $n=3$	12.1 (11.6–12.6) 46% 9.4 (9.0–9.9) 54% $n=4$	–
5-HT	9.9 (9.5–10.3) 35% 7.2 (6.8–7.0) 65% $n=3$	10.0 (9.3–10.7) 47% 8.8 (8.1–9.5) 53% $n=3$	–
5-MeOT	9.2 (8.8–9.6) 43% 6.2 (5.9–6.5) 57% $n=3$	9.6 (8.8–10.4) 32% 7.7 (7.2–8.2) 68% $n=3$	–
Sumatriptan	7.6 (7.2–8.1) 30% 5.0 (4.8–5.2) 70% $n=3$	7.0 (6.5–7.6) 46% 5.1 (4.5–5.6) 54% $n=3$	–
<i>Antagonists</i>			
Risperidone	8.3 (7.7–8.9) $n=4$	9.4 (9.0–9.8) $n=3$	8.9 (8.4–9.4)* $n=3$
LSD	8.1 (8.0–8.3) $n=4$	9.4 (8.8–10.7) $n=3$	7.6 (7.3–7.9)* $n=3$
Metergoline	8.0 (7.9–8.1) $n=4$	8.8 (8.5–9.0) $n=3$	7.8 (7.5–8.1) $n=3$
Mesulergine	8.0 (7.9–8.2)# $n=3$	7.9 (7.6–8.2)# $n=3$	7.3 (7.2–7.4) $n=3$
Ritanserin	7.6 (6.9–8.3) 51% 5.7 (5.2–6.2) 49% $n=4$	8.3 (8.1–8.5) $n=3$	7.3 (8.3–6.3)* $n=3$
Pindolol	4.5 (4.3–4.7) $n=4$	6.3 (5.8–6.4) $n=3$	6.7 (6.0–7.3)* $n=3$

*Apparent pK_B value; # pK_D value.

receptors obtained from rat or mouse and expressed in transfected cells was significant ($P < 0.05$); a Pearson correlation factor of 0.90 was obtained (Figure 3).

Saturation studies in the guinea-pig ileum were performed in the presence of the concentrations of masking drugs found to be optimum for 5-HT₇ receptor binding in the rat brain, under these conditions (see Methods). The pK_D value of [³H]-mesulergine was calculated to be 7.9 ± 0.11 and the $B_{\max}$ was 21.5 ± 4.9 fmol mg protein⁻¹ ($n = 3$).

The order of affinity of the agonist and antagonist displacers studied was the same as in the rat brain with some slightly higher pK_i values, see Table 1. All agonist ligands best fitted a two-site model whilst all antagonist ligands best fitted a one-site competition model (Figure 2c and d). The correlation between the pK_i values obtained in the guinea-pig ileum and recombinant rat or mouse 5-HT₇ receptors was significant ($P < 0.05$); a Pearson correlation factor of 0.94 was obtained (Figure 3).

Similar binding studies to those performed in the rat brain and guinea-pig ileum using [³H]-mesulergine were undertaken in the rat jejunum, but no binding to the 5-HT₇ receptor was detected.

Effect of 5-HT in the absence and presence of ondansetron in the isolated rat jejunum

In agreement with the findings of McLean & Coupar (1996) a biphasic concentration-response curve for 5-HT (10 nM –

3 μ M) induced contractions were obtained in the rat jejunum (Figure 4). A biphasic model better described the interaction of 5-HT in the rat jejunum than a monophasic model with R^2 values of 0.83 and 0.79 respectively. The pEC_{50} value of the first phase of the curve was 7.58 ± 0.12 with a maximum of $34.1 \pm 4.4\%$ of the response to acetylcholine (1 μ M). The pEC_{50}

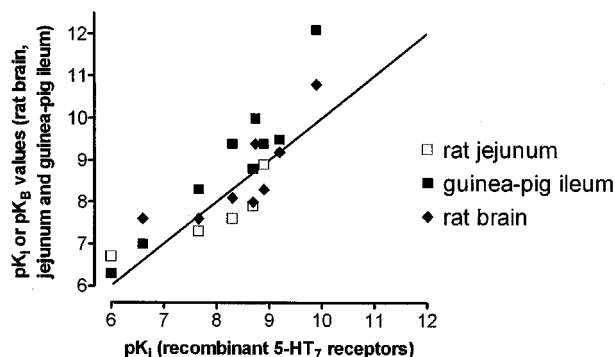


Figure 3 Correlation between pK_i values obtained in recombinant 5-HT₇ receptors from rat or mouse and expressed in transfected cells (Roth *et al.*, 1994; Plassat *et al.*, 1993; Ruat *et al.*, 1993; Shen *et al.*, 1993) and pK_i or pK_B values obtained in rat brain, guinea-pig ileum and rat jejunum. Abscissa: pK_i values for recombinant 5-HT₇ receptors, Ordinate: pK_i or pK_B values obtained in rat brain and jejunum and guinea-pig ileum. The continuous line represents a line of identity.

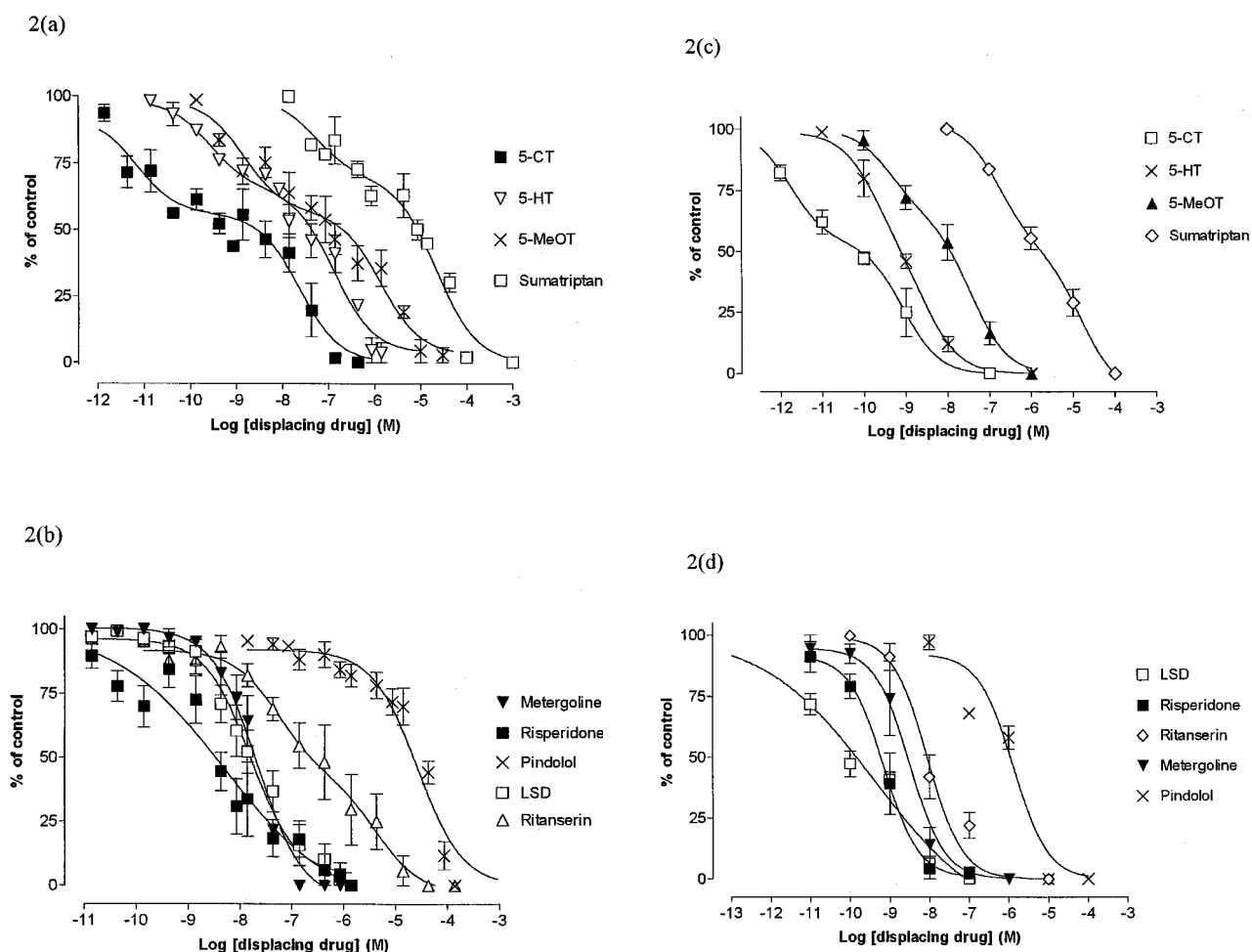


Figure 2 (a, b) inhibition of [³H]-mesulergine binding to rat brain and (c, d) guinea-pig ileum by various ligands. Abscissa: Log molar concentration of the displacing drug, Ordinate: % of specific binding. The data represent the mean $\pm$ s.e. mean per cent of maximum specific binding (defined with 1 μ M risperidone) of at least three experiments.

value of the second phase of the curve was 6.54 ± 0.17 with a maximum of 69.5 ± 8.5 of the response to acetylcholine ($1 \mu\text{M}$). However, in the presence of ondansetron (0.1 , 1 and $10 \mu\text{M}$) the curve flattened and was better defined by a monophasic curve, ($\text{pEC}_{50} = 6.79 \pm 0.07$). There was no significant difference ($P > 0.05$) between the curves obtained in the presence of 0.1 , 1 and $10 \mu\text{M}$ of ondansetron. In each case ondansetron significantly reduced the maximum response of 5-HT by *ca.* 32% ($P < 0.05$, $n = 4$). Thus ondansetron ($1 \mu\text{M}$) was included in all further experiments involving 5-HT in order to investigate the non-5-HT₃ receptor-mediated response. However, a response to 5-HT in the rat jejunum after blockade of 5-HT₃ receptors with ondansetron was not always present, *ca.* 50% of tissues failed to respond to the agonist, even though a response to acetylcholine was present.

Effect of 5-CT, 5-MeOT and sumatriptan

5-CT ($3 \text{ nM} - 3 \mu\text{M}$) resulted in a concentration-response curve similar to the monophasic curve of 5-HT. The pEC_{50} of 5-CT (7.03 ± 0.07) was significantly higher ($P < 0.05$) than that of 5-HT (6.79 ± 0.07) in the presence of ondansetron ($1 \mu\text{M}$), however the maximum contraction produced ($37.1 \pm 2.7\%$ of the contraction to Ach, $1 \mu\text{M}$) was not significantly different to that produced by 5-HT ($36.9 \pm 1.8\%$).

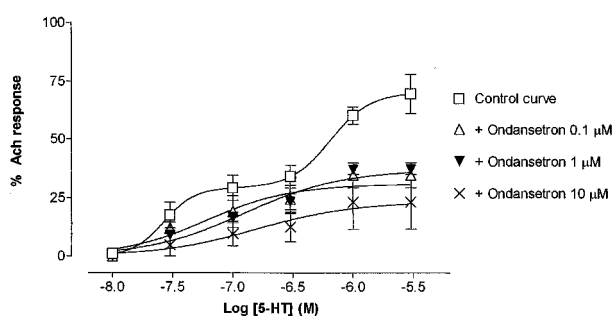


Figure 4 Concentration-response curve for the contractile effect of 5-HT in the absence and presence of ondansetron (0.1 , 1 and $10 \mu\text{M}$) in isolated rat jejunum. Abscissa: Log molar concentration of 5-HT, Ordinate: % of maximum contraction obtained with acetylcholine ($1 \mu\text{M}$). The data represent the mean $\pm$ s.e.mean of four paired experiments.

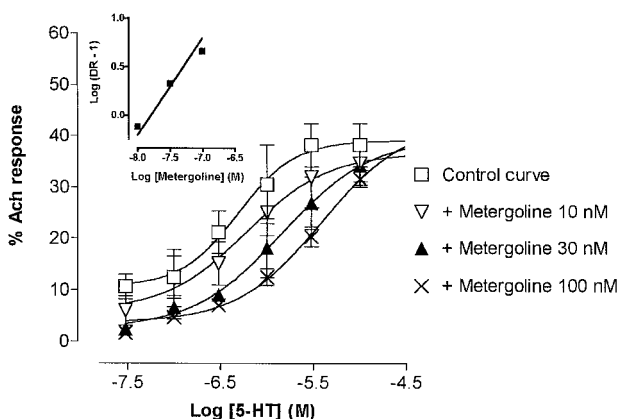


Figure 5 Concentration-response curves for 5-HT in the absence and presence of metergoline (10 , 30 and 100 nM). Abscissa: Log molar concentration of 5-HT, Ordinate: % of maximum contraction obtained with acetylcholine ($1 \mu\text{M}$). The data represent the mean $\pm$ s.e.mean of three paired experiments. All experiments were conducted in the presence of ondansetron ($1 \mu\text{M}$). The slope of the Schild regression (inset) was 0.8 ± 0.09 .

The pEC_{50} of 5-MeOT (5.33 ± 0.11) was significantly lower ($P < 0.05$) than that of 5-HT (6.79 ± 0.07) in the presence of ondansetron ($1 \mu\text{M}$), however the maximum contraction produced ($46.7 \pm 7.2\%$ of the contraction to Ach, $1 \mu\text{M}$) was greater but not significantly different to that produced by 5-HT ($P > 0.05$).

A pEC_{50} value of < 5 was obtained for sumatriptan, with a maximum contraction of only $11.3 \pm 3.1\%$ of the contraction to Ach, ($1 \mu\text{M}$).

Effect of 5-HT in the absence and presence of various antagonists

The effect of 5-HT in the presence of risperidone ($10 - 100 \text{ nM}$), ritanserin ($100 \text{ nM} - 1 \mu\text{M}$), LSD ($10 - 100 \text{ nM}$), metergoline ($10 - 100 \text{ nM}$), mesulergine ($10 - 100 \text{ nM}$) and pindolol (1 and $3 \mu\text{M}$) was investigated. At a concentration of 10 nM , risperidone and ritanserin caused both a shift in the control curve to 5-HT and a depression of the maximum response to the agonist. However, at higher concentrations of the antagonists, neither the depression of the maximum nor the shift in the control curve was increased. LSD also resulted in a depression of the maximum response with no further shift of the 5-HT curve when used at 30 nM , and this effect was increased at a concentration of 100 nM of LSD. Thus apparent pK_B values were reported for risperidone, ritanserin and LSD (Table 1). Schild slopes of 1.0 ± 0.04 (data points; 9) and 0.8 ± 0.09 (9) were obtained for mesulergine and metergoline (Figure 5) respectively. These values were not significantly different ($P < 0.05$) from unity. The correlation between the K_B and apparent pK_B values obtained in the rat jejunum and recombinant 5-HT₇ receptors from rat or mouse expressed in transfected cells (Hoyer *et al.*, 1994) was significant ($P < 0.05$); a Pearson correlation factor of 0.87 was obtained (Figure 3).

Preliminary studies showed RS 102221 ($3 \mu\text{M}$), yohimbine ($0.1 \mu\text{M}$), cinanserin (30 nM) and GR 113808 ($1 \mu\text{M}$) to have no effect on the contractile response to 5-HT in the rat jejunum, with pEC_{50} values of 5-HT in the presence of the antagonists not significantly different to that of the control curve, ($P > 0.05$). Atropine ($1 \mu\text{M}$) and tetrodotoxin ($1 \mu\text{M}$) failed to significantly modify ($P > 0.05$) the non 5-HT₃-mediated response.

Discussion

The results from the present study suggest the presence of 5-HT₇ receptor binding sites in rat brain and guinea-pig ileum, but not in rat jejunum where a functional 5-HT₇-like receptor was detected.

Receptor characterization

Due to the lack of selective and specific ligands currently available for studying the 5-HT₇ receptor, the use of rank order of agonist and antagonist affinity was used to characterize the 5-HT₇ site, as has been undertaken previously in studies using expressed and native 5-HT₇ receptors (Kalkman *et al.*, 1986; Plassat *et al.*, 1993; Monsma *et al.*, 1993; Erlander *et al.*, 1993; To *et al.*, 1995; Carter *et al.*, 1995; McLean & Coupar, 1996).

The presently described results obtained in rat brain are in agreement with findings in both guinea-pig and rat brain of 5-HT₇ sites, radiolabelled using serotonin receptor agonists (Sleight *et al.*, 1995a; To *et al.*, 1995; Gustafson *et al.*, 1996).

A number of masking drugs were used in the present study in order to inhibit the binding of [³H]-mesulergine to other receptors for which it has affinity. Mesulergine has been reported to have affinity for 5-HT_{2A} and 5-HT_{2C} receptors as well as some affinity for dopamine D₂ and α_1/α_2 -adrenoceptors (Closse, 1983; Rinne, 1983; Hoyer *et al.*, 1994), (see Methods). The masking drugs, some of which also have affinity for 5-HT₇ receptors, were chosen at concentrations which would theoretically occupy at least 90% of their targetted receptor populations (Kalkman *et al.*, 1986; Hoyer *et al.*, 1994; Sleight *et al.*, 1995a; Bonhaus *et al.*, 1997), without significantly affecting the binding of the radioligand to the 5-HT₇ receptor.

Saturation studies performed in rat brain and guinea-pig ileum provided pK_D values of 8.0 ± 0.04 and 7.9 ± 0.11 respectively for [³H]-mesulergine, which were in accordance with a previously reported pK_B value of 7.8 (Carter *et al.*, 1995) and a pK_i value of 8.15 (Hoyer *et al.*, 1994; To *et al.*, 1995) at the 5-HT₇ receptor. However, no evidence of specific binding to a 5-HT₇ site was apparent in the rat jejunum.

Displacement of [³H]-mesulergine binding in rat brain and guinea-pig ileum was observed using the agonists 5-CT ($pK_i = 11.4, 12.1$), 5-HT (9.9, 10.0), 5-MeOT (9.2, 9.6) as well as sumatriptan (7.6, 7.0) and the antagonists risperidone (8.3, 9.4), ritanserin (7.6, 8.3), metergoline (8.0, 8.8), LSD (8.1, 9.4) and pindolol (4.5, 6.3), respectively. The pK_i values of these compounds correlated well with those from other studies of the 5-HT₇ receptor in both order and magnitude (Kalkman *et al.*, 1986; Bard *et al.*, 1993; Plassat *et al.*, 1993; Ruat *et al.*, 1993; Shen *et al.*, 1993; Roth *et al.*, 1994; To *et al.*, 1995).

5-CT, 5-HT, 5-MeOT and sumatriptan displayed two-site displacement binding in the rat brain and guinea-pig ileum, which is not unusual for agonists, since they can bind to both a high and low affinity state of the receptor (Kenakin, 1984). However, the two-site binding displayed by ritanserin in the rat brain is more difficult to explain, and was not detected in the guinea-pig ileum nor reported in guinea-pig brain using [³H]-5-CT (To *et al.*, 1995).

The possibility of [³H]-mesulergine binding to non-serotonergic receptors, such as dopamine receptors, were ruled out by the addition of masking drugs such as raclopride (Rinne, 1983) and by the use of the agonists 5-CT, 5-HT and 5-MeOT which are specific for serotonin receptors. In addition, a preliminary experiment performed with haloperidol (0.01 nM to 1 μ M), a potent D₂ receptor antagonist ($pK_i = 9.3$), with little affinity for 5-HT₇ receptors ($pK_i = 6.6$; Roth *et al.*, 1994), failed to displace mesulergine binding in rat brain.

The binding site identified in both rat brain and guinea-pig ileum is unlikely to be of the 5-HT_{1A} or 5-HT_{1B} (rat brain) subtype, since pindolol was found to have a low (micromolar) affinity in this study, consistent with 5-HT₇ but not 5-HT₁ receptors, where it has nanomolar affinity (Lovenberg *et al.*, 1993; Ruat *et al.*, 1993; Hoyer *et al.*, 1994; Carter *et al.*, 1995; McLean & Coupar, 1996). The other 5-HT₁ receptor subtypes; 5-HT_{1D} (guinea-pig ileum), 5-HT_{1E}, and 5-HT_{1F} were ruled out by the high affinity of 5-CT in both the rat brain ($pK_i = 11.4$) and guinea-pig ileum ($pK_i = 12.1$, Hoyer *et al.*, 1994).

The receptor is also unlikely to be the 5-HT_{2A} or 5-HT_{2C} subtype, firstly because these receptors were excluded with cinanserin and RS 102221, respectively, and more importantly the antagonist order of affinity of risperidone ($pK_i = 8.7, 9.4$), metergoline (8.0, 8.8) and ritanserin (7.7, 8.3) in rat brain and guinea-pig ileum, respectively did not correlate with either 5-HT_{2A} (risperidone > ritanserin > metergoline) or 5-HT_{2C} (metergoline > ritanserin > risperidone) receptors (Hoyer *et al.*, 1994; Sleight *et al.*, 1995a). In addition, 5-CT was found to have nanomolar affinity in this study whilst it has micromolar

affinity or lower at both 5-HT_{2A} and 5-HT_{2C} receptor sites (Bard *et al.*, 1993; Hoyer *et al.*, 1994; Roth *et al.*, 1994). Furthermore, sumatriptan was found to have micromolar affinity in this study whilst it has only millimolar affinity at 5-HT_{2A} and 5-HT_{2C} receptors (Hoyer *et al.*, 1994).

The high affinity of 5-CT and 5-MeOT also ruled out the involvement of 5-HT₃ receptors, where the agonists are inactive (Bard *et al.*, 1993; Hoyer *et al.*, 1994; Sleight *et al.*, 1995b).

The involvement of 5-HT₄, 5-HT₅ and 5-HT₆ receptors is excluded due to the use of a nanomolar concentration of [³H]-mesulergine in displacement studies. Mesulergine has only micromolar affinity at 5-HT_{5A}, 5-HT_{5B} and 5-HT₆ receptors (Plassat *et al.*, 1993; Erlander *et al.*, 1993; Monsma *et al.*, 1993) and is inactive at 5-HT₄ receptors (Bard *et al.*, 1993). Further evidence against 5-HT_{5B} and 5-HT₆ receptor involvement is the low affinity of 5-CT ($pK_i = 7.4$ and 6.6 respectively) for these receptors (Hoyer *et al.*, 1994).

While the 5-HT orphan receptor reported by Castro *et al.* (1997) has a high affinity for 5-CT, it cannot be the receptor in question since mesulergine has only a micromolar affinity at the orphan receptor (Castro *et al.*, 1997). In addition the 5-HT orphan receptor has a similar affinity for both 5-CT and 5-HT (Castro *et al.*, 1997), a characteristic not displayed by the receptor in the present study.

Functional significance

5-HT₇ sites have been found to exist in high densities in the limbic system, hypothalamus and thalamocortical regions, hence leading to the suggestion that they may have a role in affective behaviours (Plassat *et al.*, 1993; Eglen *et al.*, 1994; Gustafson *et al.*, 1996).

This suggestion is further supported by the high affinity of atypical antipsychotics such as clozapine and risperidone for the 5-HT₇ receptor (Roth *et al.*, 1994). Moreover, the 5-HT₇ receptor has also been implicated in other affective disorders such as depression, where it was found that a down-regulation of the receptor occurs after chronic antidepressant treatment (Sleight *et al.*, 1995a). In addition 5-HT₇ receptors may have a role in the regulation of mammalian circadian rhythms (Lovenberg *et al.*, 1993).

The high affinity binding of LSD in rat brain in the present study adds further support to a 5-HT₇ site where a pK_i value similar to that reported in guinea-pig brain by To *et al.* (1995) was obtained (pK_i 8.2 compared with 7.8). This raises the possibility that the hallucinogenic action of LSD may be mediated in part *via* the 5-HT₇ receptor.

Peripheral tissue studies

The results of the binding studies in guinea-pig ileum lend further support to the functional findings of Feniuk *et al.* (1984), Kalkman *et al.* (1986) and Carter *et al.* (1995) where a postjunctional 5-HT site was seen to induce relaxation of pre-contracted guinea-pig ileum. The binding studies performed in this investigation are concordant with those of Kalkman *et al.* (1986), where [¹²⁵I]-LSD was used to characterize a postjunctional 5-HT site in guinea-pig ileum. In the study by Kalkman *et al.* (1986), cinanserin (300 nM) was used to mask 5-HT₂ receptors, a concentration which the authors claim theoretically inhibits 98% of 5-HT₂ receptors. Unfortunately, this concentration of cinanserin was also found to occupy close to 50% of the 5-HT₇-like site in a displacement study in the same investigation. This may have led to an underestimation of the K_D value of [¹²⁵I]-LSD at the 5-HT₇ site.

Nevertheless, the antagonist order of affinity reported (iodo-LSD > metergoline = mesulergine > spiperone > > haloperidol > propranolol) correlates well with a 5-HT₇ receptor.

Despite the lack of binding of [³H]-mesulergine in the rat jejunum, the functional experiments performed in the present study suggest the presence of a 5-HT₇-like receptor in this tissue, in agreement with the findings of McLean & Coupar (1996). The low affinity of pindolol and lack of antagonist affinity of yohimbine, cinanserin, RS 102221 and GR 113808 rule out a 5-HT_{1A}, α_2 , 5-HT_{2A}, 5-HT_{2C} and 5-HT₄ receptor site, in contrast to the findings of Javid & Naylor (1997), who reported the involvement of 5-HT₂ and 5-HT₄ receptors in mediating the contractile response of 5-HT in the proximal region of the rat small intestine. The use of ondansetron (1 μ M) and the relatively high potency of 5-CT rules out 5-HT₃ receptors. The presence of 5-HT₅ and 5-HT₆ receptors can also be excluded by the potency of 5-CT at this site. The relatively low potency of 5-CT compared to its high affinity in binding studies is characteristic of the 5-HT₇ receptor and was also reported by Carter *et al.* (1995), and Martin & Wilson (1995). The correlation between the pK_B and apparent pK_B values obtained in the rat jejunum and recombinant 5-HT₇ receptors from rat or mouse expressed in transfected cells (Plassat *et al.*, 1993; Ruat *et al.*, 1993; Shen *et al.*, 1993; Roth *et al.*, 1994) was very high (see Results).

It is interesting to note that the affinity values of mesulergine (7.3) and ritanserin (7.3) were lower in the present study with the rat isolated jejunum than reported in a similar study by McLean & Coupar (1996), where mesulergine and ritanserin were reported to have pA₂ values of 8.1 and 8.0 respectively. Affinity values for LSD, mesulergine and ritanserin reported in the guinea-pig ileum, receptors expressed from 5-HT₇ receptor genes (Ruat *et al.*, 1993; Carter *et al.*, 1995) and binding studies in the rat brain and guinea-pig ileum in this investigation were also slightly higher than those obtained in the rat jejunum. However, binding studies performed in the guinea-pig brain (To *et al.*, 1994), have shown a pK_D value of 7.8 for LSD, which agrees with the pK_B value obtained in the present study. In addition, risperidone, ritanserin and LSD resulted in a non-competitive antagonism of the serotonin response in the presence of ondansetron (1 μ M). At higher concentrations, risperidone (10–100 nM) and ritanserin (100 nM–1 μ M) caused a depression of the maximum response to 5-HT without further shifting the control curve. A comparison of the functional characteristics of LSD and risperidone at the 5-HT₇ receptor can not be made, as the effect of these compounds has not been investigated in other studies. However, at a concentration range of 10–100 nM, ritanserin was reported to act as a competitive inhibitor of the 5-HT₇-like response in the rat jejunum, (McLean & Coupar, 1996). The observation that an increased concentration of ritanserin and risperidone did not further shift the mean curve to 5-HT may indicate that the interaction of the antagonists with the 5-HT₇ receptor is of a non-competitive nature such as that seen with allosteric modulation. Negative allosteric modulators are known to produce parallel shifts of agonist concentration-response curves up to a limiting value (Ehlert, 1988; Lanzafame *et al.*, 1996). Often, as seen with ritanserin, they may appear to be competitive at lower concentration ranges. Further studies are required to fully define the mechanism by which ritanserin and risperidone interact with the 5-HT₇ site.

The lack of [³H]-mesulergine binding in the rat jejunum in this study may be explained by a low 5-HT₇ receptor density. This is supported by the observation that a response to 5-HT in the rat jejunum after blockade of 5-HT₃ receptors with ondansetron was not always present; (see Results). The response observed in the rat jejunum differs from other smooth muscles where activation of the 5-HT₇ receptor results in relaxation or various preparations in different species, such as porcine vena cava and myometrium (Sumner *et al.*, 1989; Kitazawa *et al.*, 1998), canine coronary artery (Cushing & Cohen, 1992), marmoset aorta (Dyer *et al.*, 1994) guinea-pig ileum (Feniuk *et al.*, 1984; Kalkman *et al.*, 1986; Carter *et al.*, 1995) and cat saphenous vein (Hoyer *et al.*, 1994). In addition, the contractile response is not consistent with the coupling mechanism of the 5-HT₇ receptor, which to date has been shown to only involve activation of adenylate cyclase, (Shenker *et al.*, 1987; Eglen *et al.*, 1997).

5-HT₇ receptor subtypes?

The possibility of 5-HT₇ receptor subtypes cannot be excluded; Ruat *et al.* (1993) isolated a 448 amino acid cDNA in the rat (5-HT_{7(a)}), while Lovenberg *et al.* (1993) isolated a 435 amino acid cDNA (5-HT_{7(b)}). The difference between the two cDNAs was only in the carboxy-terminal of the seven transmembrane structure of the expressed receptor and was said to result from alternative splicing (Boess & Martin, 1994). The generation of two receptor variants differing in the carboxy regions, can result in differential coupling to G-proteins (Lucas & Hen, 1995) and possibly a different physiological response as has been reported with the prostaglandin EP₃ (Namba *et al.*, 1993) and somatostatin SST₂ receptors (Vanetti *et al.*, 1993). In fact, the neuronal 5-HT_{7(a)} isoform has been reported to increase intracellular calcium resulting in activation of calmodulin-stimulated, adenylate cyclase isoforms AC1 and AC8, independent of phosphoinositide and protein kinase C (Baker *et al.*, 1998). This unique mechanism of coupling for any of the serotonin receptors was reported to be G_s-independent in studies with whole cells (see Wayman *et al.*, 1994; Sunahara *et al.*, 1996). It is possible some similar mechanism may exist in the rat jejunum, where a contraction instead of a relaxation to a 5-HT₇-like receptor was observed.

Furthermore, an additional rat 5-HT₇ isoform, a 470 amino acid cDNA, (5-HT_{7(c)}) resulting from a retained exon cassette, has also been reported (Heidmann *et al.*, 1997). However, unlike the other two isoforms, the rat 5-HT_{7(c)} was reported not to be present in human tissue, which suggests a species difference for the presence of various 5-HT₇ isoforms (Heidmann *et al.*, 1997). This again raises the possibility of a difference in the physiological response produced by 5-HT₇ receptors and may explain why an 'atypical' response was obtained in the rat jejunum.

In conclusion binding studies performed with [³H]-mesulergine were able to detect 5-HT₇ sites in rat brain and guinea-pig ileum, but not rat jejunum, where a functional 5-HT₇-like site was present.

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Effects of tumour necrosis factor- α on left ventricular function in the rat isolated perfused heart: possible mechanisms for a decline in cardiac function

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1 The cardiac depressant actions of TNF were investigated in the isolated perfused rat heart under constant flow (10 ml min⁻¹) and constant pressure (70 mmHg) conditions, using a recirculating (50 ml) mode of perfusion.

2 Under constant flow conditions TNF (20 ng ml⁻¹) caused an early (<25 min) decrease in left ventricular developed pressure (LVDP), which was maintained for 90 min (LVDP after 90 min: control vs TNF; 110 ± 4 vs 82 ± 10 mmHg, $P < 0.01$).

3 The depression in cardiac function seen with TNF under constant flow conditions, was blocked by the ceramidase inhibitor N-oleoylethanolamine (NOE), 1 μ M, (LVDP after 90 min: TNF vs TNF with NOE; 82 ± 10 vs 111 ± 5 mmHg, $P < 0.05$).

4 In hearts perfused at constant pressure, TNF caused a decrease in coronary flow rate (change in flow 20 min after TNF: control vs TNF; -3.0 ± 0.9 vs -8.7 ± 1.2 ml min⁻¹, $P < 0.01$). This was paralleled by a negative inotropic effect (change in LVDP 20 min after TNF: control vs TNF; -17 ± 7 vs -46 ± 6 mmHg, $P < 0.01$). The decline in function was more rapid and more severe than that seen under conditions of constant flow.

5 These data indicate that cardiac function can be disrupted by TNF on two levels, firstly *via* a direct, ceramidase dependant negative inotropic effect, and secondly *via* an indirect coronary vasoconstriction.

Keywords: TNF α ; cardiac contractility; N-oleoylethanolamine; sphingosine; sphingomyelinase; coronary circulation; Starling curve

Abbreviations: bpm, beats per minute; iNOS, inducible nitric oxide synthase; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; NOE, N-oleoylethanolamine; SMase, sphingomyelinase; TNF, tumour necrosis factor- α

Introduction

Tumour necrosis factor- α (TNF) is an essential mediator of the profound cardiovascular changes observed during the shocked-state associated with bacterial sepsis, and its experimental counterpart, endotoxic shock (Tracey *et al.*, 1986; Mathison *et al.*, 1988). Indeed, immunization of mice against TNF can protect against a lethal injection of endotoxin (Beutler *et al.*, 1985). The negative inotropic action observed during septic shock is mediated by circulating substances capable of depressing myocyte performance *in vitro* (Parrillo *et al.*, 1985). Monoclonal TNF antibodies can protect against both the hypotension and the decline in cardiac output observed upon live *Escherichia coli* administration to baboons (Tracey *et al.*, 1987), and TNF administration to dogs has been shown to have negative inotropic actions (Pagani *et al.*, 1992; Murray & Freeman, 1996). High circulating levels of TNF have also been associated with a variety of cardiac diseased states which are independent of bacterial infections (Levine *et al.*, 1990; Latini *et al.*, 1994; Vaddi *et al.*, 1994; Arbustini *et al.*, 1991; Smith & Allen, 1992) while overexpression of TNF in the murine heart leads to dilated cardiomyopathy associated with ventricular hypertrophy (Kubota *et al.*, 1997).

TNF-induced cardiac depression has been shown to have at least two phases. There appears to be an immediate depression of contractility as well as a later phase of contractile dysfunction. Cytokines, either alone or in combination, have the ability to cause upregulation of inducible nitric oxide

synthase (iNOS) in cardiac myocytes (Schulz *et al.*, 1992; Balligand *et al.*, 1994; Pinsky *et al.*, 1995), and nitric oxide from iNOS can subsequently adversely affect cardiac function. This altered cardiac function has been observed both as a direct decrease in cardiac performance (Schulz *et al.*, 1995), and as decreased responsiveness of cardiac myocytes to β -adrenergic stimulation (Balligand *et al.*, 1993). By definition iNOS is not constitutively expressed, and the protein synthesis required for its expression can take several hours. Another delayed action of TNF in the heart may involve the release of endothelins, whereby prior treatment of rats with lipopolysaccharide or TNF has been shown to cause the release of endothelin which results in a subsequent coronary constriction (Hohlfeld *et al.*, 1995; Klemm *et al.*, 1995).

In contrast to the delayed cardiac depressant effects of TNF described above, an immediate negative inotropic action has been observed within minutes of treatment, this has proven more difficult to characterize. The acute negative inotropic action of TNF has been shown to be sensitive to nitric oxide synthase inhibitors (Finkel *et al.*, 1992). Another report presented evidence to suggest that the immediate depression of function seen with TNF was *via* a nitric oxide-mediated decrease in myofilament calcium responsiveness (Goldhaber *et al.*, 1996). However, other authors have shown TNF-induced alterations in myocyte calcium handling and depressed myocyte calcium transients which are independent of nitric oxide (Yokoyama *et al.*, 1993). The same group have recently shown the potential involvement of sphingosine in TNF-

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induced changes in calcium homeostasis, and subsequent negative inotropic action (Oral *et al.*, 1997). Another recent report from the same group shows that the involvement of sphingosine in TNF-mediated cardiac depression is also evident *in vivo*, and is accompanied by a ventricular remodeling characterized as left ventricular dilation (Bozkurt *et al.*, 1998). In addition to this, short term TNF treatment has been shown to inhibit L-type calcium currents in cardiac myocytes, this was also accompanied by a decrease in calcium transients (Krown *et al.*, 1995). Using a Langendorff perfused heart preparation under constant flow conditions we have recently observed another early action of TNF in the heart. This is an immediate coronary vasoconstrictor action which could also contribute to the cardiac depressant action of TNF (Edmunds & Woodward, 1998).

The sphingomyelinase (SMase) pathway has been recognized as a major signalling pathway of TNF (Kim *et al.*, 1991; Schutze *et al.*, 1992; Dressler *et al.*, 1992; Yang *et al.*, 1993), and this pathway has recently been demonstrated in cardiac myocytes (Oral *et al.*, 1997). Sphingomyelinase can be activated by TNF to cause the breakdown of the membrane bound phospholipid, sphingomyelin to its metabolite ceramide, which can be subsequently converted to sphingosine by ceramidase. Both ceramide and sphingosine have potential second messenger functions (Kolesnick, 1991; Hannun, 1994; Merrill *et al.*, 1997). Sphingosine can depress cardiac function by decreasing calcium-induced calcium release from the sarcoplasmic reticulum (Webster *et al.*, 1994), as well as by depressing the L-type calcium current (McDonough *et al.*, 1994). N-oleylethanolamine (NOE) is a relatively specific inhibitor of the ceramidase enzyme (Sugita *et al.*, 1975), and is a compound which we have made use of in our studies.

In a previous report we have described a coronary constrictor action of TNF in the isolated perfused rat heart (Edmunds & Woodward, 1998). This appears to be mediated through an interaction between sphingosine and thromboxane A₂. In the present paper we provide evidence to show that the immediate negative inotropic action of TNF, observed in the isolated perfused rat heart also involves the sphingosine pathway. We also show that in a model where coronary flow is allowed to vary with changes in coronary tone, the vasoconstriction observed in our previous study can act in synergy with the direct negative inotropic actions of TNF to cause a further decrease in cardiac function.

Methods

Throughout this study male Wistar rats, 280–310 g, have been used. Animals were anaesthetized by an interperitoneal injection of sodium pentobarbitone (200 mg kg⁻¹) and then killed by cervical dislocation. The heart of each animal was isolated and perfused, *via* the aorta, according to the Langendorff technique under both constant flow and constant pressure conditions, with prefiltered, oxygenated (O₂ 95%, CO₂ 5%) Krebs-Henseleit solution of the following composition (mM): NaCl 118, NaHCO₃ 25, KCl 4.7, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.2, CaCl₂·6H₂O 1.2 and D-glucose 11.6, pH 7.4 and a temperature of 37°C. A fluid filled clingfilm balloon was placed in the left ventricle, and left ventricular developed pressure (LVDP) was used as a measure of cardiac contractility. Left ventricular end diastolic pressure (LVEDP) was set between 5 and 10 mmHg, with the use of a 1 ml syringe connected to a micrometer head, allowing fine adjustment of the intraventricular balloon volume. Hearts were allowed to beat spontaneously throughout the experimental procedure.

All hearts were perfused in a non-recirculating system for 30 min, before being switched to a recirculating system, with a total volume of 50 ml, for the remainder of the experiment, which lasted 90 min. The reason for recirculating the Krebs buffer was to reduce the amount of TNF used during each experiment, as well as to allow the build up of possible metabolites released by TNF. The recirculating perfusate was constantly gassed with 95% O₂, 5% CO₂. Starling curves were performed after 15, 45 and 75 min, whereby, the intraventricular balloon was emptied then slowly filled, in 0.03 ml aliquots every 30 s, to see if TNF altered the Frank-Starling response. Hearts were rejected from this study if LVDP failed to reach 100 mmHg.

For constant flow studies, hearts were perfused with a flow rate of 10 ml min⁻¹. Coronary perfusion pressure (CPP) was recorded by a pressure transducer, and used as an index of coronary tone. In another set of experiments hearts were initially perfused under constant flow conditions (10 ml min⁻¹) before being switched to a constant head of pressure of 70 mmHg. In this situation coronary flow was measured using a T 206 Transonic Systems Inc. flow probe attached to the aortic canula and represented the volume of Krebs buffer perfused into each heart with time.

Recombinant human TNF, 20 ng ml⁻¹ (stock solution: 100 µg ml⁻¹ in phosphate buffered saline with 0.1% bovine serum albumin) was added to the perfusate at 25 min, 5 min prior to recirculation. In preliminary experiments a range of concentrations, up to 100 ng ml⁻¹, were investigated, and 20 ng ml⁻¹ found to be optimum for cardiac depression (results not shown), this was a similar concentration to that used in other *in vitro* studies showing TNF-induced cardiac depression (Schulz *et al.*, 1995). The enzyme sphingomyelinase, (SMase, from *Bacillus cereus*) was added exogenously in one set of experiments to cause the breakdown of the phospholipid sphingomyelin. When added to the perfusate at 25 min, i.e. 5 min before recirculation, it showed a concentration dependant (concentrations ranging from 0.001–0.01 u l⁻¹ negative inotropic action (results not shown). In subsequent experiments a submaximal concentration of SMase, 0.003 u l⁻¹, was added 5 min prior to recirculation.

All antagonists were added directly to the Krebs buffer after 5 min, to allow 20 min for equilibration before TNF addition. The ceramidase inhibitor NOE (1 µM, dissolved in ethanol) was used to block sphingosine production from the SMase pathway (Oral *et al.*, 1997). The non-specific nitric oxide synthase inhibitor, nitro-L-arginine (100 µM, dissolved in saline) was used to block any nitric oxide release. The cyclo-oxygenase inhibitor, indomethacin (10 µM, stock solution dissolved in equimolar Na₂CO₃) was added to block prostanooid accumulation from TNF-induced AA release. Bosentan (3 µM, dissolved in saline) a combined endothelin A and B receptor antagonist (Clozel *et al.*, 1994) was used to block the actions of any TNF-induced endothelin release. Control experiments were conducted with all of the above agents in the absence of TNF. Control experiments were also conducted with vehicles for each of the drugs used, none of these altered cardiac function (data not shown).

A series of experiments was conducted to observe the actions of endothelin-1, 3 pM–3 nM, in hearts perfused under a constant head of pressure, in order to mimic the coronary constriction previously seen by us under constant flow conditions with TNF (Edmunds & Woodward, 1998), with the objective of demonstrating the dependency of LVDP on coronary flow.

In some of these constant pressure experiments, and despite having the heart surrounded by a heated water jacket,

coronary flow decreased to such an extent that there was a fall in cardiac temperature, as measured by a temperature probe inserted into the right ventricle. For this reason some experiments were performed in order to investigate the effect of drop in temperature from 37–33°C on cardiac contractility.

Statistics

All results are expressed as mean $\pm$ standard error of the mean. Statistical analysis was carried out, where appropriate, using one-way ANOVA coupled to either Dunnett's test or Tukey's multiple comparison test. For non-parametric data a Mann-Whitney U test was performed. Analysis was completed by the use of a computer programme (Minitab 10 for Windows).

Materials

Bosentan was also a gift from Dr A.G. Roach (Rhone-Poulenc Rorer, Dagenham, U.K.). rhTNF α was a kind gift from Bayer (Slough, U.K.). All of the other drugs used in this study were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.).

Results

Actions of TNF in the isolated rat heart perfused under constant flow conditions

It can be seen from the typical experimental trace shown in Figure 1a that in control hearts left ventricular developed pressure (LVDP) decreased only slightly throughout the 90 min perfusion period. Recirculation after 30 min had no direct effect on LVDP (LVDP before recirculation vs after recirculation: 127 ± 3 mmHg vs 120 ± 5 mmHg, $P = \text{NS}$, $n = 13$). Addition of TNF, 20 ng ml^{-1} , into the perfusate resulted in a depression in cardiac function which was evident within a few minutes of TNF addition (Figures 1b, 2 and 6). This depression became significant 25 min after TNF addition, and was evident for the remainder of the protocol (LVDP after 90 min: control vs TNF; 110 ± 4 mmHg vs 82 ± 10 mmHg, $P < 0.05$, $n = 10$ for TNF). TNF had no chronotropic effect, (heart rate after 90 min: control vs TNF; 285 ± 11 beats per minute (b.p.m.) vs 288 ± 12 b.p.m. $P = \text{NS}$).

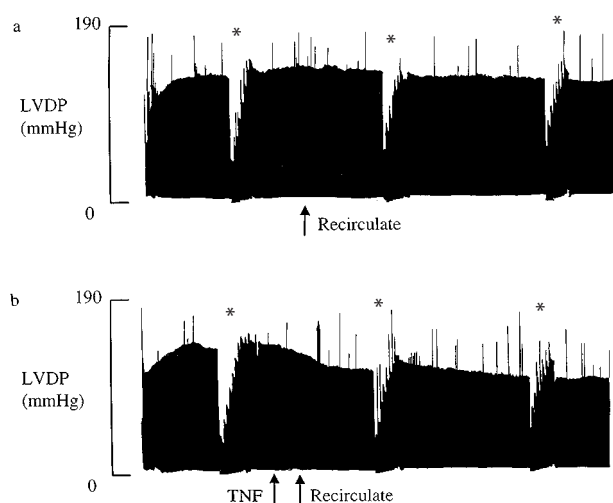


Figure 1 Experimental traces showing LVDP throughout the experiment. These traces are typical for (a) $n = 13$ control hearts and (b) $n = 10$ TNF, 20 ng ml^{-1} treated hearts. Starling curves were performed as indicated by *.

Actions of SMase in the isolated rat heart perfused under constant flow conditions

When added 5 min prior to recirculation SMase caused a negative inotropic action (Figure 3). This depression in function was seen immediately upon addition of SMase and became significant after 15 min. SMase also caused coronary constriction (increase in CPP 20 min after SMase: control, $n = 7$, vs SMase, $n = 8$; 13 ± 5 mmHg vs 75 ± 20 mmHg, $P < 0.01$). SMase did not alter heart rate (heart rate after 90 min: control vs SMase; 285 ± 11 b.p.m. vs 273 ± 25 b.p.m., $P = \text{NS}$).

Effects of antagonists and inhibitors on changes in contractility caused by TNF and SMase under constant flow conditions

N-oleoylethanolamine (NOE) Inclusion of the ceramidase inhibitor NOE, $1 \mu\text{M}$, into the perfusate 20 min before addition of TNF completely blocked the negative inotropic actions of the cytokine (Figure 2), without altering basal

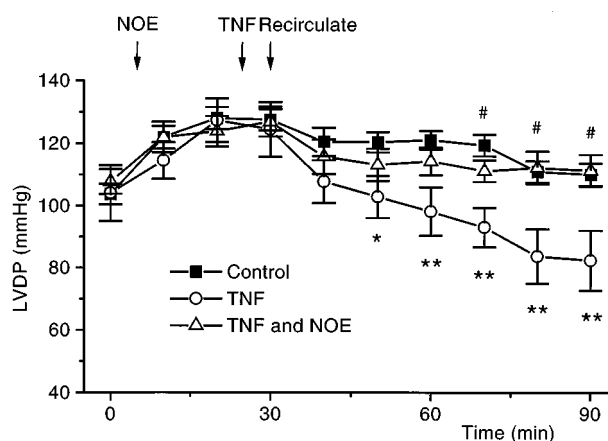


Figure 2 A graph to show changes in LVDP, in hearts, which occur with time, for control ($n = 13$) TNF treated, 20 ng ml^{-1} ($n = 10$) and TNF in the presence of NOE, $1 \mu\text{M}$ ($n = 9$). * $P < 0.05$, ** $P < 0.01$, TNF vs control. # $P < 0.05$, TNF with NOE vs TNF alone.

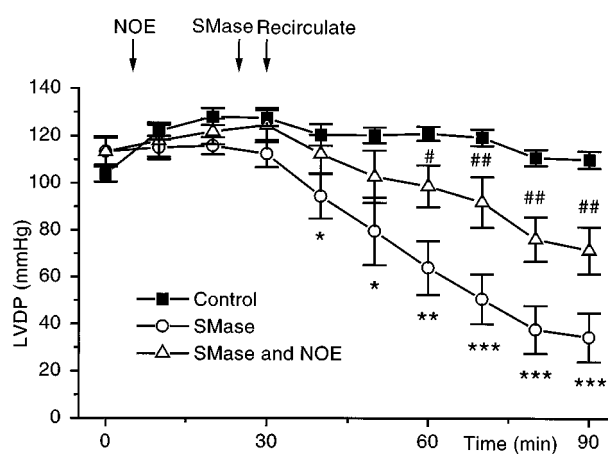


Figure 3 A graph to show the action of sphingomyelinase (SMase), alone and in combination with NOE, on the contractile parameters of the perfused heart. Control hearts ($n = 13$), SMase, 0.003 u l^{-1} , ($n = 7$), SMase, 0.003 u l^{-1} and NOE, $1 \mu\text{M}$, ($n = 8$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for SMase vs control. # $P < 0.05$, ## $P < 0.01$, for SMase with NOE vs SMase alone.

contractility (LVDP before NOE vs after NOE: 110 ± 4 mmHg vs 109 ± 4 mmHg, $P = \text{NS}$) or heart rate (heart rate before NOE vs after NOE: 267 ± 10 b.p.m. vs 263 ± 9 b.p.m., $P = \text{NS}$). NOE also caused a significant attenuation of the depression in function seen upon addition of SMase (Figure 3).

Nitro-L-arginine The nitric oxide synthase inhibitor, nitro-L-arginine, $100 \mu\text{M}$, caused a rapid and sustained increase in coronary perfusion pressure (CPP before nitro-L-arginine vs 20 min after nitro-L-arginine: 56 ± 2 mmHg vs 126 ± 9 mmHg, $P < 0.001$). However, Figure 4 shows that nitric oxide synthase inhibition did not ameliorate the decreased contractility seen with TNF. Heart rate was not altered by nitro-L-arginine (heart rate before nitro-L-arginine vs after nitro-L-arginine: 289 ± 12 b.p.m. vs 290 ± 12 b.p.m., $P = \text{NS}$).

Indomethacin We have already reported that indomethacin was able to block the increase in CPP seen with TNF (Edmunds & Woodward, 1998). However, indomethacin did not alter the cardiac depression seen with TNF (LVDP after 90 min: indomethacin vs indomethacin and TNF; 101 ± 6 mmHg vs 66 ± 6 mmHg, $P < 0.01$). Indomethacin had no effect on basal parameters of LVDP (LVDP before indomethacin vs after indomethacin: 101 ± 4 mmHg vs 105 ± 4 mmHg, $P = \text{NS}$) or heart rate (heart rate before indomethacin vs after indomethacin: 263 ± 4 b.p.m. vs 278 ± 13 b.p.m., $P = \text{NS}$).

Bosentan Addition of the ET_A - ET_B -receptor antagonist bosentan ($3 \mu\text{M}$) to the perfusate did not block the TNF-induced alterations in cardiac function (LVDP after 90 min: bosentan vs bosentan and TNF; 103 ± 6 mmHg vs 73 ± 6 mmHg, $P = 0.01$). Bosentan also did not alter the basal LVDP (LVDP before bosentan vs after bosentan: 104 ± 2 mmHg vs 106 ± 2 mmHg, $P = \text{NS}$), heart rate (heart rate before bosentan vs after bosentan: 271 ± 6 b.p.m. vs 271 ± 6 b.p.m., $P = \text{NS}$) or CPP (CPP before bosentan vs after bosentan: 50 ± 2 mmHg vs 54 ± 3 mmHg, $P = \text{NS}$).

Actions of TNF in the isolated rat heart perfused under a constant head of pressure

We have reported earlier that TNF causes coronary constriction under constant flow conditions (Edmunds &

Woodward, 1998). Under constant pressure conditions this constriction would be expected to adversely affect cardiac contractility. A set of experiments were completed, where hearts were perfused under a constant head of pressure. Upon recirculation in control hearts there was a slight decrease in coronary flow, which continued throughout the experiment (Figure 5). Addition of TNF caused a more pronounced decrease in coronary flow (Figure 5). The LVDP in hearts perfused under a constant head of pressure follow the same pattern observed in hearts perfused under constant flow conditions. Namely there was a decline in LVDP after recirculation (Figure 6). However from Figure 6 it is clear that TNF caused a more pronounced decline in cardiac function under constant pressure conditions than with constant flow perfusion. Prior addition of NOE blocked both the decrease in coronary flow (Figure 5), as well as inhibiting the depression in cardiac function observed with TNF (Figure 7).

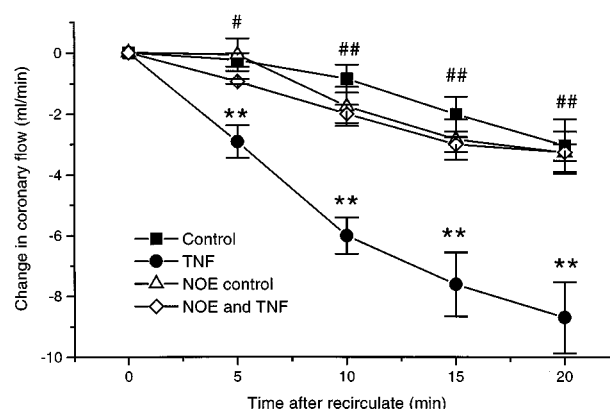


Figure 5 Effects of TNF (20 ng ml^{-1}) and NOE ($1 \mu\text{M}$), alone and in combination on coronary flow in hearts perfused at constant pressure ($n = 6-7$). ** $P < 0.01$ TNF vs control, # $P < 0.05$, ## $P < 0.01$ NOE alone vs TNF with NOE. Initial coronary flow at 0 min: $12.4 \pm 0.6 \text{ ml min}^{-1}$; $11 \pm 1.2 \text{ ml min}^{-1}$; $8.9 \pm 1 \text{ ml min}^{-1}$ and $10.3 \pm 2.4 \text{ ml min}^{-1}$ for control hearts, TNF treated, NOE treated and NOE with TNF treated hearts respectively.

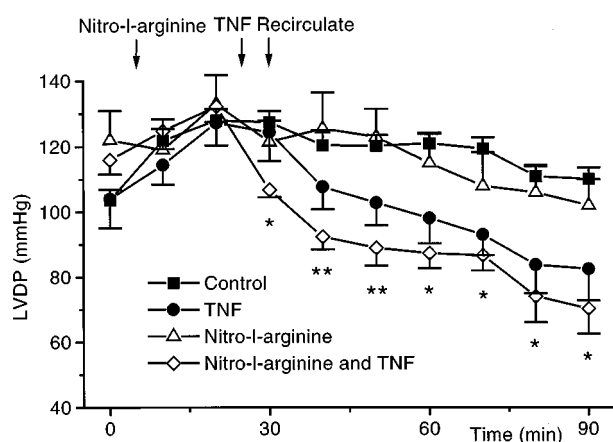


Figure 4 This graph shows the actions of nitro-L-arginine, $100 \mu\text{M}$, on the depression in LVDP seen with TNF ($n = 8$). Control hearts ($n = 13$), TNF ($n = 10$), and control hearts in the presence of nitro-L-arginine ($n = 4$) are all shown. * $P < 0.05$, ** $P < 0.01$, for TNF with nitro-L-arginine vs nitro-L-arginine alone.

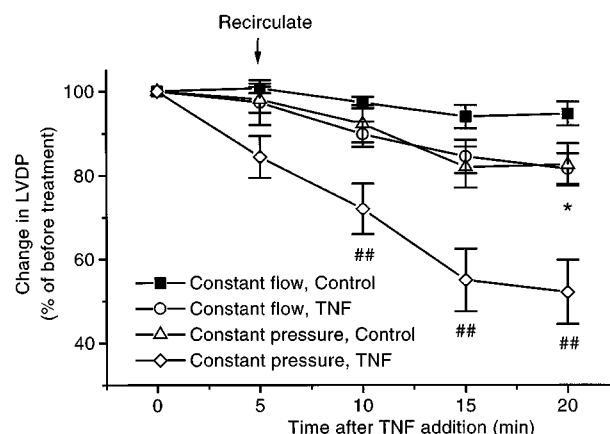


Figure 6 Effects of TNF, 20 ng ml^{-1} , in hearts perfused under constant pressure and constant flow conditions ($n = 6-7$). * $P < 0.05$, TNF vs control when perfused under constant flow conditions. * $P < 0.05$, TNF vs control when perfused under constant flow conditions. * $P < 0.05$, TNF under constant flow conditions vs TNF under constant pressure conditions. Where initial LVDP at 0 min: 122 ± 4 mmHg; 126 ± 6 mmHg; 101 ± 6 mmHg and 106 ± 3 mmHg for constant flow control and TNF treated heart and constant pressure control and TNF treated hearts respectively.

Actions of endothelin-1 in hearts perfused under a constant head of pressure

Endothelin-1, when perfused through hearts under a constant head of pressure, showed a concentration dependant decrease in coronary flow, indicative of coronary constriction. This decrease in coronary flow was accompanied by a concentration dependant depression in cardiac function. At 1 nM, endothelin-1 caused a decrease in coronary flow from 9.5 ± 0.5 ml min⁻¹ to 2.5 ± 0.7 ml min⁻¹ ($P < 0.001$, $n = 5$). At the same concentration of endothelin-1 LVDP was depressed from 114 ± 9 mmHg to 56 ± 14 mmHg ($P < 0.01$, $n = 5$).

Effects of decreased temperature on cardiac function

A drop in temperature of the Krebs buffer from 37–33°C caused a decrease in heart rate, from 286 ± 9 b.p.m. to 201 ± 9 b.p.m. ($n = 4$, $P < 0.05$) and a concomitant increase in LVDP, from 123 ± 2 – 133 ± 5 mmHg ($n = 4$, $P < 0.05$).

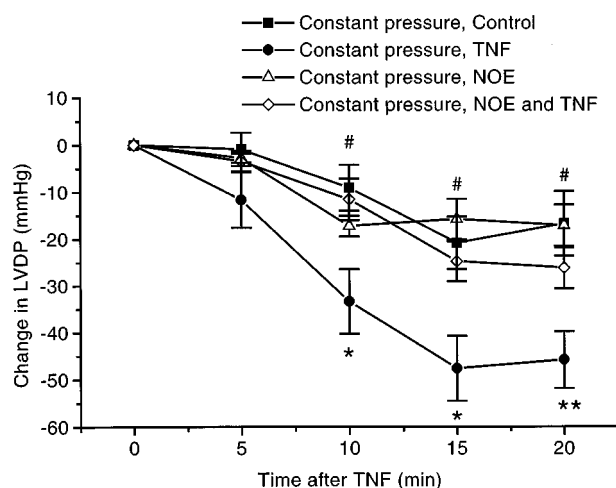


Figure 7 Effects of NOE on the depression in cardiac function seen with TNF, 20 ng ml^{-1} under constant pressure conditions. Shown as a change in LVDP after TNF administration ($n = 6-7$). * $P < 0.05$, ** $P < 0.01$ TNF vs control. # $P < 0.05$ NOE vs TNF with NOE. Where LVDP at 0 min for NOE treated controls and NOE with TNF treated animals: 107 ± 6 mmHg and 100 ± 5 mmHg respectively.

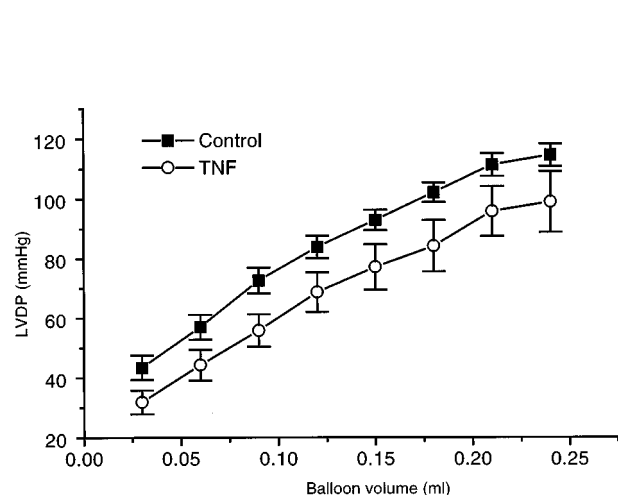


Figure 8 Starling curves for hearts perfused under constant flow conditions, 50 min after TNF addition. Control hearts ($n = 13$), and TNF, 20 ng ml^{-1} ($n = 10$).

Starling curves

Figure 8 shows a Starling curve at 75 min (50 min after TNF addition), where LVDP increases with balloon volume. It can be seen that there is a tendency for TNF to decrease LVDP for a given balloon volume, although this did not prove to be a significant effect. However in hearts perfused under a constant head of pressure there was a significant depression in the hearts ability to perform Starling curves when in the presence of TNF (Figure 9). Under these conditions, prior addition of NOE was able to block these actions of TNF (Figure 9).

Discussion

The aim of this study was to examine the mechanisms underlying the early cardiac depressant actions of TNF in the isolated perfused rat heart. We have shown an early negative inotropic action which was exacerbated by the coronary constrictor actions of TNF. The fact that these effects of TNF can be attenuated by the ceramidase inhibitor NOE implicates sphingosine in these actions. The findings of the study presented herein can be summarized as follows: isolated hearts perfused under constant flow conditions with a pathophysiologically relevant concentration of TNF (Redl *et al.*, 1993), showed a depression in ventricular contractility, as indicated by the decrease in LVDP which was evident within minutes of TNF treatment and was sustained for the remainder of the experiment. There was also a tendency for TNF to cause a depression in the Starling curves under constant flow conditions, although this was not shown to be statistically significant. The direct depression in LVDP, under constant flow conditions, was not blocked by nitro-L-arginine, bosentan or indomethacin, which inhibit nitric oxide release, endothelin actions and arachidonic acid metabolism respectively (Gross *et al.*, 1990; Clozel *et al.*, 1994; Vane, 1971). Thus NO, endothelins and arachidonic acid metabolites are unlikely to be involved in the TNF-induced decrease in LVDP. The inability of indomethacin to block the direct depression in cardiac function is interesting as indomethacin did block the increase in coronary tone previously observed by us (Edmunds & Woodward, 1998). However, the negative inotropic actions of TNF were completely blocked by the ceramidase inhibitor

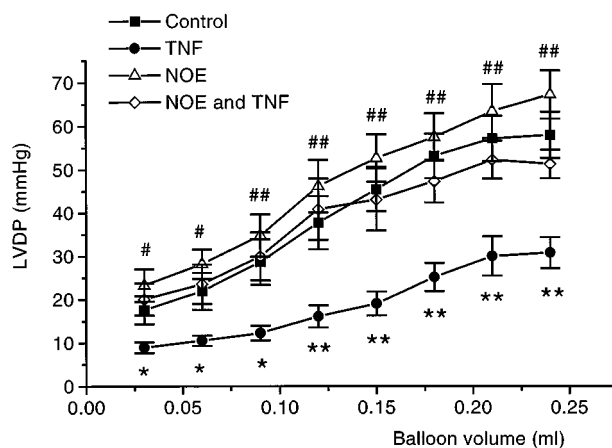


Figure 9 Starling curves in hearts perfused under a constant head of pressure, 50 min after TNF addition. Control hearts ($n = 7$), TNF, 20 ng ml^{-1} , alone ($n = 7$), NOE, $1 \mu\text{M}$, alone ($n = 6$) and TNF with NOE. * $P < 0.05$, ** $P < 0.01$, TNF vs control. # $P < 0.05$, ## $P < 0.01$, NOE alone vs TNF with NOE.

NOE. This suggests that these actions of TNF are, at least in part, dependant on ceramidase converting ceramide to sphingosine, and is in agreement with the recent data of Oral *et al.* (1997) and Bozkurt *et al.* (1998). The fact that NOE also attenuated the negative inotropic actions of SMase provides indirect evidence that activation of the sphingomyelinase pathway can depress cardiac contractility in the whole heart, and that this effect can be blocked with an inhibitor of the enzyme responsible for the conversion of ceramide to sphingosine.

We have previously reported that TNF causes coronary constriction in the rat heart (Edmunds & Woodward, 1998). The fact that TNF depressed cardiac contractility under constant flow conditions indicates that in this situation TNF is having a direct cardiac depressant action independent of changes in coronary flow. However, it is possible for the coronary circulation to alter cardiac function under constant flow condition *via* the 'garden hose' effect (Katz, 1992), which is something which we can not rule out. However, nitro-L-arginine also caused a vasoconstriction under constant flow conditions, but this was not accompanied by cardiac depression. This suggests that the garden hose effect is not important under these conditions and it provides evidence for a dissociation between cardiac depression and coronary constriction in this situation.

When hearts were perfused under a constant head of pressure, and coronary flow was allowed to vary, it was shown that TNF produced a greater, and earlier, depression of LVDP and of the Starling curves. This greater effect on contractility was coupled with a decrease in coronary flow to below 5 ml min⁻¹. Therefore under these constant pressure conditions, the TNF-induced coronary constriction appears to be contributing to its enhanced depressant actions. The TNF-induced depression in LVDP could also be blocked by prior addition of NOE. These data extend our previous observations showing a role for the coronary constriction in the cardiac depressant effects of TNF. Both depressant actions, coronary (indirect) and cardiac (direct), appear to involve the sphingomyelin pathway, but the vasoconstriction was also dependant thromboxane A₂ (Edmunds & Woodward, 1998), however the direct action of TNF on cardiac function is independent of arachidonic acid metabolism as it was not inhibited by indomethacin. Therefore there appears to be a link between the sphingosine and cyclo-oxygenase pathways in the TNF-mediated coronary constriction, but the direct cardiac depressant actions of TNF are dependant on the conversion of ceramide of sphingosine, and do not involve the cyclo-oxygenase pathway.

The TNF-induced decrease in coronary flow observed under a constant head of pressure could be completely blocked by NOE. This constriction followed a very similar time course to that seen when hearts were perfused at a constant flow, the majority of the effect being within 10 min of TNF administration. It can be seen when comparing the graphs shown in Figures 6 and 7 that the earlier depression in LVDP seen in hearts perfused under a constant head of pressure paralleled this decrease in coronary flow.

The studies utilizing the vasoconstrictor peptide endothelin-1, also show that coronary constriction under constant pressure conditions has the ability to depress contractile function. This depression in cardiac contractility induced by endothelin-1 in the whole heart under constant pressure conditions is clearly outweighing the direct positive inotropic actions that endothelin-1 has on rat cardiac myocytes (Kramer *et al.*, 1991), adding further weight to the argument that the TNF-induced coronary constriction can contribute to depressed cardiac function.

Whilst activation of the sphingomyelinase pathway by TNF has been known for some time (Dressler *et al.*, 1992; Schutze *et al.*, 1992), this has only recently been shown in cardiac tissue (Oral *et al.*, 1997). These authors demonstrated that TNF-induced sphingosine accumulation was responsible for a negative inotropic action of TNF in isolated feline cardiac myocytes, our data supports this work and indicates that similar mechanisms exist in the rat heart.

The early depression in function was not dependent on nitric oxide because it was not altered by the nitric oxide synthase inhibitor nitro-L-arginine. This is in contrast to the observations of Finkel *et al.*, 1992, but is in agreement with other reports showing that the early direct decrease in cardiac function produced by TNF was not prevented by nitric oxide synthase inhibition (Yokoyama *et al.*, 1993; Oral *et al.*, 1997; Bozkurt *et al.*, 1998). Schulz *et al.* (1995) showed that TNF-induced and IL-1 β -induced synthesis of iNOS was responsible for a late depression (> 2 h) in function in the isolated perfused rat heart. In contrast to our experiments, these authors did not observe an early depression in cardiac contractility, in fact a small increase in cardiac work was seen, we have no explanation for this apparent discrepancy. To our knowledge, alterations in cardiac function involving iNOS take place over a longer time course than used in our experiments (Schulz *et al.*, 1992; Szabo *et al.*, 1993; Shindo *et al.*, 1994; Balligand *et al.*, 1994; Pinsky *et al.*, 1995). Our results also show that the release of endothelin, which occurs after treatment of animals with lipopolysaccharide or TNF (Hohlfeld *et al.*, 1995; Klemm *et al.*, 1995), does not play a role in the early negative inotropic action of TNF. This is evidenced by the fact that the ET_{A/B} receptor antagonist, bosentan, did not alter the TNF-induced decrease in LVDP.

The ceramidase inhibitor, NOE, which attenuated the depressant actions of TNF under constant flow and constant pressure conditions, disrupts signalling through sphingomyelin breakdown by inhibiting the conversion of ceramide to sphingosine (Sugita *et al.*, 1975), an action which appears to be specific for the SMase pathway (Coroneos *et al.*, 1995). This suggests that the depressant effect of TNF is due to increased sphingosine production. Sphingosine has been shown to have the ability to disrupt cardiac myocyte calcium handling, both by a decrease in calcium release from the sarcoplasmic reticulum (Sabbadini *et al.*, 1992; Dettbarn *et al.*, 1994; Webster *et al.*, 1994), and also by a depression in the L-type calcium current (McDonough *et al.*, 1994). We have shown that the addition of SMase also caused a decrease in cardiac function, however the site of action of this exogenous SMase was not known. The sphingomyelinase enzyme used has a molecular size of 25 kDa (Matsuyama *et al.*, 1992), so it is likely that it will have access to the cardiac myocytes from the coronary circulation. A potential limitation of this study is that the site of action of this enzyme was not defined and it may have been on the endothelial cells or the smooth muscle cells of the coronary circulation and not directly on the cardiac myocytes. However, the cardiac depression seen with SMase was still attenuated by NOE, suggesting activation of the SMase pathway, and release of sphingosine is responsible for its cardiac depressant action. However, it should be noted that the source of the SMase used in this study was bacterial in origin, and hence it is possible that it contained trace amounts of endotoxin. Contamination with endotoxin, and hence subsequent activation of constitutive nitric oxide synthase by endotoxin, could possibly explain why NOE only partially blocked the SMase induced depression in cardiac function.

When perfusing under a constant head of pressure, the coronary constriction caused the flow rate to decrease, and

despite the fact that the heart was surrounded by a water jacket at 37°C, a fall in temperature of the heart occurred. However in control experiment where hearts were perfused at 33°C, a temperature comparable to that seen when coronary flow was maximally decreased by TNF, heart rate decreased and LVDP actually increased. Therefore if this was to occur upon perfusion with TNF one would expect slight protection against the decrease in myocardial contractility, and this may have masked an even more severe depressant action of TNF.

In summary, we add further evidence in confirmation of the study by Oral *et al.* (1997), showing that a direct and early

negative inotropic action of TNF in the isolated perfused rat heart is likely to be mediated *via* activation of SMase. Further to this, we have shown that the direct negative inotropic action of TNF can be accentuated by its coronary vasoconstrictor action.

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Reduction of sodium deoxycholic acid-induced scratching behaviour by bradykinin B₂ receptor antagonists

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1 Subcutaneous injection of sodium deoxycholic acid into the anterior of the back of male ddY mice elicited dose-dependent scratching of the injected site with the forepaws and hindpaws.

2 Up to 100 µg of sodium deoxycholic acid induced no significant increase in vascular permeability at the injection site as assessed by a dye leakage method.

3 Bradykinin (BK) B₂ receptor antagonists, FR173657 and Hoe140, significantly decreased the frequency of scratching induced by sodium deoxycholic acid.

4 Treatment with aprotinin to inhibit tissue kallikrein reduced the scratching behaviour induced by sodium deoxycholic acid, whereas treatment with soybean trypsin inhibitor to inhibit plasma kallikrein did not.

5 Although injection of kininase II inhibitor, lisinopril together with sodium deoxycholic acid did not alter the scratching behaviour, phosphoramidon, a neutral endopeptidase inhibitor, significantly increased the frequency of scratching.

6 Homogenates of the skin excised from the backs of mice were subjected to gel-filtration column chromatography followed by an assay of kinin release by trypsin from each fraction separated. Less kinin release from the fractions containing kininogen of low molecular weight was observed in the skin injected with sodium deoxycholic acid than in normal skin.

7 The frequency of scratching after the injection of sodium deoxycholic acid in plasma kininogen-deficient Brown Norway Katholiek rats was significantly lower than that in normal rats of the same strain, Brown Norway Kitasato rats.

8 These results indicate that BK released from low-molecular-weight kininogen by tissue kallikrein, but not from high-molecular-weight kininogen by plasma kallikrein, may be involved in the scratching behaviour induced by the injection of sodium deoxycholic acid in the rodent.

Keywords: Bradykinin; scratching behaviour; bradykinin B₂ receptor antagonist; sodium deoxycholic acid; Brown Norway Katholiek rat; kallikrein-kinin system

Abbreviations: BK, bradykinin

Introduction

Scratching behaviour is considered to be a mechanism of self-defense when pruritogens invade the body or are produced in it (McMahon & Koltzenburg, 1992). In rodents bioactive substances such as substance P and 5-hydroxytryptamine have been reported to be capable of inducing scratching behaviour when injected into the skin (Kuraishi *et al.*, 1995; Kitagawa *et al.*, 1997). Histamine has also been considered as an important mediator of itching sensations in humans (Hägermark *et al.*, 1978; Fjellner & Hägermark, 1981). Pruritus is an important symptom associated with several disorders, such as atopic eczema (Wahlgren, 1992), cholestasis (Ghent *et al.*, 1977), obstructive jaundice (Herndon & Dallas, 1972), and with dialysis for renal failure (Ståhle-Bäckdahl *et al.*, 1988) and other diseases (Greaves, 1993). In cholestasis or impaired hepatocellular secretion of bile, a marked accumulation in the plasma of bile acids was observed (Ghent *et al.*, 1977; Carey & Williams, 1961). To elucidate a mechanism for the pruritus in cholestasia, bile salts were shown to induce itching sensations in humans (Kirby *et al.*, 1974). On the other hand, antihistamine treatment is widely known to be sometimes ineffective for relief from itching in patients with such

symptoms (Krause & Shuster, 1983; Duncan *et al.*, 1984). This suggests a possible involvement of endogenous mediators other than histamine to mediate pruritus (Mettang *et al.*, 1990).

Like histamine and substance P, bradykinin (BK) is known to be one of the most potent endogenous algogenic substances (Dray & Perkins, 1993). Recently, an orally active and long-acting, non-peptide BK B₂ receptor antagonist was developed (Asano *et al.*, 1997), that would be useful for evaluating the role of BK *in vivo* (Rizzi *et al.*, 1997; Majima *et al.*, 1997; Griesbacher & Legat, 1997). Therefore in the present study, we observed the scratching behaviour following s.c. injection of the bile salt, sodium deoxycholic acid in rodents, and examined the effects of BK receptor antagonists and kallikrein inhibitors on scratching behaviour so as to elucidate the possible involvement of BK through activation of the kallikrein-kinin system in pruritus.

Methods

Behavioural experiments

Male ddY mice (Japan SLC, Hamamatsu, Japan, 40–50 g) were used in this study. They were maintained under controlled conditions of temperature (25 ± 1°C), humidity

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(55 ± 5%), and lighting (lights on only from 06.00 h to 20.00 h). Food and water were consumed freely except during the experiments. Each mouse was put in a clear acrylic cage with a floor area of 170 cm². The hair at the anterior end of the backs of the mice was cut with an electric shaver before the experiments. Sodium deoxycholic acid (Difco Laboratories, Detroit, MI, U.S.A.) was dissolved in pure water filtrated with MilliQ system (Millipore Corp., Bedford, MA, U.S.A.). Mice were slightly anaesthetized with diethyl ether for the administration of a solution of sodium deoxycholic acid into the hairless area on the back by s.c. injection with a gauge 276G × 3/4" needle. Mice recovered consciousness from the anaesthesia within about 60 s. After the s.c. injection of deoxycholic acid, the mice were replaced in their cages and their behaviours were recorded with an unmanned 8-mm video camera recorder (CCD-TR222, Sony, Tokyo, Japan) for 1 h (Kuraishi *et al.*, 1995). The frequency of scratching around the injected site with fore- and hind-paws was counted by replaying the video recording. Scratching behind the ears, but not the face, was also counted. When the mice scratched continuously for about 1 s without stopping and repeated this more than once, this episode of scratching was counted as one. Inbred female Brown Norway Katholiek rats (plasma kininogen-deficient) and Brown Norway Kitasato rats (normal rats of the same strain), both 3–4 weeks old (40–50 g), kept in the Department of Laboratory Animal Sciences, Kitasato University School of Medicine (Majima *et al.*, 1991), were also used following the same procedures as in the experiment with mice.

All the animal experiments were performed in accordance with Kitasato University School of Medicine guidelines for animal experiments.

Vascular permeability increase

Mice were anaesthetized with 50 mg kg⁻¹ of Nembutal (Abbott Laboratories, North Chicago, IL, U.S.A.) followed by an intravenous injection of pontamine sky blue (100 mg kg⁻¹). BK (Peptide Institute, Osaka, Japan) or sodium deoxycholic acid was injected subcutaneously into the backs of mice. Mice were sacrificed at 30 min after the injection. Skin was removed from the injection site and serum was collected. The excised skin specimens were incubated overnight with 0.5 ml of 1 N potassium hydrochloride at 37°C. Four ml of acetone-0.6 N phosphoric acid (13:6) solution was then added and the mixture was centrifuged at 2000 × g for 15 min. Concentrations of pontamine sky blue in the supernatant and serum were quantified by measuring the absorbance at 630 nm. The amount of dye leakage in the skin was calculated as the volume of leaked serum (Oh-ishi *et al.*, 1977).

Effects of kininase inhibitors and kallikrein inhibitors on scratching induced by sodium deoxycholic acid

Phosphoramidon (30 µg site⁻¹) or lisinopril (0.3 µg site⁻¹) was dissolved in a solution of sodium deoxycholic acid (0.3 mg ml⁻¹), and 100 µl of the mixture was injected locally. The doses of both kininase inhibitors were enough to potentiate an effect of BK, since a vascular permeability increased by s.c. injection of BK (0.1 nmol site⁻¹) into mice was significantly enhanced by co-injections of those kininase inhibitors locally. Soybean trypsin inhibitor (100 µg site⁻¹) was dissolved in a solution of sodium deoxycholic acid (1 mg ml⁻¹). The dose of soybean trypsin inhibitor was also confirmed by a dye leakage method. It inhibited a vascular permeability increase induced by s.c. injection of plasma

kallikrein (0.1 unit site⁻¹, Protogen AG., Läutelfingen, Switzerland) in mice.

Effects of kininase inhibitors on degradation of BK by skin homogenate and plasma of mice

Mice were sacrificed and perfused with 50 ml of saline through the abdominal aorta. Skin (5 mm in diameter) was excised and homogenized in 0.5 ml of Tris-HCl (50 mM) buffer (pH 7.5) containing 0.5% of sodium deoxycholic acid. The homogenate was centrifuged at 10,000 × g for 15 min and the supernatant was used as skin extract. One µl of the skin extract or 2 µl of plasma was incubated with 100 ng of BK for 10 min at 37°C in 200 µl of Tris-HCl (50 mM) buffer (pH 7.0) containing NaCl (150 mM) in the presence or the absence of lisinopril (1 µg ml⁻¹) or phosphoramidon (100 µg ml⁻¹). One fifth volume of 20% trichloroacetic acid was added to the incubation mixture to stop the reaction. Residual bradykinin in the mixture was measured using a BK enzyme immunoassay kit, Markit-M Bradykinin (Dainippon Pharmaceutical Corp., Osaka, Japan) (Majima *et al.*, 1994a).

Gel-filtration column chromatography

Mice were injected subcutaneously with 100 µl of sodium deoxycholic acid (1 mg ml⁻¹) or 100 µl of saline as vehicle control in the dorsal skin. At 60 min after the injection, the mice were sacrificed and ten pieces of skin, each 5 mm in diameter, were excised from the injection sites. The skin samples were immediately frozen in liquid nitrogen and then pulverized. The pulverized skins were solubilized with 2 ml of Tris-HCl (50 mM) buffer (pH 7.5) containing 1% of sodium deoxycholic acid, 50 µg ml⁻¹ each of aprotinin, soybean trypsin inhibitor, leupeptin, N-ethylmaleimide, phenylsulphonyl fluoride, and disodium ethylenediamine tetraacetic acid. The solutions were sonicated by Sonifier 350 (Branson, U.S.A.), with the output dial set at 6 for 1 min and were centrifuged at 10,000 × g for 15 min. The supernatant was applied to a Superose 12 HR 10/30 column (Pharmacia Biotech, Uppsala, Sweden) with a FPLC system. Proteins were eluted with Tris-HCl (20 mM) buffer (pH 7.5) containing NaCl (50 mM) at a flow rate of 0.4 ml min⁻¹. The eluate was collected in 0.4 ml fractions and kinin was released from each fraction. To estimate the kinin released from the gel-chromatography fractions, each fraction was incubated with (100 µg ml⁻¹ each of) trypsin-TPCK (Worthington Biochemical Corp., Free Hold, NJ, U.S.A.), lisinopril (Shionogi & Co. Ltd., Osaka, Japan), phosphoramidon (Peptide Institute, Osaka, Japan), and disodium ethylenediamine tetraacetic acid, at 37°C for 60 min (Hayashi *et al.*, 1984). Following the incubation, one-fifth of the resulting mixture's volume of 20% trichloroacetic acid was added to deproteinize and stop the reaction. The reaction mixture was centrifuged at 2000 × g for 10 min and then the amount of kinin released by trypsin was measured using Markit-M Bradykinin.

Materials

FR173657 ((E)-3-(6-acetamido-3-pyridyl)-N-[N-[2,4-dichloro-3-[(2-methyl-8-quinolinyloxy)methyl]phenyl]-N-methylamino-carbonylmethyl]acrylamide) and lisinopril were kindly contributed by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan), and Shionogi & Co., Ltd. (Osaka, Japan), respectively. Hoe140 (D-Arg-(Hyp³, Thi⁵, D-Tic⁷, Oic⁸)-BK) and desArg⁹-[Leu⁸]-BK were purchased from the Peptide Institute (Minoh, Osaka, Japan).

Statistical analysis

Values are expressed as means $\pm$ s.e.mean. Student's *t*-test was used to evaluate the significance of differences. When variances were heterogeneous, statistical analysis was performed by the Aspin-Welch method or Wilcoxon's rank sum test.

Results

Scratching behaviour induced by sodium deoxycholic acid in mice

Following a subcutaneous injection of 100 μ g of sodium deoxycholic acid into the anterior part of the backs of ddY mice, scratching behaviour was first observed within a few minutes after the injection (Figure 1). Although varying widely, the frequency of scratching increased over the first 20 min and then gradually decreased for the next 40 min. Intense facial scratching, although not counted, was observed several times preceding scratching around the injection site. Furthermore, the intervals between the scratching episodes were not constant over the period observed. The responses to the scratching-induction effects of different doses of sodium deoxycholic acid were observed over 60 min (Figure 2). Injection of 30 or 100 μ g of sodium deoxycholic acid caused an apparent dose-dependent increase in the frequency of scratching. These effects of sodium deoxycholic acid on the scratching were significant ($P < 0.05$ for 30 μ g, $P < 0.01$ for 100 μ g) when compared with saline alone.

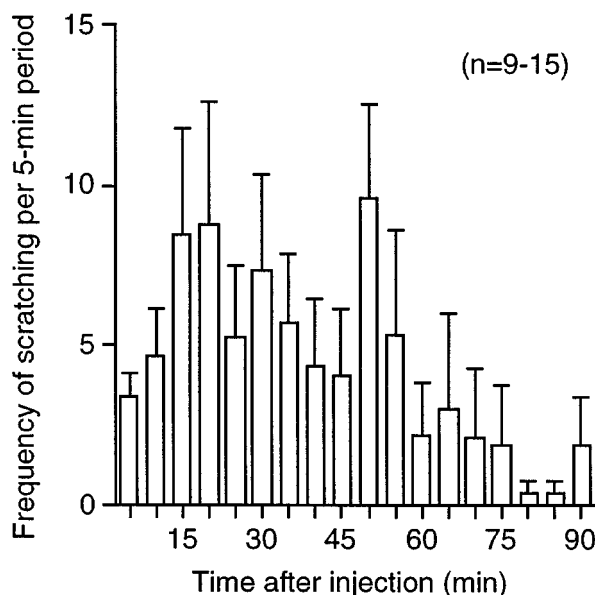


Figure 1 Time course of scratching after s.c. injection of sodium deoxycholic acid. Sodium deoxycholic acid (100 μ g site⁻¹) was injected subcutaneously into the anterior part of the back of male ddY mice under light ether anaesthesia. Each mouse was then placed separately in a cage so that its behaviour could be recorded with an unmanned video camera. Frequency of scratching around the injection site with the fore- and hind-paws was counted for every 5-min period over a 90 min duration after the injection. Each value represents mean $\pm$ s.e.mean from 9–15 animals.

Vascular permeability increase due to sodium deoxycholic acid in mouse skin

Doses between 10 and 100 μ g of sodium deoxycholic acid did not induce a significant increase in vascular permeability (Figure 3), but 300 μ g or more of it did increase vascular permeability ($P < 0.01$). BK (0.3 nmol site⁻¹) also induced dye leakage ($P < 0.01$).

Effects of BK receptor antagonists on the scratching behaviour induced by sodium deoxycholic acid

When the non-peptide antagonist FR173657 was suspended in 5% gum arabic solution and administered orally 1 h before the injection of sodium deoxycholic acid, it inhibited the scratching behaviour significantly (Figure 4, $P < 0.05$). In addition, the B₂ receptor antagonist Hoe140, injected subcutaneously with sodium deoxycholic acid, reduced the frequency of scratching by about 30%. In contrast, the B₁ receptor antagonist, des-Arg⁹-[Leu⁸]-BK did not reduce the frequency.

Effects of kininase inhibitors on scratching induced by sodium deoxycholic acid

Lisinopril, an inhibitor of kininase II, failed to augment the scratching behaviour (Figure 5a). In contrast, phosphoramidon, which inhibits neutral endopeptidase, significantly increased the frequency of scratching induced by sodium deoxycholic acid ($P < 0.01$). Although degradation of BK in

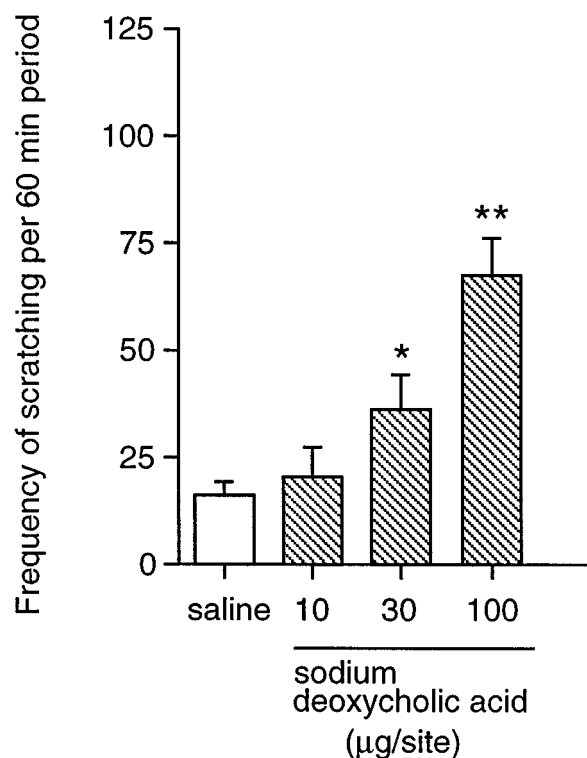


Figure 2 Dose-response for the scratch-inducing effect of sodium deoxycholic acid. Ten to 100 μ g of sodium deoxycholic acid was injected subcutaneously into the anterior part of the back of male ddY mice. The frequency of scratching around the injection site with the fore- and hind-paws was counted for 60 min after the injection. Each value represents mean $\pm$ s.e.mean from 9–15 animals. Comparisons were made with the value from mice receiving 100 μ l of saline indicated by an open column. * $P < 0.05$; ** $P < 0.01$.

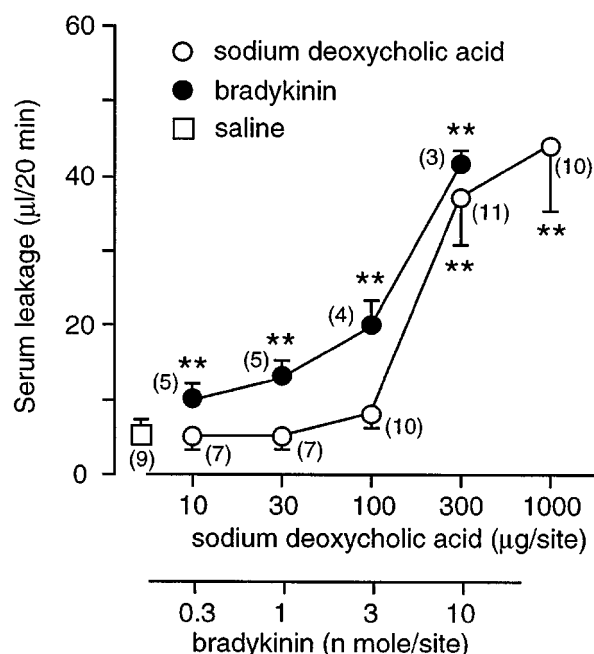


Figure 3 Vascular permeability increase due to sodium deoxycholic acid in mouse skin. Mice anaesthetized with Nembutal (50 mg kg^{-1}) were administered pontamine sky blue (100 mg kg^{-1}) intravenously followed by s.c. injections of sodium deoxycholic acid (10 – $1000 \text{ µg site}^{-1}$) intravenously followed by s.c. injections of sodium deoxycholic acid (10 – $1000 \text{ µg site}^{-1}$), BK (0.3 – $10 \text{ nmol site}^{-1}$), or saline alone (100 µl) into the dorsal skin. At 30 min after the injection, mice were sacrificed to quantify the amounts of dye that leaked into the skin as described in the text. Each value represents the mean $\pm$ s.e.mean. Numbers in parenthesis indicate the numbers of experiments. Comparisons were made with the value from mice receiving 100 µl of saline. $**P < 0.01$.

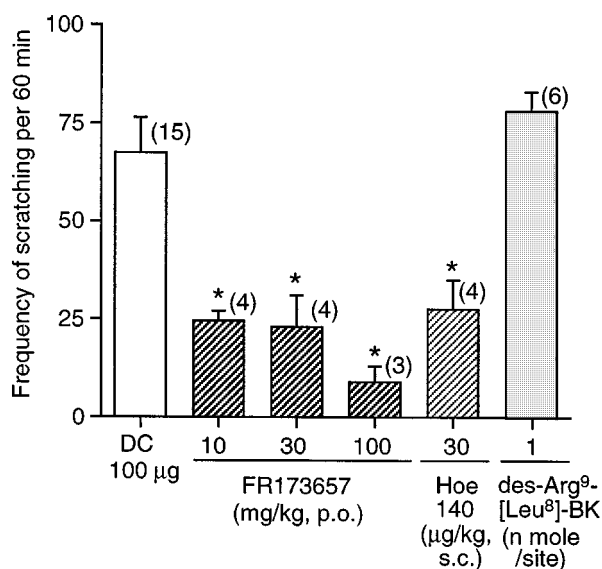


Figure 4 Effects of BK receptor antagonists on scratching induced by sodium deoxycholic acid. FR173657 (10 – 100 mg kg^{-1}) was administered orally 1 h prior to the injection of 100 µg of sodium deoxycholic acid (DC). Hoe140 (30 µg site^{-1}) or des-Arg⁹-[Leu⁸]-BK (1 nmol site^{-1}) was injected subcutaneously together with 100 µg of sodium deoxycholic acid. The frequency of scratching around the injection site by the fore- and hind-paws were counted for 60 min after the injection of sodium deoxycholic acid. Each value represents the mean $\pm$ s.e.mean from 3–15 animals. Numbers in parenthesis are the numbers of mice used. Comparisons were made with the value from mice receiving 100 µg of sodium deoxycholic acid alone, indicated by an open column. $*P \geq 0.05$.

plasma was inhibited by lisinopril *in vitro*, that in skin extract was not (Figure 5b). On the other hand, phosphoramidon inhibited the degradation in skin extract rather than in plasma.

Effects of kallikrein inhibitors on scratching induced by sodium deoxycholic acid

Simultaneous injection of soybean trypsin inhibitor, to inhibit specifically plasma kallikrein, but not tissue kallikrein, together with 100 µg of sodium deoxycholic acid, did not alter the scratching behaviour (Figure 6). Five minutes before the injection of sodium deoxycholic acid, aprotinin was administered intravenously. Aprotinin decreased the frequency of scratching by approximately 60%. The local administration of

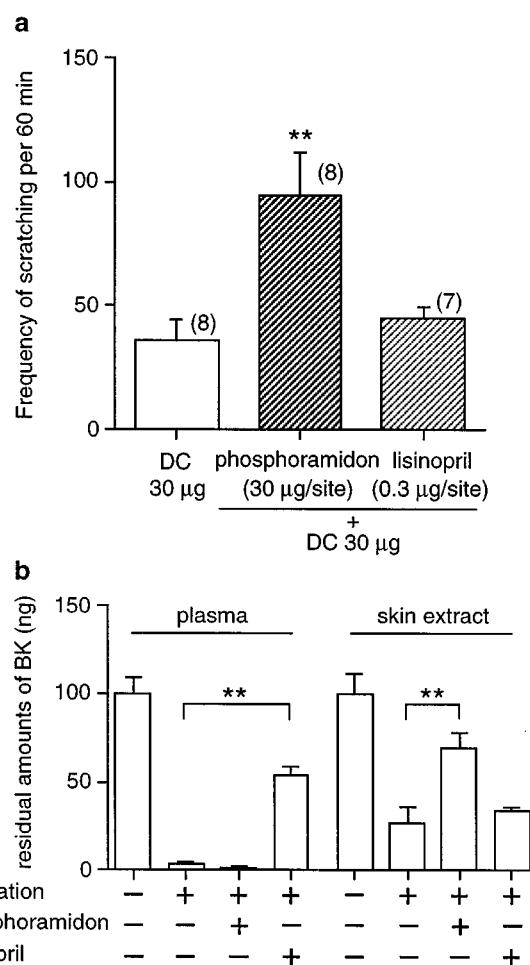


Figure 5 (a) Effects of kininase inhibitors on scratching induced by sodium deoxycholic acid. Phosphoramidon (30 µg site^{-1}) or lisinopril (0.3 µg site^{-1}) was subcutaneously injected together with 30 µg of sodium deoxycholic acid (DC) into the anterior part of the back of the mice. The frequency of scratching around the injection site by the fore- and hind-paws were counted for 60 min after the injection. Each value represents the mean $\pm$ s.e.mean from 7–8 animals. Numbers in parenthesis indicate numbers of mice used. Comparisons were made with the value from mice receiving 30 µg of sodium deoxycholic acid alone, indicated by an open column. (b) Effects of kininase inhibitors on degradation of BK by skin extract or plasma of mice. Skin extract or plasma was incubated with BK for 10 min at 37°C in the presence or the absence of lisinopril (1 µg ml^{-1}). Residual bradykinin in the mixture was measured using BK enzyme immunoassay kit. The ordinate indicates residual amounts of BK in the incubation mixture. Each column represents the mean $\pm$ s.e.mean. $**P < 0.01$.

aprotinin was abandoned, since it precipitated in 0.1% sodium deoxycholic acid.

Consumption of kininogen in the mouse skin injected with sodium deoxycholic acid

In a chromatogram of the extract of the skin injected with saline, kinin release was detected in two fractions, as indicated by the bars in Figure 7a, from fraction numbers 28–30, with higher molecular weights and fraction numbers around 39, with lower molecular weights, respectively. Much more kinin was released from the lower molecular weight fractions than from the higher molecular weight fractions. Also, in a chromatogram of the extract of the skin injection with sodium deoxycholic acid, kinin release was again detected in two categories, fractions with higher, and those with lower molecular weights (Figure 7b). Whereas the amounts of kinin released from the higher molecular weight fractions in the skin injected with sodium deoxycholic acid were almost the same as those in the skin injected with saline, the amount of kinin release from lower molecular weight fractions was markedly less for the skin injected with sodium deoxycholic acid than for that injected with saline.

Comparison of scratching behaviour induced by sodium deoxycholic acid between kininogen-deficient and normal strains of mice

Subcutaneous injection of 100 μ g of sodium deoxycholic acid into the anterior of the back of Brown Norway Kitasato rats also resulted in scratching behaviour similar to that in mice

(Figure 8). However, the frequency of scratching in the Brown Norway Katholiek rats was significantly less than that in the normal, Brown Norway Kitasato rats ($P < 0.01$). Furthermore, when 10 mg kg⁻¹ of FR173657 was administered orally 1 h before the injection of sodium deoxycholic acid to Brown Norway Kitasato rats, the frequency of scratching declined to that in the Brown Norway Katholiek rats. Pretreatment with FR173657 had no effect on scratching in the latter animals.

Discussion

The retention of bile salts is thought to be the cause of pruritus in cholestasis and obstructive jaundice (Herndon & Dallas, 1972). Although the intravenous or oral administration of exogenous cholate failed to produce any itching (Osborn *et al.*,

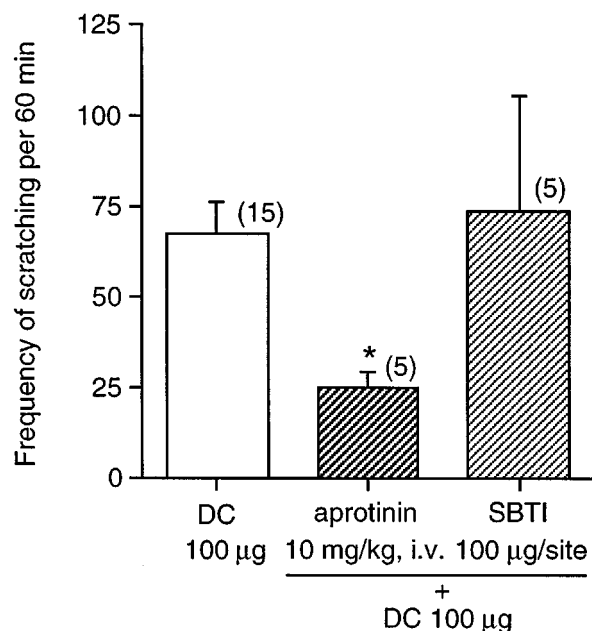


Figure 6 Effects of kallikrein inhibitors on scratching induced by sodium deoxycholic acid. Aprotinin (10 mg kg⁻¹) was administered intravenously 5 min prior to the injection of 100 μ g of sodium deoxycholic acid (DC). Soybean trypsin inhibitor (SBTI; 100 μ g site⁻¹) was subcutaneously injected together with 100 μ g of sodium deoxycholic acid. The frequency of scratching were counted for 60 min after the injection of sodium deoxycholic acid. Each value represents the mean $\pm$ s.e. mean from 5–15 animals. Numbers in parenthesis indicate the numbers of mice used. Comparisons were made with the value from mice receiving 100 μ g of sodium deoxycholic acid alone, indicated by an open column. * $P < 0.05$.

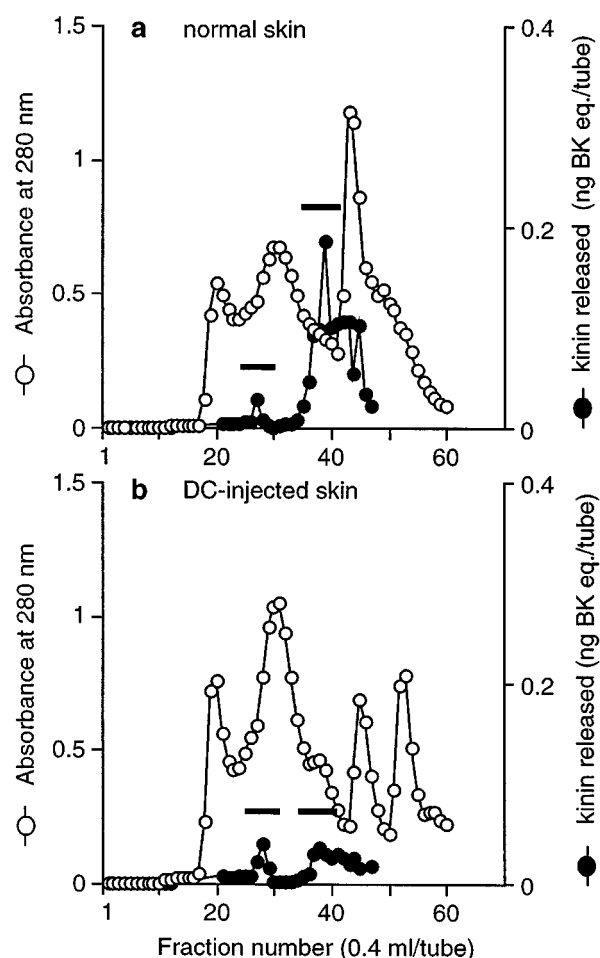


Figure 7 Superose 12 HR 10/30 column chromatography of the extracts from mouse skin. Mice were injected subcutaneously with 100 μ l of saline (a) or 100 μ g of sodium deoxycholic acid (b) into the dorsal skin. Ten pieces of skin were excised from each injection site to prepare skin extracts as described in the text. The extracts were applied to a column of Superose 12 HR 10/30 with the FPLC system to separate high-molecular-weight and low-molecular-weight kininogens in the extracts by gel-filtration column chromatography. Proteins were eluted with Tris-HCl (20 mM) buffer (pH 7.5) containing NaCl (50 mM) at a flow rate of 0.4 ml min⁻¹. A sample of 0.4 ml was fractionated. Absorbance of fractions was measured at 280 nm. The measurement of kinin production in each fraction was done by incubation with trypsin followed by BK enzyme immunoassay using Markitt-M Bradykinin. The amount of released kinin in each fraction was calculated as ng BK equivalent.

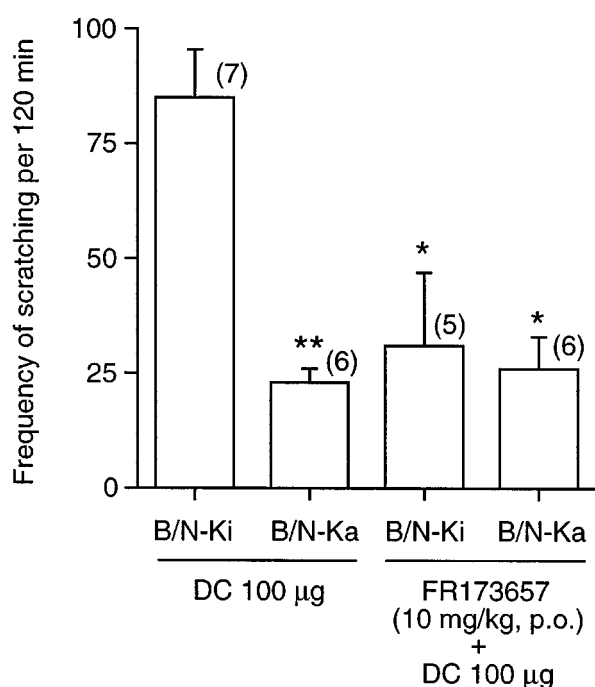


Figure 8 Scratching induced by sodium deoxycholic acid in the kininogen-deficient (B/N-Katholiek) and the normal (B/N-Kitasato) strain of rats. Sodium deoxycholic acid (DC; 100 µg site⁻¹) was injected subcutaneously into the anterior part of the back of female Brown Norway Katholiek (B/N-Ka; kininogens-deficient strain) and Brown Norway Kitasato (B/N-Ki; the normal strain) rats. The frequency of scratching around the injection site by the fore- and hind-paws was counted for 120 min after the injection. Each value represents the mean ± s.e.mean. The numbers in parenthesis indicate the numbers of rats used. The last two columns indicate the result following administered of FR173657 (10 mg kg⁻¹) orally 1 h before the injection of sodium deoxycholic acid. Comparisons were made with the value from Brown Norway Kitasato rats receiving 100 µg of sodium deoxycholic acid shown in the left-hand. * $P < 0.05$; ** $P < 0.01$.

1959; Carey, 1958), application of bile salts to keratin-stripped skin (Varadi, 1974) and to cantharidin-induced blister bases on the forearm (Kirby *et al.*, 1974) induced pruritus in human subjects. Firstly, we tested whether the administration of bile salts has the ability to induce itching in experimental animals as well as in man. Rothman (1941) defined an itch as an unpleasant cutaneous sensation which provokes the desire to scratch in man. The present results indicate that bile salts can elicit scratching in mice as well as in man. The concentrations of sodium deoxycholic acid which induced scratching correspond well with the concentrations at which purified bile salts were effective in producing pruritus in humans (Kirby *et al.*, 1974). In the present model, s.c. injection of up to 100 µg of sodium deoxycholic acid did not cause a significant increase in vascular permeability though 300 µg and greater did. Up to 100 µg of sodium deoxycholic acid had almost no effect on plasma extravasation, suggesting that the contribution of plasma proteins to the scratching behaviour is very small.

Although bile salts liberate small amounts of histamine when perfused into animal skin (Shachter, 1952), and blood histamine levels increase in experimental obstructive jaundice (Anrep & Barsoum, 1953), the poor therapeutic response to antihistamine suggests that histamine is not the major mediator of the pruritus (Krause & Shuster, 1983; Duncan *et al.*, 1984). These observations suggest that endogenous chemical mediators other than histamine might be involved in the pruritus associated with cholestasis (Jones & Bergasa,

1992; Khandelwal & Malet, 1994). Scratching behaviour is reported to be produced by s.c. injection of pruritogenic agents, such as capsaicin and formaldehyde, in mice (Kuraishi *et al.*, 1995). We have presented three pieces of evidence to suggest a partial involvement of kinin in the scratching behaviour induced by sodium deoxycholic acid. Firstly, two BK B₂ receptor antagonists markedly reduced the frequency of the scratching. Since both Hoe140 and FR173657 have been clearly characterized as specifically inhibiting the action of BK by blockade of the B₂ receptor (Wirth *et al.*, 1991; Asano *et al.*, 1997), the reduction of the scratching behaviour by these antagonists indicates the involvement of endogenous BK and the B₂ receptor in this scratching model. Secondly, treatment with phosphoramidon to inhibit neutral endopeptidase, which degrades kinin (Ura *et al.*, 1987; Kuribayashi *et al.*, 1993), significantly augmented the scratching behaviour, suggesting a potentiation of the action of kinin released due to the injection of bile salt. Thirdly, when the production of kinin was suppressed by tissue kallikrein inhibitor, aprotinin (Traut-schold *et al.*, 1967), an apparent reduction of the scratching behaviour was observed, indicating an endogenous production of kinin in this model. Furthermore, to support the participation of kinin in the scratching behaviour in another rodent, we used two strains of rats. A clearly milder scratching response was observed in the kininogen-deficient Brown Norway Katholiek rats than in normal Brown Norway Kitasato rats. In the former rats, neither high-molecular-weight kininogen nor low-molecular-weight kininogen are secreted from the hepatocytes where kininogens are normally biosynthesized, so that almost no kinin release occurs (Hayashi *et al.*, 1985, 1989, 1993; Hayashi & Oh-Ishi, 1993). In addition, the frequency of the scratching in the Brown Norway Kitasato rats was decreased by treatment with FR173657 to almost the same level as was seen in the Brown Norway Katholiek rats. These results indicate the involvement of kinin in the scratching behaviour of normal rats.

It is well known that there are two cascade reactions, i.e., the plasma kallikrein-kinin system and the tissue kallikrein-kinin system (Movat, 1979; Fiedler, 1979). We have already reported that BK released from high-molecular-weight kininogen *via* the activation of the plasma kallikrein-kinin system plays an important role in increasing vascular permeability during some acute inflammatory reactions (Oh-Ishi *et al.*, 1986, 1987; Fujie *et al.*, 1993; Majima *et al.*, 1997). On the other hand, kinin released from low-molecular-weight kininogens *via* activation of the tissue kallikrein-kinin system has the crucial function of mediating the excretion of accumulated sodium ions in the kidneys to maintain the balance of electrolytes in the body (Majima *et al.*, 1991, 1993, 1994b). Thus, these kinin-generating systems are controlled separately and function independently.

Which kinin system plays a role in the scratching behaviour induced by sodium deoxycholic acid was also clarified in the present study. Four results indicate the tissue kallikrein-kinin system is responsible for producing the kinin in the scratching behaviour. Firstly, as mentioned above, up to 100 µg of sodium deoxycholic acid did not induce a significant increase in vascular permeability when subcutaneously injected. Because of less plasma leakage into the injected site, activation of the plasma kallikrein-kinin system at the site is unlikely. Secondly, lisinopril, which inhibits kininase II, did not affect the frequency of the scratching. This result would also suggest to less plasma leakage at the injection site, since kininase II is a dominant kinin-degrading endopeptidase in the circulation rather than in the tissue. In contrast, neutral endopeptidase inhibitor, phosphoramidon

significantly increased the scratching behaviour. This endopeptidase has been reported to be a kinin-degrading enzyme localized in tissues such as the kidney of the rat (Ura *et al.*, 1987). Thirdly, the frequency of scratching was reduced when the production of kinin was suppressed following inhibition of tissue kallikrein activity by aprotinin, whereas soybean trypsin inhibitor, which inhibits plasma kallikrein activity (Wunderer *et al.*, 1972), did not have any effect. Fourthly, gel-filtration experiments showed less kinin release from the fractions eluted as a lower molecular weight of skin extract after sodium deoxycholic acid injection. This suggests the consumption of low-molecular-weight kininogen rather than high-molecular-weight kininogen as this eluted in the fractions with a higher molecular weight. If all of these results are taken together, kinin released from low-molecular-weight kininogen by tissue kallikrein could be involved in part of the scratching behaviour induced by sodium deoxycholic acid.

Bradykinin has often been characterized as a mediator of pain and hyperalgesia through its effects on various neural tissues and sensory neurons (Keele & Armstrong, 1964; Steranka *et al.*, 1988; Dray & Perkins, 1993). On the other hand, intradermal injection of BK or kallikrein on the upper arms was also reported to cause itching in human subjects

(Hägermark, 1974). Whereas one site of action of BK in producing pain has been considered to be the C-fibre nociceptors (Steranka *et al.*, 1988; Lang *et al.*, 1990), the excitation of afferent C-fibres in the skin by the stimulation of spicules of cowhage, which causes itching, has been reported (Tuckett & Wei, 1987a,b). Therefore, it is possible that BK mediates not only pain production but also itch production. The details of the mechanisms of tissue kallikrein-kinin system activation by bile salts and the precise mechanisms that mediate the scratching behaviour remain to be elucidated. However, the observations in the present study also raise the possibility of a therapeutic use of a BK B₂ receptor antagonist as an antipruritic drug.

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Antagonist activity of α -substituted 4-carboxyphenylglycine analogues at group I metabotropic glutamate receptors expressed in CHO cells

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1 We have investigated the antagonist properties of 6 α -substituted phenylglycine analogues based on the structure of 4-carboxyphenylglycine (4-CPG) for group I metabotropic glutamate receptors (mGlu₁ and mGlu₅) permanently expressed in CHO cells.

2 (S)-4-CPG and (S)-MCPG were the most selective mGlu₁ receptor antagonists. Longer chain α -carbon substitutions resulted in a progressive loss of antagonist affinity at mGlu₁ receptors but not at mGlu₅ receptors. Thus mGlu₁ receptor antagonists require small aliphatic groups at the α -position. α -cyclopropyl-4-CPG showed a tendency towards mGlu₅ selectivity, suggesting that bulky groups at this position may favour mGlu₅ receptor antagonism.

3 We demonstrate that the mGlu₅ receptor displays agonist-dependent antagonism. L-glutamate-induced Ca²⁺ release in mGlu₅ receptor expressing cells was more susceptible to antagonism by cyclic α -carbon derivatives than (S)-3,5-dihydroxyphenylglycine (DHPG)-induced Ca²⁺ release in the same cell line.

4 The data presented suggests that mGlu₁ and mGlu₅ receptors have different steric and/or conformational requirements for the binding of antagonists and different amino acids which could interact with agonists.

5 These phenylglycine analogues could provide leads for the development of subtype selective antagonists.

Keywords: Metabotropic glutamate receptor; mGluR; phenylglycine; excitatory amino acid; CHO

Abbreviations: (S)-4-CPG, (S)-4-carboxyphenylglycine; (S)-4C3HPG, (S)-4-carboxy-3-hydroxyphenylglycine; (RS)-CHPG, (RS)-2-chloro-5-hydroxyphenylglycine; (RS)-CyCPG, (RS)- α -cyclopropyl-4-carboxyphenylglycine; (S)-3,5-DHPG, (S)-3,5-dihydroxyphenylglycine; DMEM, Dulbecco's Modified Eagle's Medium; (RS)-ECPG, (RS)- α -ethyl-4-carboxyphenylglycine; FBS, foetal bovine serum; (S)-MCPG, (S)- α -methyl-4-carboxyphenylglycine; mGlu receptor, metabotropic glutamate receptor; NMDA, N-methyl-D-aspartate; (RS)-PeCPG, (RS)- α -pentyl-4-carboxyphenylglycine; (RS)-PhCPG, (RS)- α -phenyl-4-carboxyphenylglycine

Introduction

The mGlu family of glutamate receptors are G-protein-linked receptors currently comprising eight members. These are divided into three groups based on structural homology, pharmacology and signal transduction mechanisms when expressed in clonal cell lines (for review, see Pin & Duvoisin, 1995). Group I mGlu receptors (mGlu₁ and mGlu₅) are linked to phosphoinositide turnover and thus diacylglycerol production and Ca²⁺ mobilization (Tanabe *et al.*, 1992; Abe *et al.*, 1992). These receptors are specifically activated by (S)-3,5-dihydroxyphenylglycine ((S)-3,5-DHPG, (Ito *et al.*, 1992; Schoepp *et al.*, 1994)) and may play a role in long-term potentiation and long-term depression (e.g. Bashir *et al.*, 1993; Bashir & Collingridge, 1994; Cohen & Abraham, 1996; Davis & Laroche, 1996; Fukuda *et al.*, 1997; Manahan-Vaughan, 1997). (S)-3,5-DHPG has also been shown to potentiate NMDA induced depolarizations in the CA1 region of the hippocampus (Harvey & Collingridge, 1993; Fitzjohn *et al.*, 1996) and to induce a novel form of long term depression in the same region (Palmer *et al.*, 1997).

A number of pharmacological tools are available with which to distinguish groups of mGlu receptors and much effort has gone into the synthesis and analysis of phenylglycine derivatives as potential selective agonists and antagonists (Watkins & Collingridge, 1994; Roberts, 1995). (S)-4-carboxyphenylglycine ((S)-4-CPG) and many of its derivatives display antagonist activity at group I mGlu receptors while (S)- α -methyl-4-carboxyphenylglycine ((S)-MCPG) is a well known antagonist at all groups of metabotropic glutamate receptors (Kemp *et al.*, 1994; Bedingfield *et al.*, 1995; Thomsen *et al.*, 1994; Sekiyama *et al.*, 1996). However, only a few compounds have been synthesized which discriminate between individual subtypes within a group (S)-4-CPG and (S)-MCPG have been shown to be more potent antagonists at mGlu₁ than mGlu₅ receptors cloned from both rat and human cDNA libraries (Brabet *et al.*, 1995; Kingston *et al.*, 1995) and (+)-2-methyl-4-carboxyphenylglycine was recently shown to be an antagonist at mGlu₁ receptors (Clark *et al.*, 1997). Such work has also lead to the discovery of (S)-4-carboxy-3-hydroxyphenylglycine ((S)-4C3HPG), a partial agonist selective for mGlu₅ and an antagonist of mGlu₁ receptors (Brabet *et al.*, 1995) as well as (RS)-2-chloro-5-hydroxyphenylglycine ((RS)-CHPG), an mGlu₅ selective agonist (Doherty *et al.*, 1997).

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Most of the phenylglycine derivatives described to date which have activity on the group I mGlu receptors have either hydroxyl or carboxyl substitutes on the phenyl ring. Relatively little work, however, has been carried out using α -substituted analogues (Bedingfield *et al.*, 1995; Sekiyama *et al.*, 1996). These studies have suggested that alkylation of the α -carbon with a methyl or an ethyl group increases the affinity of (S)-4-CPG for mGlu₁ receptors. However, with the exception of (S)-MCPG, no studies to date have been reported showing any discrimination between mGlu₁ and mGlu₅ receptors by such compounds. We have therefore tested a variety of α -substituted derivatives of 4-CPG for their antagonist effects on recombinant mGlu_{1 α} and mGlu_{5 α} receptors permanently expressed in CHO cells (Figure 1). We report that short-chain alkyl substituents maintain the mGlu₁ receptor selectivity of (S)-4-CPG, but that this selectivity is lost when bulkier α -substituents are used. In addition, we report that, in common with the rat mGlu_{1 α} receptor (Brabet *et al.*, 1995), the mGlu_{5 α} receptor displays agonist-dependent antagonism by some α -substituted phenylglycine analogues.

Methods

Synthesis of phenylglycine analogues

(RS)- α -Ethyl-4-carboxyphenylglycine ((RS)-ECPG) was synthesized by the previously reported method (Bedingfield *et al.*, 1995). The α -cyclopropyl-, α -pentyl- and α -phenyl-analogues of 4-carboxyphenylglycine were synthesized in a similar manner. The new phenylglycine analogues had ¹H and ¹³C n.m.r. spectra and elemental analytical data consistent with the proposed structure.

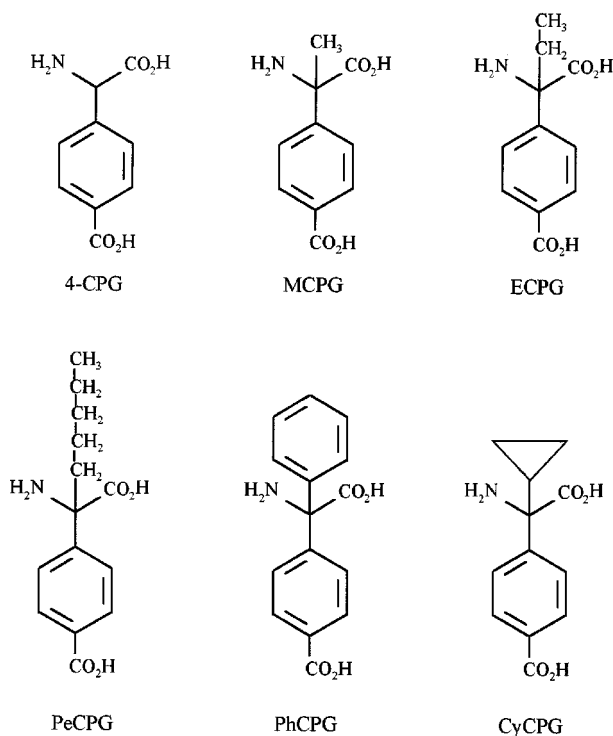


Figure 1 Structures of α -substituted phenylglycine derivatives. Analogues are 4-carboxyphenylglycine (4-CPG); α -methyl-4-CPG (MCPG); α -ethyl-4-CPG (ECPG); α -pentyl-4-CPG (PeCPG); α -phenyl-4-CPG (PhCPG), and α -cyclopropyl-4-CPG (CyCPG).

Cell culture

CHO cells stably expressing mGlu_{1 α} and mGlu_{5 α} receptors were kindly provided by Professor S. Nakanishi and were cultured in Dulbecco's Modified Eagle's Medium (DMEM, 4.5 g l⁻¹ D-glucose) supplemented with 10% dialysed foetal bovine serum (FBS, 10,000 M_r cut-off, Sigma), glutamine (2 mM), 1% L-proline, penicillin (100 U ml⁻¹) and streptomycin (100 μ g ml⁻¹). Cells were maintained at 37°C in a 5% CO₂ humidified atmosphere. For imaging experiments, cells were plated onto glass coverslips (22 mm diameter, 1 thickness, 1–2 $\times$ 10⁵ cells coverslip⁻¹) and used within 48 h.

Ca²⁺ imaging

Cells were washed three times in Krebs buffer (in mM, NaCl 124, KCl 2.9, NaHCO₃ 25, NaH₂PO₄·H₂O 1.4, MgSO₄·7H₂O 1, to which is added 4.5 g l⁻¹ D-glucose and CaCl₂ (2 mM) and loaded with 5 μ M of the membrane permeable Ca²⁺ indicator Fluo3-AM made up in 1 mg ml⁻¹ bovine serum albumin/Krebs at 37°C for 20 min. Cells were then washed three times in Krebs buffer and incubated for 30 min in a 5% CO₂ atmosphere at 22°C in order to allow for de-esterification of the fluorophore. Cells were then viewed on a Bio-Rad MRC600 confocal microscope equipped with an argon ion laser using standard green filter sets and perfused continuously with Krebs buffer at a rate of approximately 2 ml min⁻¹. Kalman integrations of five individual images were obtained every 10 s. Agonists (2 ml) were bath applied while antagonists were pre-incubated for 1 min prior to co-application with agonist. The fluorescence of between five and ten individual cells in each preparation was measured on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nihimage/>) and expressed relative to baseline. The mean peak fluorescence was taken as a measure of mGlu receptor activation.

Data analysis

Dose response profiles were analysed by non-linear least-squares analysis and EC₅₀ values derived using SigmaPlot (Jandel Scientific). K_B values were calculated using the equation $K_B = L/r - 1$ where L is the antagonist concentration and r is the ratio of EC₅₀ values in the presence and absence of antagonist. Unless stated otherwise, results are given as means $\pm$ s.e.mean (n). Statistical analysis was by unpaired *t*-test.

Materials

(S)-4-CPG and (S)-MCPG were obtained from Tocris-Cookson (Bristol, U.K.), DMEM and penicillin/streptomycin solution from GibcoBRL (Paisley, U.K.), and dialysed FBS, L-proline, L-glutamine (cell culture grade) and Fluo3-AM from Sigma Chemical Co. (Poole, U.K.). All other chemicals used were of analytical grade.

Results

Effect of a single concentration of antagonist on L-glutamate-mediated Ca²⁺ release

The ability of 1 mM of each phenylglycine analogue to antagonize L-glutamate (10 μ M)-evoked Ca²⁺ release in cells

expressing mGlu_{1α} and mGlu_{5α} receptors was analysed. In each experiment, control responses to L-glutamate at 1, 10 and 100 μ M were carried out to ensure that the concentration of agonist used was within the linear phase of the dose response profile. The EC₅₀ for L-glutamate-evoked Ca²⁺ release in both these cell lines using this methodology has previously been shown to be 8 μ M (Doherty *et al.*, 1997). The results are shown in Figure 2a,b. (S)-4-CPG was the most selective compound tested, depressing responses to 0.9 ± 0.4 (5) and $73 \pm 10\%$ (4) of control ($P < 0.01$) in mGlu_{1α} and mGlu_{5α} receptor-expressing cells followed by (RS)-ECPG, which reduced responses to 2 ± 1 (4) and $34 \pm 8\%$ (3) of control, respectively ($P < 0.01$). (S)-MCPG was an effective antagonist on both cell lines, completely blocking the responses in mGlu_{1α} receptor-expressing cells in all experiments ($n = 4$) and reducing responses to $6 \pm 3\%$ (4) of control in cells expressing the mGlu_{5α} receptor. Conversely, (RS)-CyCPG was a more effective antagonist in cells expressing mGlu_{5α} receptors than in mGlu_{1α} receptor-expressing cells, depressing responses to 7 ± 4 (3) and $20 \pm 7\%$ (3) of control, respectively, although this did not reach statistical significance.

Effect of a single concentration of antagonist on (S)-3,5-DHPG-mediated Ca²⁺ release in mGlu_{5α} receptor expressing cells

It has previously been reported that the group I mGlu receptors display agonist-dependent antagonism (Brabet *et al.*, 1995). It is therefore of great interest to determine if the antagonist characteristics of these phenylglycine analogues is retained when the commonly used group I mGlu selective agonist (S)-3,5-DHPG is used to elicit the functional response. As with the experiments using L-glutamate as the agonist, a single concentration of antagonist (1 mM) was used to block mGlu_{5α} receptor function mediated by an agonist concentration (50 μ M) near its EC₅₀ value (40 ± 8 μ M (4)). The results are shown in Figure 2c. The ability of (RS)-PhCPG and (RS)-CyCPG to antagonize mGlu_{5α} receptor-mediated Ca²⁺ release was significantly reduced when using (S)-3,5-DHPG as the agonist compared to that evoked by L-glutamate. Responses were reduced to 63 ± 5 (3) and $46 \pm 11\%$ (3) of control respective ($P < 0.05$). In contrast, (S)-4-CPG appeared to a more effective antagonist when (S)-3,5-DHPG rather than L-glutamate was used as agonist, reducing the maximal Ca²⁺ release to $35 \pm 18\%$ (3) of control, although this did not reach statistical significance. No difference was detected in the ability of any other analogue to antagonize mGlu_{5α} receptor function evoked by either agonist.

K_B value determination

The experiments carried out above suggest that modification of the α -carbon alters the ability of the phenylglycine derivative to antagonize mGlu_{1α} and mGlu_{5α} receptor function. In order to investigate this in more detail, the aliphatic series of compounds (S)-4-CPG, (S)-MCPG, (RS)-ECPG and (RS)-PeCPG were chosen for further study. Increasing concentrations of L-glutamate were used to evoke Ca²⁺ release in the absence and presence of 1 mM of each compound. Agonist applications were made in a random fashion. EC₅₀ values derived from the resulting dose response profiles were then used to calculate a K_B value as described in Methods. The results from a representative experiment are shown in Figure 3. (RS)-PeCPG caused a parallel shift in the dose response profile of L-glutamate in cells expressing the mGlu_{5α} receptor with no loss of maximal activity, a response consistent with

competitive antagonism. Similar parallel shifts were shown by all the compounds which showed antagonism. The EC₅₀ values derived from the dose response curves in Figure 3 were 5 ± 2 and 27 ± 11 μ M in the absence and presence of (RS)-PeCPG, respectively giving a K_B value of 312 μ M.

Similar experiments were carried out for each phenylglycine analogue at least three times with cells expressing either mGlu_{1α} or mGlu_{5α} receptors. The results from these experiments are shown in Figure 4. (The K_B values quoted are those for the single active enantiomer of each compound; ECPG and PeCPG were only available as racemic mixtures in a 50:50

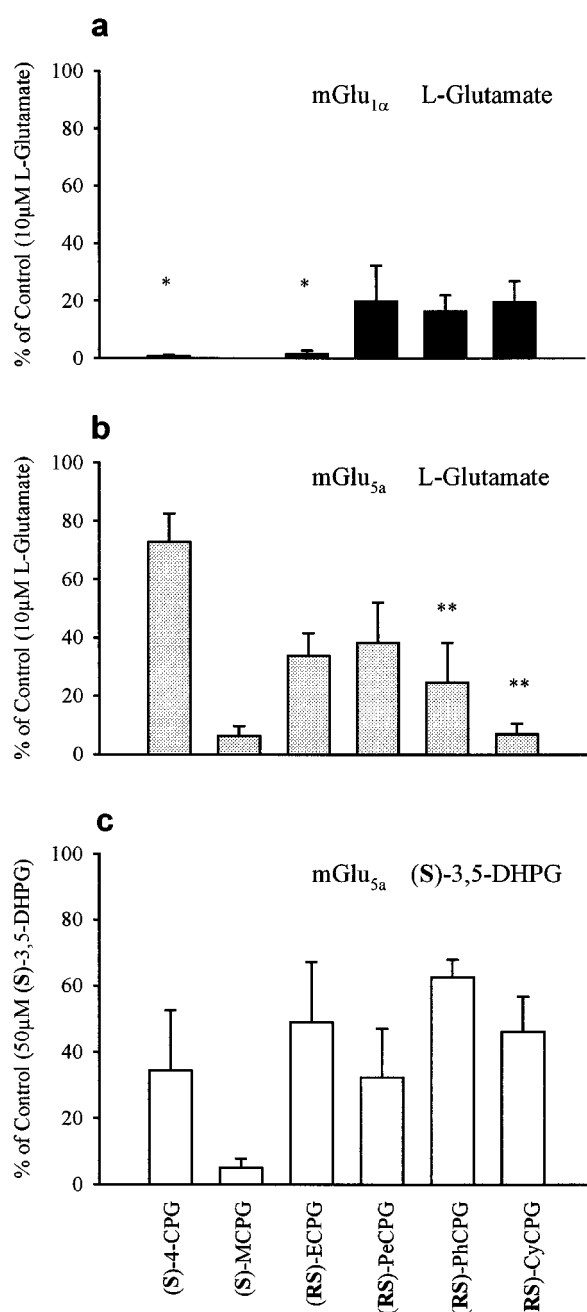


Figure 2 Effects of α -substituted phenylglycine derivatives on agonist-induced Ca²⁺ release in CHO cells expressing either mGlu_{1α} receptors (a) or mGlu_{5α} receptors (b,c). Results are displayed as percentage of control responses (10 μ M glutamate (panels a,b) or 50 μ M (S)-3,5-DHPG (panel c)) following incubation with 1 mM of each antagonist. *Denotes significant difference between mGlu_{1α} and mGlu_{5α} receptor-expressing cells, $P < 0.01$. **Denotes significant difference between effects on L-glutamate and (S)-3,5-DHPG-induced Ca²⁺ release, $P < 0.05$.

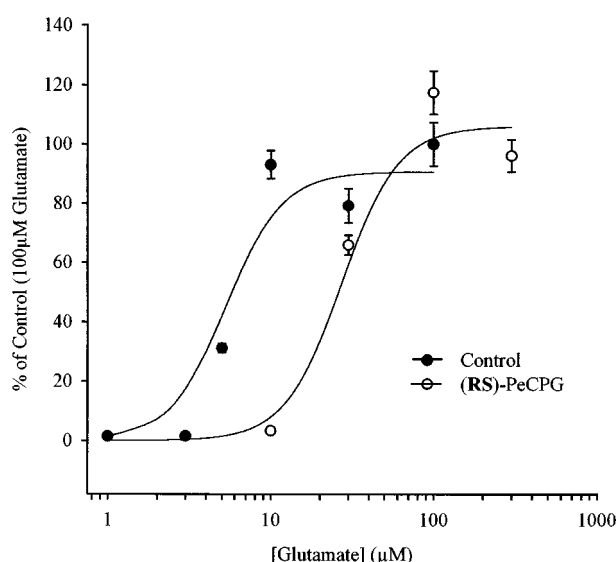


Figure 3 Effect of (RS)-PeCPG on L-glutamate-evoked Ca^{2+} release in CHO cells expressing the mGlu_{5a} receptor. Representative experiment demonstrating the method for determining the K_B value for a single phenylglycine derivative. Increasing concentrations of L-glutamate used to evoke a Ca^{2+} release in the absence and presence of 1 mM (RS)-PeCPG. EC_{50} values derived using non-linear least squares analysis were 5 ± 2 and $21 \pm 11 \mu\text{M}$, respectively giving a K_B value of $312 \mu\text{M}$.

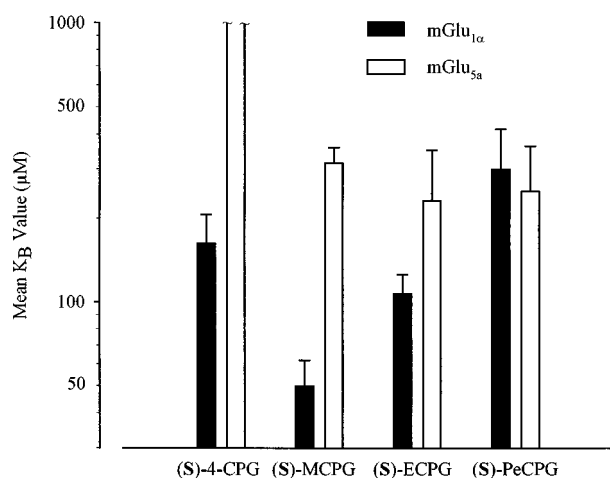


Figure 4 Graphical representation showing K_B values derived for the aliphatic series of α -substituted phenylglycine analogues when used with $\text{mGlu}_{1\alpha}$ receptor- and mGlu_{5a} receptor-expressing cells. K_B values were derived from EC_{50} values calculated from dose responses carried out in the absence and presence of 1 mM of each antagonist. Results are means $\pm$ s.e.mean from three independent experiments carried out on each cell line.

ratio). In common with the results for using a single concentration of antagonist, (S)-4-CPG demonstrated selectively for $\text{mGlu}_{1\alpha}$ -receptor expressing cells ($P < 0.01$), with a K_B value of $163 \pm 43 \mu\text{M}$. In mGlu_{5a} receptor-expressing cells, no shift in the L-glutamate dose response was detected in two out of three preparations. Methylation of (S)-4-CPG to (S)-MCPG induced antagonist activity at mGlu_{5a} receptor-expressing cells ($K_B = 316 \pm 43 \mu\text{M}$) while maintaining the selectivity for $\text{mGlu}_{1\alpha}$ receptors, (K_B value of $50 \pm 12 \mu\text{M}$, $P < 0.05$). Increasing the chain length of the α -carbon substituent had

no further effect on the ability of the phenylglycine analogues to antagonize mGlu_{5a} receptor function. However, in $\text{mGlu}_{1\alpha}$ receptor-expressing cells, increasing the chain length of the substituent group to two and then five carbon atoms results in a progressive lowering of antagonist affinity when used such that (S)-PeCPG has a 6 fold higher K_B value than (S)-MCPG.

Discussion

Selectivity of α -substituted phenylglycine analogues on $\text{mGlu}_{1\alpha}$ and mGlu_{5a} receptors

The aim of this study was to investigate the effects of substitution on the α -carbon of 4-CPG on the ability of this compound to discriminate between $\text{mGlu}_{1\alpha}$ and mGlu_{5a} receptors. The results show that, in common with previous studies (Kingston *et al.*, 1995; Brabet *et al.*, 1995; Bedingfield *et al.*, 1995), both (S)-4-CPG and (S)-MCPG selectively antagonize $\text{mGlu}_{1\alpha}$ receptors over mGlu_{5a} receptors and that (RS)-ECPG is a good antagonist of the $\text{mGlu}_{1\alpha}$ receptor. In addition, we have demonstrated that this $\text{mGlu}_{1\alpha}$ receptor selectivity is maintained in (RS)-ECPG (Figures 2 and 4).

It has previously been reported that (S)-MCPG is a poor antagonist of L-glutamate-induced rat mGlu_{5a} receptor function (Brabet *et al.*, 1995). In contrast, we have found that although (S)-MCPG displays selectivity for $\text{mGlu}_{1\alpha}$ receptors, it is also an effective antagonist of mGlu_{5a} receptors expressed in CHO cells. The reasons for this discrepancy may well be due to the methodologies used in these two studies. Here, we have used cell lines permanently expressing the appropriate receptor, whereas Brabet *et al.* (1995) used receptors transiently expressed in LLC-PK₁ cells, thus potentially giving much higher expression levels of receptor. The K_B for (S)-MCPG antagonism of L-glutamate-evoked $\text{mGlu}_{1\alpha}$ receptor activation derived in that study was 10 fold higher than we find here. In addition, only $500 \mu\text{M}$ (S)-MCPG was used to antagonize the functional response. Thus, under those conditions, any effect of (S)-MCPG on the mGlu_{5a} receptor would probably be insignificant although a small shift is discernible in their data. Interestingly, Kingston *et al.* (1995) found that (S)-MCPG acted as an antagonist at the human mGlu_5 receptor, with an affinity similar to that derived in this study, when using quisqualate as the agonist.

As well as confirming the $\text{mGlu}_{1\alpha}$ receptor selective nature of (S)-4-CPG and (S)-MCPG, this study has extended the analysis to analogues substituted at the α -position with longer chain aliphatic, cyclopropyl or phenyl groups. The data presented here demonstrates that the ability of α -substituted derivatives of 4-CPG to antagonize mGlu receptor activity is dependent on the nature of the substituent group and on the particular receptor subtype involved. Short chain aliphatic substitutions of the α -carbon of 4-CPG produce effective antagonists of $\text{mGlu}_{1\alpha}$ receptors, while bulky cyclic substituent groups lead to analogues with greater mGlu_{5a} selectivity (Figure 2). Thus, $\text{mGlu}_{1\alpha}$ and mGlu_{5a} receptors have different steric and/or conformational requirements for the binding of these analogues and it should therefore be possible to design selective antagonists for each of these subtypes.

A question remains regarding the effects of these compounds on the other subgroups of mGlu receptors. Many phenylglycine derivatives have both agonist and antagonist actions when used with different mGlu receptor subgroups. For example, (S)-4-CPG and (S)-4-carboxy-3-hydroxyphenylglycine ((S)-4C3HPG) are antagonists of group I mGlu receptors but agonists of group II receptors (Hayashi *et al.*,

1994). Previous work has indicated that, in addition to the effects on group I mGlu receptors, (RS)-ECPG is able to antagonize mGlu₂ but not mGlu₆ receptors when expressed in CHO cells (Sekiyama *et al.*, 1996). However, the ability of (RS)-ECPG to antagonize mGlu₂ receptors was reduced compared to mGlu_{1α} receptors, unlike (S)-MCPG which has a similar affinity for both receptors (Hayashi *et al.*, 1994) and is also known to antagonize group III mGlu receptors (Kemp *et al.*, 1994; Bushell *et al.*, 1996). This may make (RS)-ECPG a more useful antagonist for mGlu_{1α} receptors than (S)-MCPG. Little data is available regarding the effects of the other compounds tested here on individual mGlu receptor subtypes, although all the α -substituted compounds used in this study are able to antagonize group II and group III receptors in spinal cord (unpublished observations).

Agonist dependent antagonism

A further important question regarding the ability of compounds to antagonize the group I mGlu receptors is the phenomenon of agonist-dependent antagonism. It has previously been reported that (S)-4-CPG, (S)-MCPG and (S)-4C3HPG were found to display higher affinities for the antagonism of (1S,3R)-ACPD-mediated phosphoinositide turnover in mGlu_{1α}-expressing cells than the same functional response mediated by L-glutamate (Brabet *et al.*, 1995). It was therefore of great interest to determine if the same phenomenon was displayed by the mGlu_{5α} receptor. The antagonist effects of the phenylglycine analogues on (S)-3,5-DHPG-mediated Ca²⁺ release were measured (Figure 2c) and compared to the effects on L-glutamate-mediated Ca²⁺ release (Figure 2b). The results indicate that indeed the ability of phenylglycines to antagonize mGlu_{5α} receptor function is also dependent on the agonist used to elicit the functional response. (S)-4-CPG appeared to be a better antagonist of (S)-3,5-DHPG-evoked Ca²⁺ release in this cell line than of L-glutamate-mediated release (65 and 28% inhibition of control responses, respectively). Conversely, (RS)-CyCPG showed a greater ability to antagonize L-glutamate-evoked Ca²⁺ release than that mediated by (S)-3,5-DHPG (93 and 46% inhibition of control responses, respectively) as did (RS)-PhCPG. This effect is unlikely to be simply a matter of a bulky substituent on the α -carbon as (RS)-PeCPG showed no tendency towards agonist dependency. Thus the cyclic α -substituents may block the interaction of L-glutamate with amino-acids which are critical for binding, but which are less important for the binding of (S)-3,5-DHPG. It may well be, therefore, that the ligand binding domain of subgroup I mGlu receptors contains a number of sites within it capable of interacting with different agonists, thus allowing different modes of agonist binding and giving rise to agonist-dependent antagonism.

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- These data demonstrate the complexity of the pharmacology of the group I mGlu receptors which may provide greater opportunities for the development of subtype selective antagonists. It is also clear that the phenomenon of agonist-dependent antagonism must be taken into consideration in studies using phenylglycine analogues.
- Effects of α -alkylation on phenylglycine antagonism of the mGlu_{1α} receptor*
- In order to investigate the effects of α -alkylation in more detail, experiments were undertaken to measure the affinity of the alkyl series of analogues at both mGlu_{1α} and mGlu_{5α} receptors. The affinities of (S)-4-CPG, (S)-MCPG and (RS)-ECPG for the mGlu_{1α} receptor measured here are in line with previous work using different methodologies (Thomsen *et al.*, 1994; Bedingfield *et al.*, 1995; Kingston *et al.*, 1995). K_B values for the α -alkylphenylglycines showed that alkylation of the α -carbon of 4-CPG with a methyl group increases its affinity at both mGlu_{1α} and mGlu_{5α} receptors, but substitution with longer alkyl chains results in a chain length-dependent loss of affinity at the mGlu_{1α} receptor (Figure 4). In contrast, at the mGlu_{5α} receptor, no significant effect of either ethyl- or pentyl-alkylation could be detected over that produced by methylation. These results show that the effectiveness of phenylglycine analogues as antagonists of group I mGlu receptors is considerably influenced by α -carbon substitution. In this light, it is interesting that the phenyl- and cyclopropyl-derivatives of 4-CPG also showed a reduced ability to antagonize mGlu_{1α} receptor function (Figure 2a) while showing greater effectiveness against the mGlu_{5α} receptor. Thus the loss of antagonist affinity at mGlu_{1α} receptors may be due to steric hindrance caused by bulky α -substituents or may be due to changes to the preferred conformation of the phenylglycines induced by such α -substituents while such changes may increase mGlu_{5α} selectivity.
- The data presented in this paper suggest that the mode of binding of the 4-CPG moiety and thus the ligand binding domains of the mGlu_{1α} and mGlu_{5α} receptors may be very different. The mode of binding and antagonist potency of α -substituted 4-carboxyphenylglycine analogues is dependent on the particular modification made, the agonist chosen to mediate the functional response and the receptor subtype used. In general, short chain aliphatic substitutions retain the mGlu_{1α} receptor selectivity of 4-CPG itself. But this is lost when long chain aliphatic groups or bulky cyclic groups are used. Indeed it may well be that these latter compounds will provide lead compounds from which mGlu₅ selective antagonists may be developed.
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Accelerated intimal hyperplasia and increased endogenous inhibitors for NO synthesis in rabbits with alloxan-induced hyperglycaemia

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1 We examined whether endogenous inhibitors of NO synthesis are involved in the augmentation of intimal hyperplasia in rabbits with hyperglycaemia induced by alloxan.

2 Four weeks after the endothelial denudation of carotid artery which had been performed 12 weeks after alloxan, the intimal hyperplasia was greatly augmented with hyperglycaemia. The degree of hyperplasia was assessed using three different parameters of histopathological findings as well as changes in luminal area and intima:media ratio.

3 There were positive and significant correlations between intima:media ratio, plasma glucose, and concentrations of N^G-monomethyl-L-arginine (L-NMMA) and N^G, N^G-dimethyl-L-arginine (ADMA) in endothelial cells, that is, the intima:media ratio became greater as plasma glucose and endothelial L-NMMA and ADMA were increased. Furthermore, endothelial L-NMMA and ADMA were increased in proportion to the increase in plasma glucose.

4 In contrast, there were inverse and significant correlations between cyclic GMP production by carotid artery strips with endothelium and plasma glucose, between cyclic GMP production and endothelial L-NMMA and ADMA, and between the intima:media ratio and cyclic GMP production.

5 Exogenously applied L-NMMA and ADMA inhibited cyclic GMP production in a concentration-dependent manner. IC₅₀ values were determined to be 12.1 µM for the former and 26.2 µM for the latter. The cyclic GMP production was abolished after the deliberate removal of endothelium from the artery strips.

6 These results suggest that the augmentation of intimal hyperplasia with hyperglycaemia is closely related to increased accumulation of L-NMMA and ADMA with hyperglycaemia, which would result in an accelerated reduction in NO production/release by endothelial cells.

Keywords: Hyperglycaemia; alloxan; accelerated intimal hyperplasia; L-NMMA; ADMA; NO production; endothelial denudation

Abbreviations: ADMA, asymmetric dimethylarginine; IBMX, 3-isobutyl-1-methylxanthine; LCA, left carotid artery; L-NMMA, N^G-monomethyl-L-arginine; RCA, right carotid artery; SMC, smooth muscle cell; SDMA, symmetric dimethylarginine

Introduction

Diabetes mellitus is associated with several cardiovascular complications, predisposing the patient to diseases such as hypertension, atherosclerosis and thrombosis (Christlieb *et al.*, 1976). These diseases have been studied in streptozotocin-induced hyperglycaemic rats or alloxan-induced hyperglycaemic rabbits as diabetic animal models (Taylor *et al.*, 1992; Tesfamariam *et al.*, 1989). It has been reported that atherosclerotic changes, brought about by the accelerated growth of smooth muscle cells (SMCs) are observed in the aorta of alloxan-induced diabetic rabbits (Miller & Wilson, 1984), and that cultured aortic SMCs from alloxan-induced diabetic rabbits grow faster than control SMCs (Alipui *et al.*, 1991). As one of the mechanisms of accelerated cell growth, Kawano *et al.* (1993) and Kanzaki *et al.* (1994) reported that the overexpression of platelet-derived growth factor (PDGF) β -receptors in SMCs of the medial layer played an important role.

Nitric oxide (NO) is reportedly generated in endothelial cells from L-arginine by constitutive NO synthase (Moncada & Higgs, 1993; Förstermann *et al.*, 1994). NO is well known as a potent vasodilator (Furchgott & Zawadzki, 1980), an anti-

aggregatory substance (Azuma *et al.*, 1986; Radomski *et al.*, 1987) and an inhibitor of SMC proliferation (Garg & Hassid, 1989). Azuma and coworkers (1990, 1992; Niimi *et al.*, 1994) have suggested that the neointima formation which follows endothelial denudation of the rabbit carotid artery might be caused, at least partly, by impairment of the ability of endothelial cells to produce/release NO. On the other hand, it has been reported that hyperglycaemia causes endothelial dysfunction (Kamata *et al.*, 1989; Cameron & Cotter, 1992; Taylor *et al.*, 1992). However, few studies have investigated the relationship between accelerated intimal hyperplasia and endothelial dysfunction in diabetes mellitus (Poston & Taylor, 1995).

Vallance *et al.* (1992) have obtained evidence that N^G-monomethyl-L-arginine (L-NMMA) and N^G, N^G-dimethyl-L-arginine (ADMA) play a role as endogenous inhibitors of NO synthesis. Recently, it has been reported that the accumulation of endogenous inhibitors in regenerated endothelial cells is associated with decreased NO production/release and occurrence of intimal hyperplasia after endothelial denudation of the rabbit carotid artery (Azuma *et al.*, 1995) and that the concentration of these inhibitors were increased in plasma of patients with peripheral arterial occlusive disease including diabetes mellitus (Böger *et al.*, 1997).

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Thus, the present experiments were designed to investigate whether hyperglycaemia modifies intimal hyperplasia after endothelial denudation of rabbit carotid artery and whether endogenous inhibitors for NO synthesis contribute to the modification.

Methods

Experimental animals

Thirty-three Japanese White male rabbits were used. These rabbits were purchased at 8 weeks of age and housed in a temperature ($23 \pm 1^\circ\text{C}$) and humidity ($50 \pm 20\%$) controlled room and were fed regular chow (RC-4, Oriental Yeast, Tokyo, Japan) throughout the experimental periods. A single intravenous bolus of alloxan (100 mg kg^{-1}) in $0.9\% \text{ w v}^{-1}$ saline were given *via* the marginal ear vein at 10 weeks of age. Twelve weeks after alloxan, nine normoglycaemic rabbits and 24 hyperglycaemic rabbits were distinguishable. Experiments were performed on these two groups. Animals underwent a unilateral endothelial denudation 12 weeks after alloxan as described below. This study complied with the Animal Welfare Regulation of Tokyo Medical and Dental University and the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society.

Endothelial denudation

Endothelial denudation of the rabbit carotid artery was performed according to the method described previously (Azuma *et al.*, 1990, 1992, 1994; Niimi *et al.*, 1994). In brief, the right common carotid artery was exposed under anaesthesia with sodium pentobarbitone (25 mg kg^{-1} , i.v.) by aseptic surgery. An embolectomy catheter (12-040-3F/40cm/3F; Baxter Healthcare Corporation, Santa Ana, CA, U.S.A.) was then inserted into the artery in retrograde fashion through a small nick made near the carotid bifurcation. The luminal surface of the artery from the origin to the balloon entrance was gently rubbed back and forth with the balloon filled with 0.15 ml of air. This method of removing endothelial cells did not cause deep injury of the vascular wall (Azuma *et al.*, 1992, 1994; Niimi *et al.*, 1994; Hamasaki *et al.*, 1997). After the balloon was removed, the arterial incision was sutured with 10-0 nylon thread, keeping patency of the artery. The left carotid artery (LCA) underwent a sham operation and served as a control. Four weeks after the balloon denudation, animals were anaesthetized with sodium pentobarbitone (25 mg kg^{-1} , i.v.) and exsanguinated *via* the femoral arteries.

Determination of plasma glucose

Blood (0.9 ml) was withdrawn *via* the central ear artery by using a syringe containing 0.1 ml sodium citrate solution ($3.8\% \text{ w v}^{-1}$) once a week for 12 weeks after alloxan and for 4 weeks after endothelial denudation of the carotid artery. Plasma glucose was determined using the OTB (O-Toluidine Boric acid) method (Glucose-Test Wako kit, Wako Pure Chemicals, Tokyo, Japan).

Morphological examination

Approximately 5-mm pieces of the control left (LCA) and hyperplastic right carotid arteries (RCA) which had been isolated from normoglycaemic and hyperglycaemic rabbits were fixed in a $10\% \text{ v v}^{-1}$ neutral solution of formaldehyde

and embedded in paraffin after dehydration with ethanol. Thin sections were stained with hematoxylin-eosin or by the Elastica Van Gieson method for microscopic analysis (Azuma *et al.*, 1990, 1992, 1994; Niimi *et al.*, 1994). To provide a semiquantitative parameter for assessing intimal hyperplasia, the luminal area, intimal area and medial area were determined by means of an image analyzer (Quadra-950, Macintosh) and image scanner (ScanJet IIC, Hewlett Packard) (Obayashi *et al.*, 1996). The ratios (%) of the luminal area to whole area surrounded by the border between medial layer and adventitia, and of the intimal area to the medial area (intima:media ratio) (Obayashi *et al.*, 1996) were calculated.

Determination of L-NMMA and ADMA

The concentrations of N^G -monomethyl-L-arginine (L-NMMA) and N^G , N^G -dimethyl-L-arginine (ADMA) in endothelial cells were determined by means of automated high-performance liquid chromatography according to the method described previously (Azuma *et al.*, 1995, 1997; Hamasaki *et al.*, 1997). To estimate intracellular concentrations of L-NMMA and ADMA, we obtained the equation between the number of endothelial cells (y) and DNA concentration (x) by the least-square method as follows (Hamasaki *et al.*, 1997):

$$y = (14.3x + 6) \times 10^4 \quad (r = 0.9840)$$

From a determination of the DNA concentration in endothelial cells, the number of endothelial cells was calculated. If the intracellular water space is assumed to be 2 pl endothelial cell $^{-1}$ (Baydoun *et al.*, 1990), intracellular concentrations of L-NMMA and ADMA can be calculated.

Determination of cyclic GMP

Ring preparations weighing approximately 4 mg with intact endothelium were cut off from the carotid artery with a razor blade. Cyclic GMP level was determined according to the method described previously (Masuda *et al.*, 1997; Kamikawatoko *et al.*, 1998). In brief, preparations which had been processed without damaging the luminal surface were preincubated in modified Krebs solution for 20 min at 37°C , transferred into fresh Krebs solution and followed by a further 30 min incubation. Preparations were then rapidly transferred into $10\% \text{ w v}^{-1}$ trichloroacetic acid (TCA) with liquid nitrogen in order to stop the reaction. Noradrenaline (NA, 10^{-6} M) and acetylcholine (ACh, $3 \times 10^{-6} \text{ M}$) were added immediately and 15 min after transferring the preparations into the fresh Krebs solution, respectively. All experiments were performed in the presence of 10^{-4} M 3-isobutyl-1-methylxanthine (IBMX) as a non-selective inhibitor of phosphodiesterases. The net production of cyclic GMP was expressed as the difference between the production with NA (10^{-6} M) plus ACh ($3 \times 10^{-6} \text{ M}$) and that with NA (10^{-6} M) plus ACh ($3 \times 10^{-6} \text{ M}$) plus N^G -nitro-L-arginine (10^{-4} M) as an inhibitor of nitric oxide synthase (Kobayashi & Hattori, 1990). The basal cyclic GMP level was taken as the value without any agonist and antagonist expect for IBMX. The protein concentration of the sample was determined by use of the protein assay reagent (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The composition of the modified Krebs solution was as follows (mM): NaCl 118.0 , KCl 4.7 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2 , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 2.5 , KH_2PO_4 1.2 , NaHCO_3 25.0 and glucose 10.0 (pH 7.4).

In order to examine the inhibitory activities of exogenously applied L-NMMA and ADMA, cyclic GMP production

stimulated by NA (10^{-6} M) plus ACh (3×10^{-6} M) was determined in the presence of L-NMMA or ADMA in concentrations of 10^{-6} M– 10^{-4} M using carotid artery strips from an additional three normal rabbits without any treatment. In order to remove endothelial cells, the luminal surface was gently rubbed with a wet cotton swab (Niimi *et al.*, 1994).

Determination of DNA

The microassay of DNA was performed according to the fluorometric method described by Kissane & Robins (1958).

Chemicals

The following chemicals were used in the present experiments: alloxan (Tokyo Kasei Kogyo Co., Tokyo, Japan), acetylcholine chloride (ACh) (Daiichi Pharmaceutical Co., Tokyo, Japan), N^G -nitro-L-arginine (Research Biochemicals Inc., Natick, MA, U.S.A.), and –noradrenaline bitartrate (NA), 3-isobutyl-1-methylxanthine (IBMX), N^G -monomethyl-L-arginine (L-NMMA) and N^G , N^G -dimethyl-L-arginine (ADMA) (all from Sigma Chemical Co. St. Louis, MO, U.S.A.).

Statistical analysis

Results are given as means $\pm$ s.e.mean. The statistical analysis was carried out by Student's *t*-test. Correlation was examined by Pearson's rank correlation coefficient (*r*) and Fisher's *z*-translation (analysis of statistical significance). Regression equation was obtained by least square method.

Results

Baseline data

As shown in Table 1, plasma glucose levels 12 and 16 weeks after the intravenous bolus injection of alloxan at a dose of 100 mg kg^{-1} were not different from the level before alloxan in nine rabbits out of 33 (normoglycaemic group). On the other hand, the remaining 24 rabbits showed significant ($P < 0.005$) hyperglycaemia (hyperglycaemic group), in which plasma glucose was approximately 3 fold higher than the corresponding value in normoglycaemic group and the value prior to alloxan injection. The body weight gain was significantly ($P < 0.005$) decreased in the hyperglycaemic group.

Table 1 Body weight and plasma glucose levels in experimental rabbits immediately before (0) and after alloxan

Group	Weeks	Body weight (kg)	Plasma glucose (mg dl ⁻¹)
NG	0	1.78 ± 0.04 (9)	113 ± 3 (9)
	12	2.75 ± 0.06 (9)	117 ± 11 (7) [†]
	16	2.87 ± 0.07 (9)	104 ± 6 (9)
HG	0	1.82 ± 0.02 (24)	116 ± 3 (24)
	12	$1.88 \pm 0.08^*$ (24)	$379 \pm 21^*$ (24)
	16	$2.00 \pm 0.09^*$ (24)	$373 \pm 17^*$ (24)

Results are given as means $\pm$ s.e.mean of 7–9 determinations for normoglycaemia (NG) and 24 for hyperglycaemia (HG).

*Significant difference at $P < 0.005$ vs corresponding value in the NG group. [†]Two determinations were omitted because of assay trouble.

Intimal hyperplasia

At week 16 (4 weeks after the endothelial denudation), rabbits were killed by exsanguination under anaesthesia with sodium pentobarbitone (25 mg kg^{-1} , i.v.) for the following experiments. A significant intimal hyperplasia, assessed by comparing the percentage luminal area and intima:media ratio (%) between the sham-operated left carotid artery (LCA) and the previously denuded right carotid artery (RCA), and by histopathological examination, was produced after endothelial denudation. The luminal area of RCA in normoglycaemic group was $49.1 \pm 1.1\%$ ($n = 5$), which was significantly ($P < 0.005$) different from the corresponding value in the LCA ($60.0 \pm 2.4\%$, $n = 5$). In the hyperglycaemic group, the reduction of luminal area in RCA was more pronounced ($44.9 \pm 1.1\%$, $n = 5$, $P < 0.05$) than the corresponding value in the normoglycaemic group, although the value of LCA ($56.7 \pm 1.9\%$, $n = 5$) remained unaffected. As shown in Figure 1, the intima:media ratios in LCA and RCA were significantly ($P < 0.005$) greater in the hyperglycaemic group compared with corresponding values in the normoglycaemic group. There were no changes in the medial thickness of both arteries from the two groups.

In the sham-operated LCA, no noticeable histopathological changes could be detected in the normoglycaemic group. However, light microscopy revealed a diffuse and slight proliferation of smooth muscle cells in the intima of LCA from the hyperglycaemic group. In contrast, the proliferation of smooth muscle cells and elastic fibres in the intima was observed clearly in the previously denuded RCA of normoglycaemic group. These changes in RCA became severe in the hyperglycaemic group.

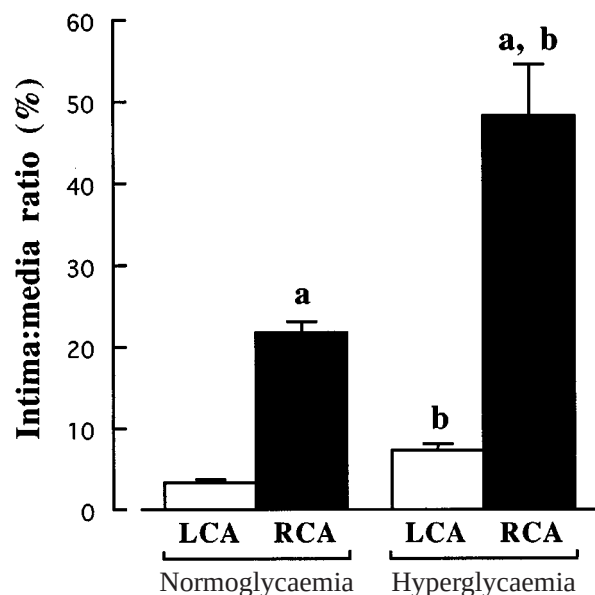


Figure 1 Effect of hyperglycaemia on the intimal hyperplasia after endothelial denudation of rabbit carotid artery. Intimal hyperplasia after endothelial denudation was assessed by comparing the intima:media ratio (%). Results are given as mean of five determinations and vertical bars show s.e.mean. ^aSignificant difference at $P < 0.005$ vs corresponding value in LCA. ^bSignificant difference at $P < 0.005$ vs corresponding value in the normoglycaemia group. LCA, Sham operated left carotid artery; RCA, Previously denuded right carotid artery.

Concentrations of L-NMMA and ADMA in endothelial cells

Concentrations of L-NMMA and ADMA were 5.2 ± 0.1 and $5.0 \pm 0.3 \mu\text{M}$, respectively in the control endothelial cells of the normoglycaemic group. The concentrations in the regenerated endothelial cells (Azuma *et al.*, 1990) from hyperplastic carotid arteries were approximately 2 fold higher than the corresponding values in the control endothelial cells ($P < 0.005$). In hyperglycaemic rabbits, concentrations of L-NMMA and ADMA were significantly increased even in the control endothelial cells. Although the concentrations in the regenerated endothelial cells from hyperplastic arteries tended to be higher than the corresponding values in the normoglycaemic group, there was no significant difference between the corresponding two values. L-NMMA and ADMA were detectable at similar concentrations in endothelial cells of rabbit carotid arteries. These results are shown in Table 2.

Cyclic GMP levels in the carotid artery

In normoglycaemic rabbits, basal levels and stimulated production of cyclic GMP with NA and ACh were 66.9 ± 8.6 and 83.6 ± 12.0 pmoles mg^{-1} protein, respectively. The stimulated production of cyclic GMP was significantly ($P < 0.05$) decreased in the hyperplastic artery strips of this group. In hyperglycaemic rabbits, on the other hand, both basal level and stimulated production of the nucleotide were significantly ($P < 0.005$) decreased even in the sham-operated LCA strips compared with corresponding values in normoglycaemic rabbits. Basal level in the hyperplastic artery strips (RCA) of hyperglycaemic group was significantly ($P < 0.05$) more decreased compared with corresponding value in LCA of the same group (Table 3).

Inhibitory effects of exogenously applied L-NMMA and ADMA on the cyclic GMP production

Cyclic GMP production when stimulated by NA (10^{-6} M) and ACh (3×10^{-6} M) was assessed in the presence of L-NMMA (10^{-6} – 10^{-4} M) or ADMA (10^{-6} – 10^{-4} M). Both L-NMMA and ADMA inhibited cyclic GMP production in a concentration-dependent manner (Figure 2). The 50% inhibitory concentrations (IC_{50}) were $12.1 \mu\text{M}$ for the former and $26.2 \mu\text{M}$ for the latter. In addition, the basal levels and stimulated

production of cyclic GMP with NA and ACh were greatly decreased or abolished after the deliberate removal of endothelium from the artery strips (data not shown).

Relationships between plasma glucose concentration, intima:media ratio, endothelial ADMA or L-NMMA concentration and cyclic GMP production

When there are four different parameters, six combinations to estimate correlation between two parameters should be considered. Results are shown in Figure 3. On the one hand, there were positive and significant correlations between intima:media ratio and plasma glucose, between endothelial ADMA and plasma glucose, and between intima:media ratio and endothelial ADMA, that is, the intima:media ratio became greater as plasma glucose, and endothelial ADMA concentration increased. Furthermore, the endothelial ADMA concentration was increased in proportion to the

Table 3 Basal level and net production of cyclic GMP in the carotid artery (CA) strips from normoglycaemic (NG) and hyperglycaemic (HG) rabbits following alloxan

Group	Blood vessel	Basal level	Net production
NG	LCA	66.9 ± 8.6	83.6 ± 12.0
	RCA	44.5 ± 4.4	$43.6 \pm 3.1^*$
HG	LCA	$17.9 \pm 2.2^\dagger$	$24.8 \pm 1.5^\dagger$
	RCA	$8.3 \pm 3.1^{*\dagger}$	22.7 ± 10.7

Results (pmoles mg^{-1} protein) are given as means $\pm$ s.e.-mean of four determinations. Net production of cyclic GMP was expressed as the difference between the production with 10^{-6} M adrenaline (NA) plus 3×10^{-6} M acetylcholine (ACh) and that with NA (10^{-6} M) plus ACh (3×10^{-6} M) plus N^G -nitro-L-arginine (10^{-4} M) as an inhibitor of nitric oxide synthase. The basal level was given as the value without any agonist and antagonist except for 10^{-4} M 3-isobutyl-1-methylxanthine (IBMX) as a non-selective inhibitor of phosphodiesterases. *Significant difference at $P < 0.05$ vs corresponding value in LCA. † Significant difference at $P < 0.005$ vs corresponding value in the NG group. LCA, Sham operated left carotid artery; RCA, Previously denuded right carotid artery.

Table 2 Concentrations of L-NMMA and ADMA in the endothelial cells from carotid arteries of normoglycaemic (NG) and hyperglycaemic (HG) rabbits

Group	Blood vessel	Concentration (μM)	
		L-NMMA	ADMA
NG	LCA	5.2 ± 0.1	5.0 ± 0.3
	RCA	$11.4 \pm 0.6^*$	$11.0 \pm 0.6^*$
HG	LCA	$12.1 \pm 1.1^{\dagger\dagger}$	$12.1 \pm 1.4^\dagger$
	RCA	13.6 ± 1.7	14.1 ± 2.3

Results (μM) are given as means $\pm$ s.e.-mean of three determinations. The concentrations of methylarginine derivatives were estimated from the equation between DNA concentration and the number of endothelial cells (Hamasaki *et al.*, 1997), and the intracellular water space reported by Baydoun *et al.* (1990). *Significant difference at $P < 0.005$ vs corresponding value in LCA. † and †† Significant difference at $P < 0.01$ and $P < 0.005$ vs corresponding value in the NG group. LCA, Sham operated left carotid artery; RCA, Previously denuded right carotid artery.

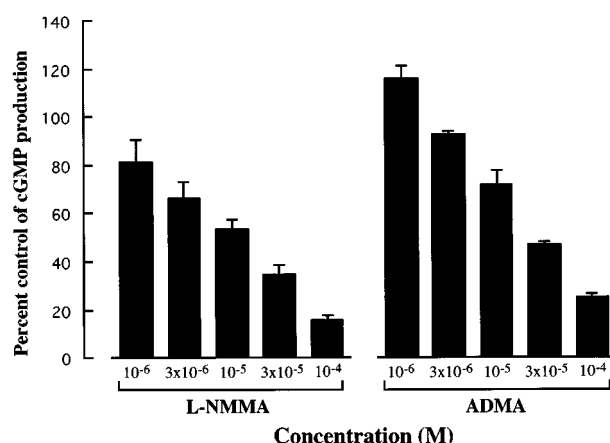


Figure 2 Inhibitory activities of L-NMMA and ADMA on the cyclic GMP production. Results are given as means $\pm$ s.e.-mean of five determinations in the rabbit carotid artery strips with intact endothelium. Cyclic GMP production was stimulated by NA (10^{-6} M) plus ACh (3×10^{-6} M) in the presence of IBMX (10^{-4} M). Control production of cyclic GMP in the absence of NOS inhibitors was determined to be 55.9 ± 1.8 pmoles mg^{-1} protein ($n = 6$).

increase in plasma glucose concentration. There were, on the other hand, inverse and significant correlations between cyclic GMP production and plasma glucose, between cyclic GMP production and endothelial ADMA, and between intima:media ratio and cyclic GMP production, that is, cyclic GMP production was decreased as plasma glucose and endothelial ADMA concentration elevated. In addition, intima:media ratio was increased as cyclic GMP production

was decreased. These correlations could be observed in the previously denuded RCA and sham-operated LCA.

There were also positive and significant ($P < 0.05$ and $P < 0.005$) correlations between endothelial L-NMMA concentration and plasma glucose concentration, and between intima:media ratio and endothelial L-NMMA concentration. In contrast, correlation between cyclic GMP production and endothelial L-NMMA concentration was inverse and sig-

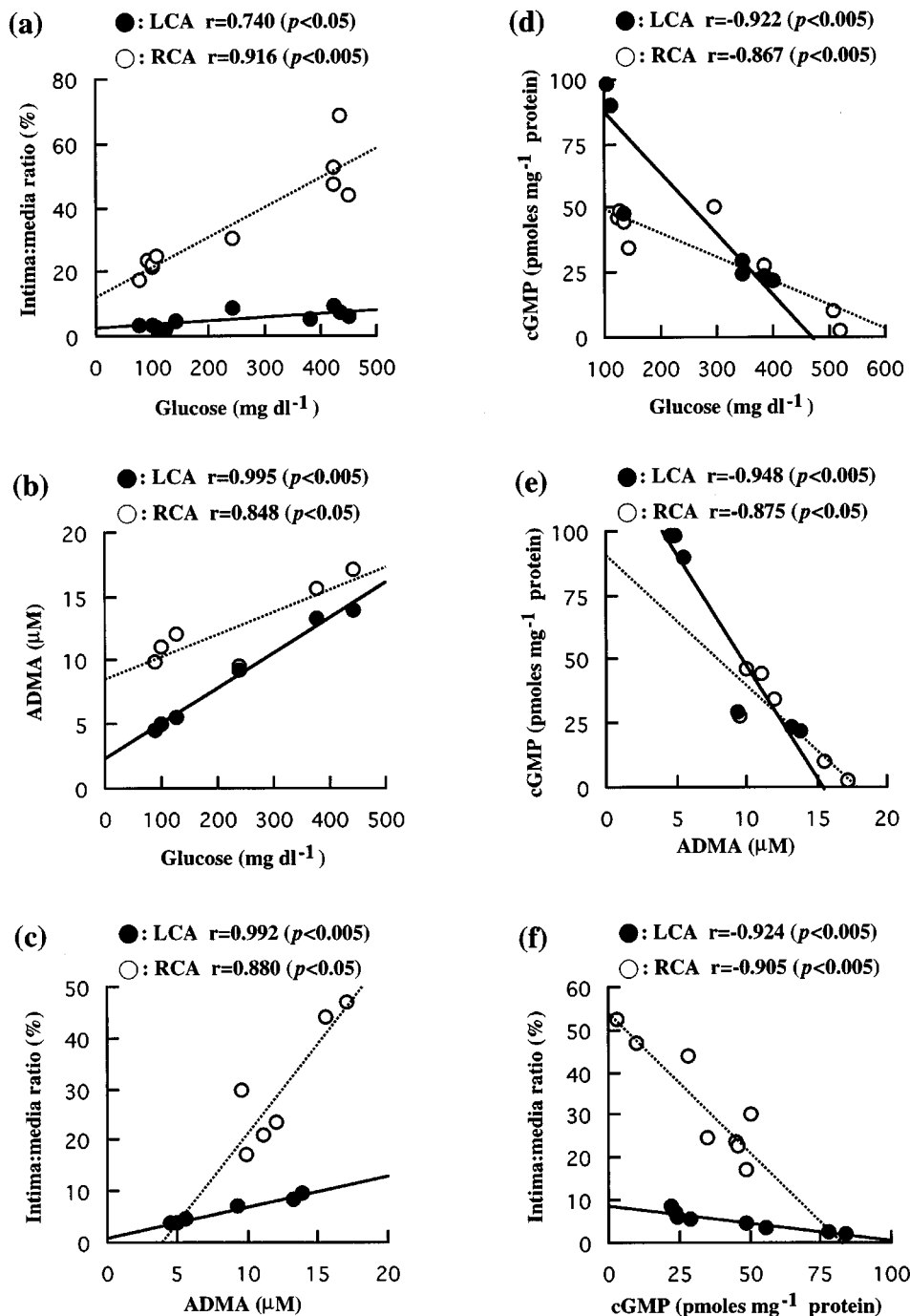


Figure 3 Relationships among four parameters of plasma glucose, intima:media ratio, endothelial ADMA concentration and cyclic GMP production in normoglycaemic and hyperglycaemic rabbits. Results are given as the values at 16 weeks after alloxan (4 weeks after the endothelial denudation). Each value was obtained from an individual rabbit except for ADMA concentration, which was determined in endothelial cells collected from carotid arteries of three rabbits each. The mean values of plasma glucose and intima:media ratio in corresponding three rabbits were used for assessing the correlations to ADMA. Correlation was examined by Pearson's rank correlation coefficient (r) and Fisher's z -translation. LCA, Sham operated left carotid artery; RCA, previously denuded right carotid artery.

nificant ($P < 0.05$ and $P < 0.005$) as in the case of ADMA (data not shown).

Discussion

An intravenous bolus injection of alloxan (100 mg kg^{-1}) induced hyperglycaemia in 24 rabbits out of 33. The remaining nine rabbits showed normoglycaemia, indicating variability in the susceptibility of rabbits to alloxan (Zhao *et al.*, 1987). These two groups of rabbits were used in the present experiments to investigate the effect of hyperglycaemia on the intimal hyperplasia after endothelial denudation of carotid artery.

Intimal hyperplasia was significantly augmented in alloxan-induced hyperglycaemic rabbits (Figure 1). Similar results have been reported by Miller & Wilson (1984), Kawano *et al.* (1993) and Kanzaki *et al.* (1994). Some of these investigators (Kawano *et al.*, 1993; Kanzaki *et al.*, 1994) reported that augmentation of intimal hyperplasia due to hyperglycaemia was accompanied by an overexpression of platelet-derived growth factor (PDGF) β -receptors in the medial smooth muscle cells. However, the mechanism of the augmentation of intimal hyperplasia associated with hyperglycaemia remains obscure.

We have demonstrated that intimal hyperplasia after endothelial denudation may be caused, at least partly, through impairment of the ability of endothelial cells to produce/release NO and that the accumulation of endogenous L-NMMA and ADMA as inhibitors of NO synthesis is associated with decreased NO production/release and, in turn, with the occurrence of intimal hyperplasia (Azuma *et al.*, 1990, 1992, 1994, 1995; Niimi *et al.*, 1994). In contrast, there are many reports that hyperglycaemia causes an endothelial dysfunction in the production of NO (Kamata *et al.*, 1989; Cameron & Cotter, 1992; Taylor *et al.*, 1994). As far as we know, there are no reports investigating whether the impaired endothelial function which might be brought about by increased L-NMMA and ADMA in endothelial cells is involved in the occurrence of the accelerated intimal hyperplasia with hyperglycaemia. In the present experiments, it was found that there were positive and significant correlations between intima:media ratio, plasma glucose, and concentrations of L-NMMA and ADMA in endothelial cells, that is, the intima:media ratio became greater as plasma glucose, and concentrations of L-NMMA and ADMA in endothelial cells increased. Furthermore, the concentrations of L-NMMA and ADMA were increased in proportion to the increase in plasma glucose concentration. In addition to these findings, there were significant and inverse correlations between cyclic GMP production and concentrations of L-NMMA and ADMA in endothelial cells, and between intima:media ratio and cyclic GMP production. Thus, it may be suggested that accelerated intimal hyperplasia associated with hyperglycaemia is closely related to increased concentrations of ADMA and L-NMMA in endothelium and decreased cyclic GMP production resulting from the increase in plasma glucose concentration.

Exogenously applied L-NMMA and ADMA inhibited cyclic GMP production in a concentration-dependent manner (Figure 2), which presumably reflects decreased NO biosynthesis

with these inhibitors. It is well established that NO binds the haem group of soluble guanylate cyclase (Ignarro, 1990a; Chinkers & Garbers, 1991), thereby stimulating the production of cyclic GMP. Consequently, the generation of cyclic GMP is widely used as an index of NO biosynthesis (Moncada *et al.*, 1988; 1991; Ignarro, 1990b; Lüscher *et al.*, 1990). Indeed, the basal level and the stimulated cyclic GMP production with NA and ACh were greatly decreased or abolished after the deliberate removal of endothelium from the artery strips in the present experiments. NO inhibits platelet adhesion and aggregation (Azuma *et al.*, 1986; Radomski *et al.*, 1987) which are considered as an initiating event for the intimal hyperplasia (Ross, 1986; Schwartz *et al.*, 1981). It has also been demonstrated that NO inhibits vascular smooth muscle cell proliferation (Garg & Hassid, 1989). If all of these findings are considered together, they strongly suggest that an increased accumulation of L-NMMA and ADMA as endogenous inhibitors of NO synthesis with hyperglycaemia results in the accelerated decrease in the NO production/release by endothelial cells, which, in turn, brings about the augmentation of intimal hyperplasia.

The mechanisms which regulate the concentrations of L-NMMA and ADMA in endothelial cells are not clarified in the present experiments, although several possibilities can be considered. It has been reported that the transmembrane transport (system y^+) of endogenous methylarginines might alter their concentrations in NO-generating cells (Bogle *et al.*, 1995). Therefore, it may be suggested that hyperglycaemia may increase the uptake of L-NMMA and ADMA by endothelial cells, which, in turn, increases the intracellular concentration of the methylarginines and effectively inhibit NO biosynthesis. This speculation seems to be supported in part by the demonstration that system y^+ activity is increased by high glucose in umbilical vein endothelial cells cultured from gestational diabetic pregnancies (Sobrevia *et al.*, 1998). However, further experiments will be necessary to confirm this hypothesis. Dimethylarginine dimethylaminohydrolase (DDAH), an enzyme that metabolizes L-NMMA and ADMA, is localized in endothelial cells (MacAllister *et al.*, 1996). Hyperglycaemia may inhibit the activity of this enzyme, resulting in a high level of L-NMMA and ADMA in endothelial cells. This possibility may be supported partly by findings that the concentration in endothelial cells of N^G , N^G -dimethyl-L-arginine (SDMA), which is not a substrate for the metabolizing enzyme (Ogawa *et al.*, 1989), remained unaltered in all groups examined in the present experiments (data not shown).

In conclusion, the augmentation of intimal hyperplasia associated with hyperglycaemia seems to be closely related to the increased accumulation of endothelial endogenous inhibitors of NO synthesis which may result in reduced NO production/release by endothelial cells.

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Acetylcholine-induced relaxation in blood vessels from endothelial nitric oxide synthase knockout mice

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1 Isometric tension was recorded in isolated rings of aorta, carotid, coronary and mesenteric arteries taken from endothelial nitric oxide synthase knockout mice (eNOS(–/–) mice) and the corresponding wild-type strain (eNOS(+ / +) mice). The membrane potential of smooth muscle cells was measured in coronary arteries with intracellular microelectrodes.

2 In the isolated aorta, carotid and coronary arteries from the eNOS(+ / +) mice, acetylcholine induced an endothelium-dependent relaxation which was inhibited by N^ω-L-nitro-arginine. In contrast, in the mesenteric arteries, the inhibition of the cholinergic relaxation required the combination of N^ω-L-nitro-arginine and indomethacin.

3 The isolated aorta, carotid and coronary arteries from the eNOS(–/–) mice did not relax in response to acetylcholine. However, acetylcholine produced an indomethacin-sensitive relaxation in the mesenteric artery from eNOS(–/–) mice.

4 The resting membrane potential of smooth muscle cells from isolated coronary arteries was significantly less negative in the eNOS(–/–) mice (-64.8 ± 1.8 mV, $n=20$ and -58.4 ± 1.9 mV, $n=17$, for eNOS(+ / +) and eNOS(–/–) mice, respectively). In both strains, acetylcholine, bradykinin and substance P did not induce endothelium-dependent hyperpolarizations whereas cromakalim consistently produced hyperpolarizations (-7.9 ± 1.1 mV, $n=8$ and -13.8 ± 2.6 mV, $n=4$, for eNOS(+ / +) and eNOS(–/–) mice, respectively).

5 These findings demonstrate that in the blood vessels studied: (1) in the eNOS(+ / +) mice, the endothelium-dependent relaxations to acetylcholine involve either NO or the combination of NO plus a product of cyclo-oxygenase but not EDHF; (2) in the eNOS(–/–) mice, NO-dependent responses and EDHF-like responses were not observed. In the mesenteric arteries acetylcholine releases a cyclo-oxygenase derivative.

Keywords: Cyclo-oxygenase products; EDHF; endothelium; eNOS knockout mice; nitric oxide; smooth muscle

Abbreviations: EDHF: endothelium-derived hyperpolarizing factor; eNOS: endothelial nitric oxide synthase; eNOS(–/–) mice: endothelial nitric oxide synthase knock-out mice; eNOS(+ / +) mice: wild-type strain mice; L-NA: N^ω-nitro-L-arginine

Introduction

The production by the endothelial cells of vasoactive substances, such as nitric oxide (NO; Furchgott & Zawadzki, 1980; Palmer *et al.*, 1987), prostacyclin (Moncada & Vane, 1979) and a still unidentified molecule, endothelium-derived hyperpolarizing factor (EDHF; Chen *et al.*, 1988; Félétou & Vanhoutte, 1988; Taylor & Weston, 1988), contributes to the local regulation of vascular tone. The relative importance of these factors for endothelium-dependent vasodilation depends on the size of the blood vessels and the vascular bed investigated. In resistance arteries, the contribution of EDHF to endothelium-dependent vasodilation may be major (Nagao *et al.*, 1992; Bauersachs *et al.*, 1996; Garland & Plane, 1996; Shimokawa *et al.*, 1996).

Chronic inhibition of nitric oxide synthase (NOS) has increased vascular resistance in all species studied. This was associated with an increase in blood pressure in rats and pigs but no such changes in rabbits and dogs (Oliveira *et al.*, 1992; Cayatte *et al.*, 1994; Ito *et al.*, 1995; Puybasset *et al.*, 1995).

However, since inhibitors of NO production are not selective, and the three isoforms of NOS are inhibited during chronic treatment with those drugs, the cardiovascular changes observed are not necessarily linked to the inhibition of the endothelial isoform. In contrast, disruption of the gene encoding the eNOS in mice produces a specific suppression of endothelial NO production and an increase in systemic blood pressure (Huang *et al.*, 1995).

EDHF may act as a back-up mechanism when the endothelial production of NO is impaired (Kilpatrick & Cocks, 1994; Corriu *et al.*, 1998). Conversely, NO may regulate the production and the effect of EDHF (Olmos *et al.*, 1995; Mombouli *et al.*, 1996a; Popp *et al.*, 1996; Kristof *et al.*, 1997). The purpose of the present work was to address these two possibilities by studying endothelium-dependent relaxations and hyperpolarizations in isolated arteries of mutant eNOS(–/–) mice and the corresponding control wild-type strain (eNOS(+ / +) mice).

Methods

C57BL6 mice (wild-type strain; eNOS(+ / +) mice) and homozygous mutant mice lacking the gene for endothelial

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NOS (eNOS(-/-), Iffa Credo, L'Arbresle, France), 8–10 weeks of age, were used for the study. The generation of eNOS deficient mice has been described elsewhere (Huang *et al.*, 1995). The mice were maintained under pathogen-free conditions in filter-topped cages in an air-conditioned room at $21 \pm 1^\circ\text{C}$, fed a standard laboratory diet and given water *ad libitum*.

Contraction

eNOS (+/+) and eNOS(-/-) mice were killed by CO_2 inhalation. The aorta and the carotid, left anterior coronary and mesenteric (first order) arteries were removed and dissected free of adherent connective tissues. Aortic rings (4 mm in length) were suspended in organ chambers and segments of the other vessels (2 mm in length for carotid and mesenteric arteries, 1.5–3 mm for the coronary artery depending on the branching pattern) were mounted in a microvessel myographs for isometric tension recording (Mulvany & Halpern, 1977). Blood vessels were immersed in Krebs' Ringer bicarbonate solution (37°C , aerated with a 95% O_2 /5% CO_2 gas mixture, pH 7.4) of the following composition (in mM): NaCl 118.3; KCl 4.7; CaCl_2 2.5; MgSO_4 1.2; KH_2PO_4 1.2; NaHCO_3 25; calcium-disodium EDTA 0.026 and glucose 11.1 (control solution). Segments were stretched step by step and contracted repeatedly with KCl (60 mM) until maximal and reproducible contractions were obtained (Félétou and Teissere 1990). A passive tension of approximately 1000 mg was exerted on the aortas and 100–200 mg on the other arteries. Under these conditions, the imposed diameter of the carotid, mesenteric and coronary arteries was 250, 120 and 70 μm , respectively. After a 45 min equilibration period, endothelium-dependent relaxations to acetylcholine were studied. Aortic rings were contracted with noradrenaline (1–10 μM) and carotid arteries with U 46619 (10–30 nM), in order to achieve a level of tension representing 50–80% of the reference contraction to KCl (60 mM). Cumulative concentration-response curves (1 nM or 10 nM to 100 μM) were performed in these two arteries. Successive concentrations of acetylcholine were applied after stabilization of the relaxation or every 5 min. Murine mesenteric and coronary arteries were contracted with noradrenaline (3 μM) and U 46619 (100 nM), respectively. In preliminary experiments, complete acetylcholine-relaxation curve in mesenteric and coronary arteries were tentatively performed. A sustained level of tension, in all the murine arteries studied, was difficult to obtain. In contrast to the aorta and the carotid artery, in which acetylcholine-induced relaxation could easily be differentiated from a spontaneous decrease in tone, in the mesenteric and coronary arteries the maximal amplitude of acetylcholine-induced relaxation represented only 30–50% of the precontracted level. By using a single, supramaximal concentration of acetylcholine (10 μM), it was easier to evaluate the effects of N^ω -nitro-L-arginine (L-NA) or/and indomethacin. Indeed, the effect of this single concentration of acetylcholine produced fast and sharp relaxation which could not be mistaken for a spontaneous decrease in tone. The incubation time with the various inhibitors studied (L-NA and/or indomethacin) was at least 30 min. At the end of the experiment, maximal relaxation was obtained with sodium nitroprusside (1 mM). In some arterial rings the endothelium was mechanically removed (aorta: with jeweller forceps; other arteries: by inserting a human hair into the lumen) to verify the endothelium-dependency of the relaxation induced by acetylcholine (10 μM).

Electrophysiology

Segments of left anterior descending coronary artery (luminal diameter: 70–90 μm) were pinned down to the bottom of an organ chamber (0.5 ml in volume) and superfused at a constant flow (2.5 ml min^{-1}) with modified Krebs' Ringer bicarbonate solution. Care was taken to preserve the integrity of the endothelium. In preliminary experiments, the preservation of the endothelial lining was verified histologically in preparations fixed in Bouin solution and stained with either haematein and eosin or Masson trichrome at the end of the electrophysiological study (data not shown). Smooth muscle cell impalements were performed from the adventitial side of the vessels. The transmembrane potential was recorded with glass capillary microelectrodes (tip resistance of 30–90 M Ω) filled with KCl (3 M) connected to the headstage of a recording amplifier (Intra 767, World Precision Instruments Ltd, New Haven, CT, U.S.A.) equipped with capacitance neutralization; an Ag/AgCl pellet, in contact with the bath solution and directly connected to the amplifier, served as the reference electrode. The electrophysiological signal was continuously monitored on an oscilloscope (3091 Nicolet, Madison, WI, U.S.A.) and simultaneously recorded on paper (Gould, Valley View, OH, U.S.A.) and on a video recorder (TEAC XR310, Tokyo, Japan). Successful impalements were signalled by a sudden negative drop in potential from the baseline (zero potential reference) followed by a stable negative potential for at least 3 min.

Drugs

The drugs used were: noradrenaline hydrochloride, acetylcholine chloride, bradykinin, indomethacin, N^ω -nitro-L-arginine (L-NA), sodium nitroprusside (Sigma, La Verpillère, France); substance P (Bachem, Voisins-Le-Bretonneux, France); U 46619 (9,11-dideoxy-9 α , 11 α -methanoepoxyprostaglandin $\text{F}_{2\alpha}$, Cayman Chemical Company, Ann Arbor, MI, U.S.A.). Cromakalim and SIN-1 were synthesized in the chemistry department of the Servier Research Center (Suresnes, France). Indomethacin was dissolved in deionized water with an equimolar concentration of Na_2CO_3 . Cromakalim was dissolved in equivalent volumes of deionized water and propylene glycol. SIN-1 was prepared in methanol. U 46619 was solubilized in ethanol. Final concentrations of propylene glycol, methanol and ethanol in the bath were respectively 6.8, 25 and 0.2 mM. The other drugs were dissolved in distilled water.

Statistics

Data are shown as mean $\pm$ s.e.mean. *n* indicates the number of animals from which arteries were taken. Statistical analysis was performed using Student's *t*-test for paired or unpaired observations. Differences were considered to be statistically significant when *P* was less than 0.05.

Results

Contraction

The contractions induced by KCl (60 mM) were not significantly different in arteries from eNOS(+/+) and eNOS(-/-) mice (contraction, in arteries from eNOS(+/+) and eNOS(-/-) mice, respectively: aorta: $910 \pm 110 \text{ mg}$, $n = 13$ and $890 \pm 210 \text{ mg}$, $n = 11$; carotid arteries: $52 \pm 13 \text{ mg}$, $n = 5$ and $77 \pm 18 \text{ mg}$, $n = 6$; coronary arteries: $20 \pm 3 \text{ mg}$, $n = 17$

and 21 ± 3 mg, $n=17$; mesenteric arteries: 54 ± 5 mg, $n=18$ and 54 ± 5 mg, $n=20$).

Isolated rings of aorta and carotid arteries from both strains were studied in control solution or in the presence of L-NA (100 μ M), indomethacin (5 μ M), or the combination of both inhibitors. Aortic rings were contracted with noradrenaline (1–10 μ M) and carotid arteries with U 46619 (10–30 nM), in order to achieve a level of tension representing 50–80% of the reference contraction to KCl (60 mM). Acetylcholine (10 nM to 100 μ M) induced endothelium-dependent relaxations in aortas and carotid arteries from the eNOS(+/+) mice but not in those from eNOS(–/–). In the eNOS(+/+), these relaxations were unaffected by indomethacin but were blocked by L-NA or by the combination of the two inhibitors (Figure 1 and 2). In the eNOS(–/–), the decrease in tension was not significantly different from that observed in time-matched controls (data not shown).

Mesenteric arteries were contracted with noradrenaline (3 μ M). The contractile response was significantly larger in mesenteric arteries from the eNOS(–/–) mice (48 ± 4 mg, $n=33$ and 67 ± 7 mg, $n=31$, in eNOS(+/+) and eNOS(–/–) mice, respectively, $P<0.05$). The addition of L-NA (100 μ M) induced a further increase in tension in mesenteric arteries from eNOS(+/+) but not in eNOS(–/–) mice ($+14 \pm 7$ mg, $n=7$ and -8 ± 6 mg, $n=8$ in eNOS(+/+) and eNOS(–/–) mice, respectively). Indomethacin (5 μ M) decreased the contractile response to noradrenaline in eNOS(+/+) mice but did not influence the level of tension in the eNOS(–/–) mice. The combination of L-NA and indomethacin provoked an increase in tension in arteries in eNOS(+/+) mice but not

in eNOS(–/–) mice ($+13 \pm 4$ mg, $n=10$ and -3 ± 11 mg, $n=7$ in eNOS(+/+) and eNOS(–/–) mice, respectively). Acetylcholine (10 μ M) induced relaxation in the mesenteric arteries from both strains. In the wild-type strain the relaxation to acetylcholine was not significantly affected by indomethacin or L-NA but was abolished by the combination of the two inhibitors. In mesenteric arteries from eNOS(–/–) mice, acetylcholine-induced relaxation was not affected by L-NA but was significantly inhibited either by indomethacin or the combination of both inhibitors (Figure 3).

Coronary arteries were contracted with U 46619 (100 nM). The contractile response was significantly larger in coronary arteries from the eNOS(–/–) mice (27 ± 3 mg, $n=25$ and 45 ± 7 mg, $n=28$, in eNOS(+/+) and eNOS(–/–) mice, respectively). The addition of L-NA (100 μ M) induced a further increase in tension in coronary arteries in eNOS(+/+) but not in eNOS(–/–) mice ($+17 \pm 4$ mg, $n=5$ and $+2 \pm 1$ mg, $n=6$ in eNOS(+/+) and eNOS(–/–) mice, respectively). Indomethacin (5 μ M) did not significantly affect the contractile response to U 46619 in either strain. The combination of L-NA and indomethacin provoked an increase in tension in eNOS(+/+) mice but not in eNOS(–/–) mice ($+8 \pm 1$ mg, $n=7$ and -6 ± 2 mg, $n=6$ in eNOS(+/+) and eNOS(–/–) mice, respectively). Acetylcholine (10 μ M) induced relaxation in the coronary artery from eNOS(+/+) mice. This relaxation was unaffected by indomethacin but was inhibited by L-NA or by the combination of both inhibitors. In coronary arteries from eNOS(–/–) mice, acetylcholine did not produce any significant relaxations either in control or in presence of inhibitors (Figure 4).

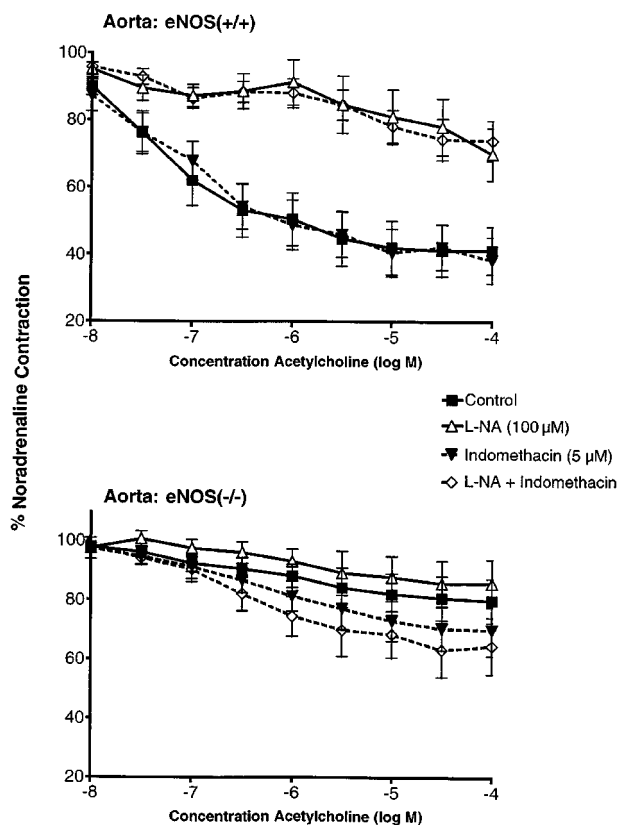


Figure 1 Concentration-relaxation curves to acetylcholine in aortas with endothelium from eNOS(+/+) (upper panel; $n=13$) and eNOS(–/–) mice (lower panel; $n=11$) in the absence and presence of indomethacin (5 μ M) and/or N^G -nitro-L-arginine (L-NA, 100 μ M). Data are shown as means $\pm$ s.e.mean.

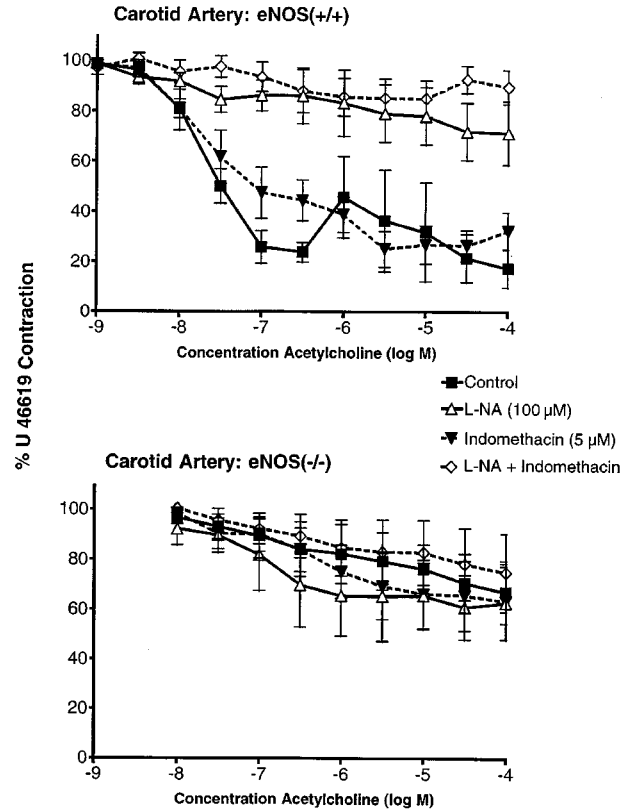


Figure 2 Concentration-relaxation curves to acetylcholine in carotid arteries with endothelium from eNOS(+/+) (upper panel; $n=5-6$) and eNOS(–/–) mice (lower panel; $n=4-5$) in the absence and presence of indomethacin (5 μ M) and/or N^G -nitro-L-arginine (L-NA, 100 μ M). Data are shown as means $\pm$ s.e.mean.

Sodium nitroprusside (1 mM) induced complete relaxation in all the arteries studied from both strains (data not shown).

Electrophysiology

Under control conditions, the resting membrane potential of eNOS(+/+) mice coronary arterial smooth muscle cells was not altered significantly by the presence of L-NA and/or indomethacin (Table 1). In eNOS(-/-) mice, under control conditions, the resting membrane potential of the coronary arterial smooth muscle cells was significantly less negative. This value was not altered significantly by the presence of L-NA and/or indomethacin (Table 1).

In coronary smooth muscle cells from eNOS(+/+) mice, acetylcholine (1 μ M) did not modify the resting membrane potential in the absence or the presence of L-NA and/or indomethacin. At a higher concentration (10 μ M), acetylcholine induced a depolarization of the smooth muscle cells in approximately 50% of the cells (five depolarizations in nine blood vessels studied) in control solution and in the presence of

L-NA (Table 2) which was in some cases accompanied by a rhythmic activity (Figure 5). The depolarization was observed in only one artery treated with indomethacin or L-NA plus indomethacin (one depolarization in seven blood vessels studied; Table 2). Substance P (100 nM) and bradykinin (100 nM) did not influence the resting membrane potential. In two smooth muscle cells out of twelve, SIN-1 (10 μ M) induced a marked hyperpolarization (-5 and -9 mV) but no significant changes in the other cells (data not shown). In contrast, cromakalim (10 μ M) induced a significant hyperpolarization under the various experimental conditions (Figure 5, Table 2).

In coronary smooth muscle cells from eNOS(-/-) mice, the effects of acetylcholine (1 μ M) bradykinin (100 nM), substance P (100 nM), SIN-1 (10 μ M) and cromakalim (10 μ M) were qualitatively and quantitatively similar to those observed in eNOS(+/+) mice (Figure 5, Table 2). Acetylcholine (10 μ M) induced a depolarization of the smooth muscle cells in six out of seven blood vessels studied, either under control conditions or in the presence of L-NA (Table 2), which was in

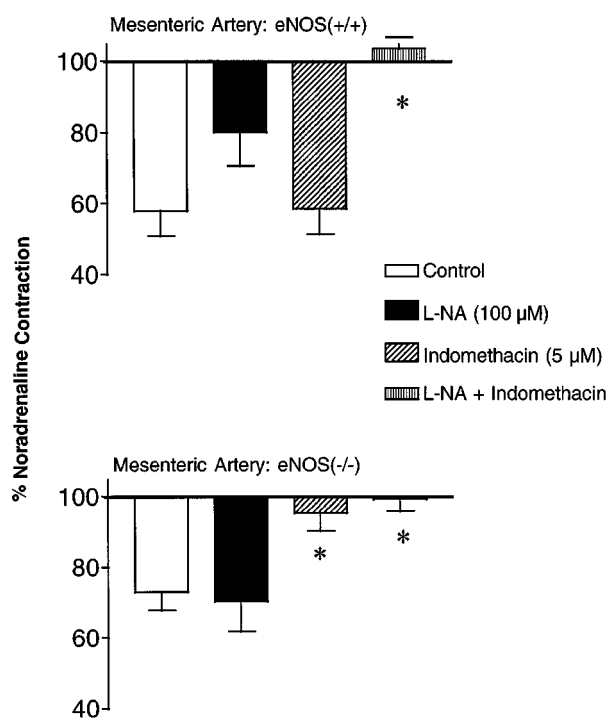


Figure 3 Acetylcholine (10 μ M)-induced relaxations in mesenteric arteries with endothelium from eNOS(+/+) (upper panel; $n=8-10$) and eNOS(-/-) mice (lower panel; $n=8-9$), in the absence and presence of indomethacin (5 μ M) and/or N^G-nitro-L-arginine (L-NA, 100 μ M). Data are shown as means $\pm$ s.e.mean. Asterisks indicate a statistically significant difference with the corresponding control value.

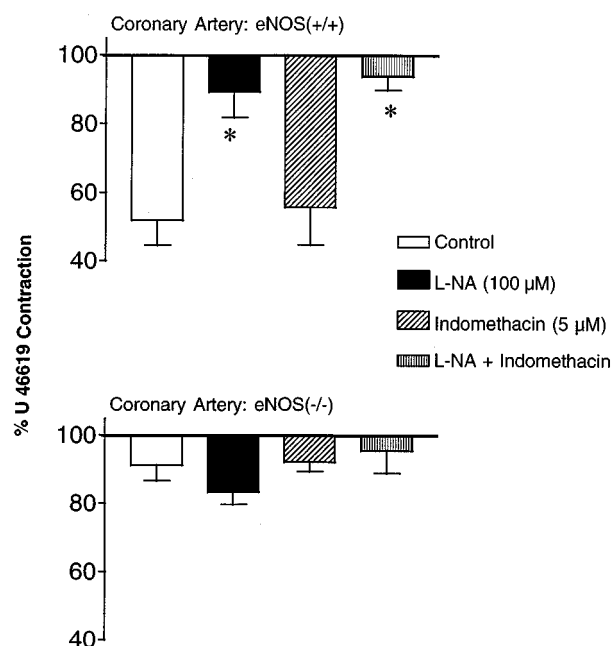


Figure 4 Acetylcholine (10 μ M)-induced relaxations in coronary arteries with endothelium from eNOS(+/+) (upper panel; $n=6-8$) and eNOS(-/-) mice (lower panel; $n=6-8$), in presence or not of indomethacin (5 μ M) and/or N^G-nitro-L-arginine (L-NA, 100 μ M). Data are shown as means $\pm$ s.e.mean. Asterisks indicate a statistically significant difference with the corresponding control value.

Table 1 Resting membrane potential (mV) in coronary artery smooth muscle cells from eNOS(+/+) and eNOS(-/-) mice

	Control (mV)	L-NA (mV)	Indo. (mV)	L-NA + Indo. (mV)
eNOS (+/+)	-64.8 ± 1.8 ($n=10$)	-65.5 ± 3.3 ($n=5$)	-61.4 ± 1.8 ($n=7$)	-63.9 ± 1.3 ($n=5$)
eNOS (-/-)	$-58.4 \pm 1.9^*$ ($n=12$)	-63.3 ± 1.6 ($n=6$)	-58.5 ± 2.5 ($n=5$)	-60.3 ± 2.0 ($n=5$)

Data are shown as means $\pm$ s.e.mean. n indicates the number of tissues taken from different mice. Experiments were performed in the presence or absence of N^G-L-nitro-arginine (L-NA, 100 μ M) or/and indomethacin (Indo, 5 μ M). The asterisk indicates a statistically significant difference between eNOS(+/+) and eNOS(-/-) mice.

Table 2 Changes in membrane potential induced by acetylcholine SIN-1 and cromakalim in coronary artery smooth muscle cells from eNOS (+/+) and eNOS (-/-) mice

		Control (mV)	L-NA (mV)	Indo. (mV)	L-NA + Indo. (mV)
eNOS (+/+)	Acetylcholine (10 μ M)	+4.2 $\pm$ 2.6 (n=5)	+3.5 $\pm$ 2.5 (n=4)	-0.3 $\pm$ 1.7 (n=3)	-1.0 $\pm$ 1.1 (n=4)
eNOS (-/-)		+6.3 $\pm$ 1.1 (n=4)	+7.7 $\pm$ 3.0 (n=3)	+0.7 $\pm$ 1.7* (n=3)	+2.0 $\pm$ 2.1 (n=3)
eNOS (+/+)	SIN-1 (10 μ M)	+1.7 $\pm$ 0.3 (n=3)	-3.0 $\pm$ 2.1 (n=4)	-0.5 $\pm$ 2.5 (n=2)	-1.3 $\pm$ 2.0 (n=3)
eNOS (-/-)		+4.0 $\pm$ 2.5 (n=3)	0.0 $\pm$ 1.5 (n=3)	-2.3 $\pm$ 2.0 (n=3)	-1.3 $\pm$ 2.8 (n=3)
eNOS (+/+)	Cromakalim (10 μ M)	-7.9 $\pm$ 1.1 (n=8)	-8.3 $\pm$ 2.3 (n=4)	-12.0 $\pm$ 2.7 (n=5)	-10.6 $\pm$ 1.6 (n=4)
eNOS (-/-)		-13.8 $\pm$ 2.6 (n=4)	-11.3 $\pm$ 1.5 (n=6)	-12.3 $\pm$ 5.2 (n=4)	-17.8 $\pm$ 4.3 (n=4)

Data are shown as means $\pm$ s.e.mean. *n* indicates the number of tissues taken from different mice. Experiments were performed in the presence or absence of N^ω-L-nitro-arginine (L-NA, 100 μ M) or/and indomethacin (Indo, 5 μ M). The asterisk indicates a statistically significant differences between control and treated tissues.

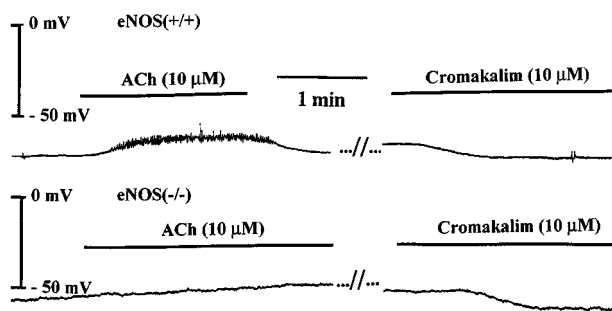


Figure 5 Original traces showing the effects of acetylcholine (ACh, 10 μ M) and cromakalim (10 μ M) on the resting membrane potential of smooth muscle cell from isolated coronary artery with endothelium taken from eNOS(+/+) (upper panel) or eNOS(-/-) mice (lower panel) mice.

some cases accompanied by a rhythmic activity. The depolarization was inhibited significantly by indomethacin.

Discussion

The disruption of the eNOS gene did not alter the intrinsic contractile properties of the smooth muscle cells, since isolated rings of aorta, carotid, mesenteric and coronary arteries from eNOS(-/-) mice developed a similar level of tension in response to KCl (60 mM) to that of corresponding arterial rings from eNOS(+/+) mice. The absolute value of the resting membrane potential of the coronary artery smooth muscle cells from eNOS(-/-) mice was less negative than that observed in eNOS(+/+) mice. The modest depolarization observed in smooth muscle cells from eNOS(-/-) mice is unlikely to be attributed to the disappearance of endothelial NO *per se*. Indeed, in both eNOS(-/-) and eNOS(+/+) mice as well as in various arteries from different species, treatment of the isolated artery rings with either L-NA or indomethacin or their combination did not affect the resting membrane potential. Furthermore, in other species, endothelial denudation does not affect vascular smooth muscle membrane potential indicating that basal production of NO or/and cyclo-oxygenase products does not exert any modulatory role on the membrane potential of vascular smooth muscle cells (Parkington *et al.*, 1993; 1995; Zygmunt *et al.*,

1994; Corriu *et al.*, 1996). eNOS(-/-) mice have systemic arterial and moderate pulmonary hypertension (Huang *et al.*, 1995; 1996; Steudel *et al.*, 1997). Depolarization of the arterial smooth muscle cells has been associated with various experimental models of hypertension and may be linked to the physical strain exerted by high blood pressure on the blood vessel wall (Tomobe *et al.*, 1991; Fujii *et al.*, 1992; Morel *et al.*, 1996; Vanheel & Van De Voorde, 1996).

In mesenteric and coronary arteries from eNOS(+/+) mice, the addition of L-NA after stabilization of the contractile response produced either by noradrenaline or U 46619, the analogue of thromboxane A₂, resulted in a further increase in tension indicating a basal endothelial release of NO and possibly the release of NO by activation of endothelial α_2 -adrenoceptor (Cocks & Angus 1983; Thorin *et al.*, 1998). In the corresponding arteries from eNOS(-/-) mice, the addition of L-NA did not produce a contraction, indicating the absence of endothelial NO production in these blood vessels. However, when compared with eNOS(+/+) mice, the contractile responses to noradrenaline and U 46619 observed in the mesenteric and the coronary arteries from eNOS(-/-) mice were significantly larger. The difference observed was abolished after L-NA treatment demonstrating the role of endothelial NO release in modulating agonist-induced contraction of vascular smooth muscle.

The endothelium-dependent relaxations induced by acetylcholine in the aorta, carotid and left anterior descending coronary arteries from eNOS(+/+) mice were abolished by acute inhibition of NOS indicating that, in these blood vessels, NO is the sole endogenous endothelium-derived vasodilator (Ku *et al.*, 1996). This conclusion is strengthened by the disappearance of these relaxations in eNOS(-/-) mice, in agreement with previous studies (Huang *et al.*, 1995; Faraci *et al.*, 1998). In the isolated perfused murine heart, the vasodilatation produced by acetylcholine involved both NO and a cyclo-oxygenase derivative in eNOS(+/+) mice and only the latter in eNOS(-/-) mice (Godecke *et al.*, 1998) which may indicate that the cyclo-oxygenase component is more prominent in distal coronary arteries than in the left anterior descending coronary artery studied in the present work. Acetylcholine, bradykinin and substance P did not hyperpolarize the smooth muscle cells of the murine coronary artery, which, in conjunction with the absence of relaxations resistant to inhibitors of NOS and cyclo-oxygenase, indicates that EDHF does not contribute to endothelium-dependent relaxations in the various murine blood vessels studied. NO

could suppress or regulate the expression of EDHF (Olmos *et al.*, 1995; Mombouli *et al.*, 1996a; Popp *et al.*, 1996; Kristof *et al.*, 1997) which has been described in various species as a back-up mechanism in case of dysfunction of the endothelial NO pathway (Kilpatrick & Cocks, 1994; Corriu *et al.*, 1998). However, studies performed in eNOS(+/+) mice in presence of an inhibitor of NOS and eNOS(-/-) mice did not unmask an EDHF-like response. To date, the mouse, appears to be the only mammal studied so far that appears to lack an endothelium-dependent hyperpolarizing mechanism in isolated coronary arteries (for review: Félétou & Vanhoutte, 1996). Compensatory mechanisms have been described in isolated perfused heart from eNOS(-/-) mice, in response to reactive hyperaemia, but the origin of these mechanisms is not known at present (Godecke *et al.*, 1998).

Although endothelium-dependent hyperpolarization could not be observed in murine coronary smooth muscle cells, cromakalim induced hyperpolarization of the coronary arteries of both murine strains, under all the experimental conditions tested. However, cromakalim activates ATP-sensitive potassium channels while EDHF in most blood vessels, taken from other species, activates a different but yet undetermined potassium conductance (Corriu *et al.*, 1996). Therefore, this observation does not allow to determine whether the absence of EDHF-mediated responses lies in the absence of EDHF-synthesis/release by the endothelial cells or in the absence of the target for EDHF such as a specific population of potassium channel on the vascular smooth muscle cells.

Exogenous NO released by SIN-1 produced a hyperpolarization in depolarized smooth muscle cells. Repolarization of the smooth muscle cells by endogenous release of NO or by the addition of nitrovasodilators in the absence of any hyperpolarizing effect has been described in other species (Tare *et al.*, 1990; Vanheel *et al.*, 1994). Sodium nitroprusside, another nitrovasodilator, consistently induced maximal relaxations in all the vessels studied from both murine strains. This is in agreement with previous studies demonstrating that the relaxing effect of exogenous NO, which can be attributed to activation of soluble guanylate cyclase, was not altered (and even enhanced) in eNOS(-/-) mice (Huang *et al.*, 1995; Faraci *et al.*, 1998).

The endothelium-dependent relaxation induced by acetylcholine in the mesenteric arteries from eNOS(+/+) mice was partially blocked by the NOS inhibitor, as previously shown in the murine perfused mesenteric bed (Berthiaume *et al.*, 1997). However, in contrast with the aorta and carotid arteries, a combined exposure to L-NA plus indomethacin was necessary to inhibit acetylcholine-induced relaxation fully. The cyclo-oxygenase component is unmasked only after NOS inhibition and the effects of the two endothelial mediators are not additive. Similar observations have been previously reported concerning the relative participation of NO and prostacyclin in the endothelium-dependent relaxations and hyperpolarizations of rat and guinea-pig arteries (Corriu *et al.*, 1996; Zigmunt & Högestätt, 1996). In the mesenteric arteries

from eNOS(-/-) mice, acetylcholine induced a relaxation which was insensitive to L-NA but abolished by indomethacin indicating that nitric oxide is not involved in this relaxation and that the products of cyclo-oxygenase present in mesenteric arteries from eNOS(+/+) mice can still be produced in eNOS(-/-) mice. An upregulation of the endothelial cyclo-oxygenases has been shown in dogs (COX-1) and rats (COX-2) chronically treated with inhibitors of NOS (Puybasset *et al.*, 1996; Beverelli *et al.*, 1997; Henrion *et al.*, 1997). The present study was not designed to determine whether or not cyclo-oxygenase is upregulated in eNOS(-/-) mice. However, intravenous indomethacin injection does not modify systemic haemodynamic parameters either in eNOS(+/+) or in eNOS(-/-) mice (Steudel *et al.*, 1997). In isolated mesenteric artery from both eNOS(+/+) and eNOS(-/-) mice, Thorin *et al.* (1998) have recently shown that an α 2-adrenergic agonist could elicit NO-sensitive, endothelium-dependent relaxations which are resistant to cyclo-oxygenase inhibition. These experiments suggest that an endothelium-derived hyperpolarizing factor, whose release and/or production is reduced by NO, can be produced by the murine mesenteric endothelial cells. Whether or not this relaxation is due to the hyperpolarization of the underlying vascular smooth muscle cells remains to be determined. The specificity of this response upon α 2-adrenergic stimulation and the unusual occurrence of an endothelium-dependent relaxation markedly potentiated by a NOS inhibitor has to be further explored.

Neuronal NO may compensate for the absence of endothelial NO during acetylcholine-induced dilatation of cerebral arterioles of eNOS(-/-) mice (Meng *et al.*, 1996, 1998) and contribute to the increase in regional cerebral blood flow in response to hypercapnia (Ma *et al.*, 1996). The present study experiments did not reveal a L-NA sensitive component in the various peripheral arteries from the eNOS(-/-) mice. Thus the compensatory role of neuronal NOS might be restricted to the cerebral circulation.

Acetylcholine-induced depolarizations were observed in coronary smooth muscle cells from both eNOS(-/-) and eNOS(+/+) mice. The depolarization to acetylcholine could be due to a direct effect of acetylcholine on the smooth muscle cells. Alternatively, acetylcholine may release endothelial-derived depolarizing factor (Corriu *et al.*, 1996; Mombouli *et al.*, 1996b), or vasoconstricting endothelial products of cyclo-oxygenase observed in several models of hypertension (Lüscher & Vanhoutte, 1986; Zanchi *et al.*, 1995). The depolarization was not affected by L-NA but was inhibited by indomethacin suggesting the latter possibility.

In conclusion, in the murine eNOS(+/+) blood vessels studied, endothelium-dependent relaxations involve either NO or the combination of NO and a product of cyclo-oxygenase, but EDHF is not involved. In the eNOS(-/-) mice, NO-dependent responses and EDHF-like responses were not observed suggesting that the latter pathway is not upregulated by eNOS gene disruption. The only back-up mechanism which was observed in mesenteric arteries from the eNOS(-/-) mice was a cyclo-oxygenase-dependent response.

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Effects of heptanol on the neurogenic and myogenic contractions of the guinea-pig *vas deferens*

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1 The effects of the putative gap junction uncoupler, 1-heptanol, on the neurogenic and myogenic contractile responses of guinea-pig *vas deferens* were studied *in vitro*.

2 Superfusion of 2.0 mM heptanol for 20–30 min produced the following reversible changes in the biphasic neurogenic contractile response (8 trials): (i) suppression of both phases; (ii) delayed development of both the first as well as the second phase, accompanied by complete temporal separation of the two phases; (iii) prominent oscillations of force during the second (noradrenergic) phase only.

3 To eliminate prejunctional effects of heptanol, myogenic contractions were evoked by field stimulation of the *vas* in the presence of suramin (200 μ M) and prazosin (1 μ M). Heptanol (2.0 mM) abolished these contractions reversibly.

4 These results show that (i) heptanol inhibits both excitatory junction potential (EJP)-dependent and non EJP-dependent contractions of the *vas*; (ii) a postjunctional site of action of heptanol, probably intercellular uncoupling of smooth muscle cells, contributes to the inhibition of contraction.

Keywords: Neurogenic contraction; myogenic contraction; heptanol; *vas deferens*; smooth muscle; gap junctions

Abbreviations: ATP, adenosine 5'-triphosphate; EJP, excitatory junction potential; NA, noradrenaline; SEJP, spontaneous excitatory junction potential

Introduction

Smooth muscle cells form 'functional syncytia' (Tomita, 1975), in which biochemical or electrical changes originating in one cell are communicated to neighbouring cells through pathways of intercellular communication (Tomita, 1967; Christ *et al.*, 1996). These pathways, or 'gap junctions', are believed to be located at regions of close apposition of the surface membranes of neighbouring cells, in the form of intercellular ion channels connecting their interiors (Unwin & Zampighi, 1980; Loewenstein, 1981).

The contractile response of mammalian smooth muscle may depend upon the spread of excitation through gap junctions. In neurogenic contractions such as those evoked in the guinea-pig *vas deferens* by stimulation of the innervating hypogastric nerve, only a small proportion of cells are directly influenced by transmitter released from the sympathetic motor innervation, because of two reasons. (1) Only about a fifth of the cells receive direct innervation by close-contact axonal varicosities (Merrillees, 1968; Bennett, 1973); (2) Varicosities do not release transmitter in response to every invasion by the axonal action potential because of the low probability (≈ 0.01) of evoked transmitter release (Cunnane & Stjarne, 1984; Brock & Cunnane, 1988). Therefore, spread and co-ordination of excitation from the few directly activated cells to other cells probably requires the involvement of gap junctions.

Contraction of smooth muscle cells is triggered finally by an increase in concentration of cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$, Himpens & Somlyo, 1988). Activation leading up to elevation of $[\text{Ca}^{2+}]_i$ and the ensuing contraction in the guinea-pig *vas deferens* is of two kinds. The first is primarily electrical activation, mediated

by adenosine 5'-triphosphate (ATP) which is released as a co-transmitter from the sympathetic innervation along with the 'classical' sympathetic neurotransmitter noradrenaline (NA) (Burnstock, 1995; von Kugelgen & Starke, 1994). NA itself acts primarily *via* biochemical activation. Corresponding to its activation by the two transmitters, the contraction of the guinea-pig *vas* in response to tetanic (8–20 Hz) motor nerve stimulation is biphasic, consisting of a rapid twitch, mediated mainly by ATP and a slower tonic phase, mediated mainly by NA (Sneddon & Westfall, 1984; Kennedy *et al.*, 1996). The purinergic phase is triggered *via* P_{2x} purinoceptor-mediated excitatory junction potentials (EJPs) leading to regenerative muscle action potentials associated with Ca^{2+} influx (Blakeley *et al.*, 1981; Sneddon & Westfall, 1984; Sneddon, 1992; Driessen *et al.*, 1994), whereas the noradrenergic phase is mediated by elevation of $[\text{Ca}^{2+}]_i$ through the inositol phosphate (IP_3) second messenger pathway following α_1 adrenoceptor activation (Berridge, 1993).

The proposal that gap junctions modulate contractile activity has been explored, and strengthened, in vascular smooth muscle by the use of substances that appear selectively to block gap junctional communication, in particular the alkanol 1-heptanol (Christ, 1995; Christ *et al.*, 1996). These studies have concentrated on the effects of heptanol on contractions evoked by externally applied agonists. Neurogenic contractions, however, which may involve considerably different spatial patterns of directly excited and indirectly recruited cells, have not been similarly investigated.

The aim of the present investigation was to assess the contribution of cell-to-cell communication to the neurogenic contractile response of the guinea-pig *vas deferens*. It was found that both phases of the neurogenic contraction were reversibly suppressed by heptanol. To clarify the site of

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heptanol action, we also explored its effects on myogenic contractions, and present evidence supporting a postjunctional mechanism of action of heptanol.

Methods

Contractile studies

Male guinea-pigs (350–450 g) were sacrificed by stunning and exsanguination. The abdomen was opened by a midline incision and the vasa on either side were exposed by pulling the testes out from the scrotal sacs. Both the vasa were dissected out along with the innervating hypogastric nerve. The isolated vas was suspended in a vertical organ bath of 25 ml capacity. The prostatic end was attached to an isometric force transducer using a silk suture. The epididymal end was anchored immovably to the bottom of the organ bath. The tissue was continuously superfused with carbogen (95% O₂, 5% CO₂) bubbled Krebs solution of the following composition (mM): NaCl 118.4, KCl 4.7, MgCl₂ 1.2, CaCl₂ 2.5, NaHCO₃ 25.0, NaH₂PO₄ 0.4 and glucose 11.1. Experiments were conducted at room temperature (25–30°C).

The force transducer was connected to an amplifier (Polyrite, Biodevices, Chandigarh, India) whose output was directly displayed on a digital storage oscilloscope (1425, L&T Gould, Mumbai, India) and simultaneously stored on a digital tape recorder (DTR-1204, Biologic, Claix, France). The tissue was mounted under a resting tension of 0.1–0.5 g. The hypogastric nerve was stimulated *via* a pair of Ag/AgCl ring electrodes (separation about 3 mm), through which its proximal end was passed, using rectangular voltage pulses delivered by an electronic stimulator. A second pair of Ag/AgCl ring electrodes was placed surrounding the muscle, one electrode at the epididymal end and the other at the prostatic end, for direct stimulation of the muscle. Supramaximal tetanic nerve stimulation was carried out at 8 Hz, using a pulse amplitude of 10 V and pulse width 5 ms. A gap of 10 min was provided between consecutive tetani (~20 s duration each) to allow for recovery of the tissue. The muscle was stimulated selectively using pulses of amplitude 3 V and width 200 ms at 1 Hz, these parameters of stimulation being known to result in direct muscle stimulation (Holman *et al.*, 1977).

Electrophysiological studies

EJPs were recorded as described earlier (Manchanda & Venkateswarlu, 1997), using intracellular glass capillary microelectrodes with tip impedances of 40–60 M Ω interfaced with a high input impedance electrometer (IE 201, Warner Instruments, U.S.A.). The output of the amplifier was observed on an oscilloscope (TDS 310, Tektronix, U.S.A.) and stored simultaneously on the digital tape recorder.

Drugs

Salts used for the Krebs solution and 1-heptanol were obtained from S.D. Fine Chemicals Ltd., Mumbai, India. Suramin sodium was a gift from Bayer plc, U.K. and prazosin HCl was a gift from Pfizer Ltd, Mumbai, India. Prazosin stock solution was prepared in N,N-dimethyl acetamide (LOBA Chemicals, Mumbai, India; see Brock & Cunnane, 1988) and suramin stock in distilled water. Experimental drug solutions were prepared by dilution of stock solutions in Krebs just before application. Solution of 1-heptanol were made by vigorous shaking with Krebs at the time of application.

Data collection and analysis

Signals played back on the tape recorder were amplified as required and digitized using an analogue to digital conversion card (PCL 209, Dynalog Microsystems, Mumbai, India) interfaced with an IBM compatible PC-AT 80486 using SCAN (part of Strathclyde Electrophysiology Software kindly supplied by Dr J. Dempster, Strathclyde University, Glasgow, U.K.).

All data are expressed as mean $\pm$ s.e. mean number of observations). Student's unpaired *t*-test was used to assess the statistical significance of the difference between means, a *P* value of <0.05 being taken to indicate a statistically significant difference.

Results

Effects of heptanol on neurogenic contractions

The contractile response of the guinea-pig *vas deferens* to tetanic stimulation of the hypogastric nerve was biphasic, as reported earlier (Sneddon & Westfall, 1984; Kennedy *et al.*, 1996) with the rising phase of the slow phase overlapping the decay of the fast phase (Figure 1). The response had the expected pharmacological profile, the fast phase being abolished selectively by the P_{2x} purinoceptor antagonist suramin (200 μ M; Sneddon, 1992), the slow phase selectively by the α_1 adrenoceptor antagonist prazosin (1 μ M) and the entire response by a combination of prazosin and suramin (see Figure 4A).

Suppression of force

The effects of heptanol on contractile force were investigated in most trials at a concentration of 2.0 mM. Effects at lower (0.5, 1.0 mM) or higher (4.0 mM) concentrations were qualitatively similar, though rates of onset were variable. 2.0 mM seems to be the optimal concentration of heptanol for the reversible abolition of EJPs in the organ under investigation, the guinea-pig *vas deferens* (see Discussion), hence this concentration was used so that effects on contractile and electrical activity could be compared.

In general, superfusion of 2.0 mM heptanol solution for about 20 min gradually suppressed both phases of the neurogenic contraction (Figure 1). In the majority of trials (seven out of nine), both phases were inhibited similarly. The peak force of the first phase was suppressed without change of shape by $83 \pm 7\%$ ($n=7$) on 20 min exposure to heptanol. Inhibition of the second phase was more complex, as it developed irregular fluctuations during its suppression, as described in greater detail below.

Figure 1A shows results of a trial in which the inhibition was complete for both phases at 20 min (Figure 1A, c). The effect was completely reversed on washout with normal Krebs for about 20 min (Figure 1A, e). In the other two trials the purinergic phase was partly resistant to the action of heptanol, its force being suppressed by only 50% following 20 min of heptanol exposure, whereas the adrenergic phase was completely abolished during this interval (see Figure 1B, c).

Increase in latency of force and separation of the phases

In control contractions the latency between the start of stimulation and the onset of the purinergic phase was 0.83 ± 0.11 s ($n=9$). Exposure to 2.0 mM heptanol for 20 min

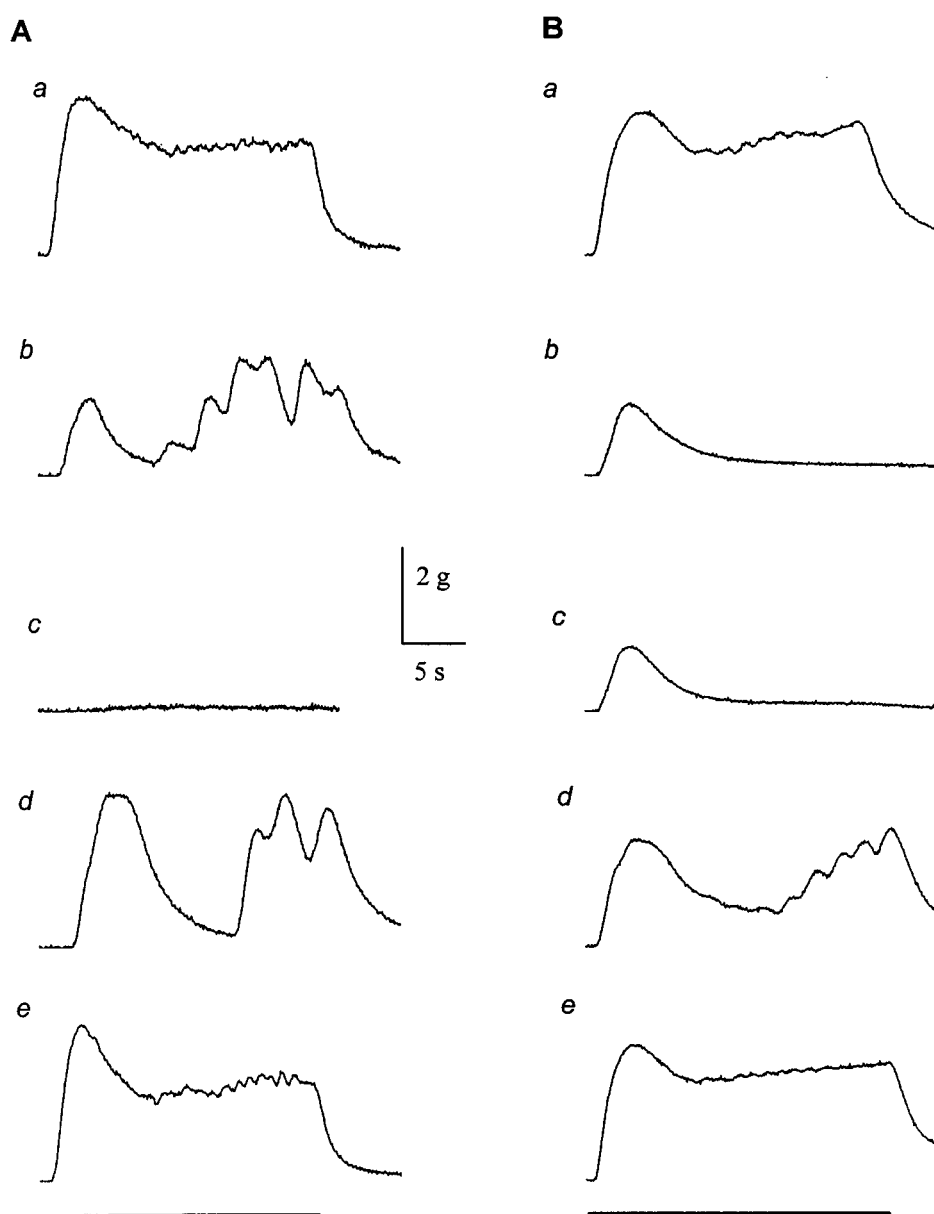


Figure 1 Effect of 2.0 mM heptanol on neurogenic contractions of the guinea-pig *vas deferens* in two tissues. (A) Tissue showing complete suppression of two phases and (B) showing only partial suppression of purinergic phase a: Control; b: after 10 min exposure to heptanol; c: after 20 min exposure to heptanol; d,e: 10 and 20 min respectively after start of heptanol washout. Note the separation of two phases and oscillations in second phase in presence of heptanol. The thick line at the bottom indicates the period of hypogastric nerve stimulation (10-V, 5-ms pulses at 8 Hz).

increased the latency of the first phase significantly ($P < 0.05$) to 1.58 ± 0.30 s ($n = 7$). The change in latency is displayed in the superimposed and expanded traces of Figure 2A and B, which show responses recorded during the progressive inhibition of contractions by heptanol, before their complete abolition.

The onset of the noradrenergic phase was more strikingly delayed by heptanol (Figure 2A). The latency of the second phase in control contractions is difficult to determine with certainty because its onset is obscured by the preceding purinergic phase. However, the latency of the second phase can reasonably be assumed to be similar to that of the first since both ATP and NA, the two neurotransmitters mediating the phases, are known to be released simultaneously from the innervation following stimulation, though the noradrenergic phase is slower to develop (von Kugelgen & Starke, 1994; von

Kugelgen *et al.*, 1994; Stjarne & Stjarne, 1995). On the basis of this assumption the latency of the second phase was increased from 0.83 ± 0.11 s in controls to 14.93 ± 1.23 s ($n = 7$) following 20 min exposure to heptanol (Figure 2A).

Since the increase of latency of the second phase was much greater than that of the first phase, the induced suppression of contractile force was accompanied by a progressive temporal separation of the two phases. The extent of separation at 20 min exposure to heptanol was such that there was no longer any overlap between the phases (Figure 2A).

Since the first phase of contraction is EJP-dependent, the increase in latency of this phase suggests that EJPs inhibited by heptanol (Manchanda & Venkateswarlu, 1997) take longer to reach threshold for action potential generation. To test this, we recorded EJPs in the presence of heptanol at 8 Hz, the same frequency of stimulation as that employed in the contractile

studies. In control conditions, EJPs at ≥ 5 Hz readily summate to reach action potential threshold and produce contractions (Blakeley *et al.*, 1981). In the presence of heptanol, the development of threshold depolarization was considerably delayed, and in one trial EJPs at 8 Hz did not reach threshold in 8 s of stimulation (Figure 2C). When the frequency of stimulation was increased gradually beyond 8 Hz to about 70 Hz, an action potential and contraction were finally generated (Figure 2D). Threshold for regenerative excitation is achieved at higher frequencies of stimulation since a larger degree of summation results in a larger mean depolarization.

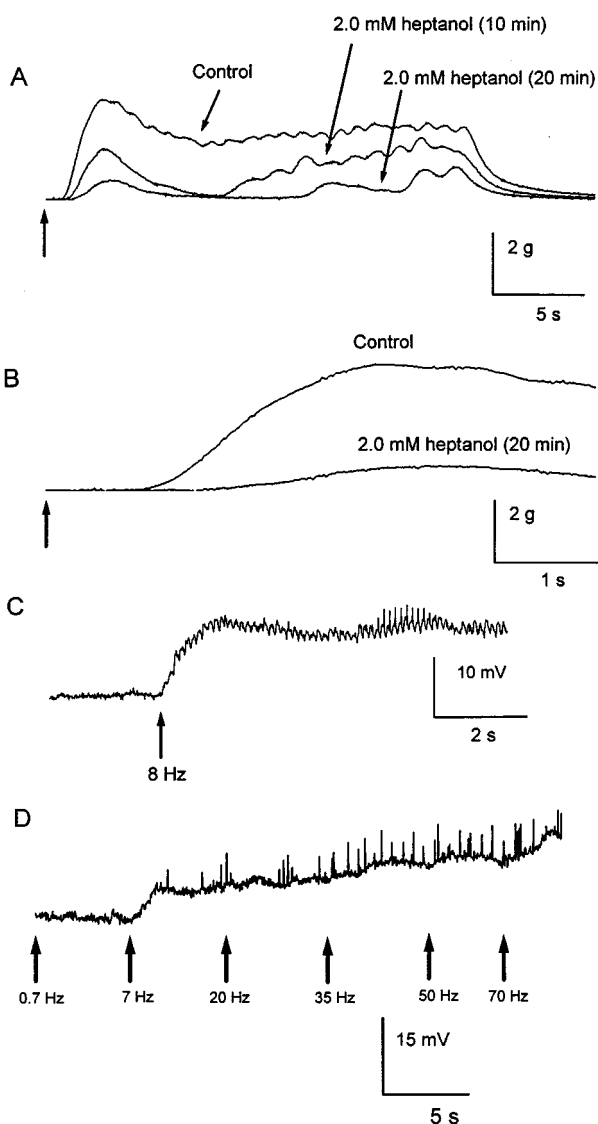


Figure 2 (A,B) Comparison of control neurogenic response with that in the presence of heptanol. Note the increase in latency, separation of the two phases and oscillations in the second phase induced by heptanol. Stimulation carried out at 8 Hz (10 V, 5 ms pulses). (B) Upper and lower traces of A expanded to show increase of latency of the first phase induced by heptanol. (C) Intracellularly recorded transmembrane potential in response to hypogastric nerve stimulation at 8 Hz (10 V, 0.1 ms pulses) in the presence of 2.0 mM heptanol. Note the slow development of depolarization and failure to reach threshold for action potential generation even after about 50 stimuli. (D) Transmembrane potential in response to hypogastric nerve stimulation in the presence of 2.0 mM heptanol at various frequencies from 0.7 to 70 Hz, indicated by arrows. At 70 Hz, the electrode was expelled, possibly due to contraction of the tissue. Spiky transients in C and D are stimulus artifacts.

Correspondingly in contractile studies, the heptanol inhibited neurogenic contraction, too, was partially restored by increasing the stimulus frequency to 70 Hz (not shown).

Oscillations in the noradrenergic phase

In addition to the remarkable increase of latency in the presence of heptanol, the second phase also developed, during its suppression, prominent, irregular oscillations in force, being split into an uncoordinated series of alternate contractions and relaxations. Examples of these oscillations are evident in Figures 1A, b,d and 2A. The extent of variation of force during these oscillations could be as much as $\sim 50\%$ of the peak force developed (Figure 1A, b), or just enough to cause a significant ripple during the second phase (e.g. in Figure 1B, d). The effect was specific to heptanol action, since control contractions either before or after application of heptanol were seen not to possess such pronounced fluctuations, though a slight, but regular ripple could occasionally be observed (Figures 1A, a,e and 2A).

A possible interpretation of the oscillations observed in the presence of heptanol is that they are the result of muscle action potentials triggered intermittently by residual purinergic EJPs which may not have been inhibited completely by heptanol. To confirm the nature of the transmitter responsible for the delayed response in the presence of heptanol, the *vas* was superfused with suramin (200 μ M) before the application of heptanol. Suramin inhibited powerfully the first phase of contraction and a monophasic contraction remained, corresponding to the second, noradrenergic phase of the normal biphasic neurogenic contraction (Figure 3). These suramin-resistant contractions were affected by heptanol in the same way as the second (noradrenergic) phase of the normal neurogenic contraction, i.e. in suppression of force, induction of oscillations in force during suppression, and marked increase of latency of the same order (Figure 3). Thus these oscillations are likely to be due to effects exerted specifically on noradrenergic mechanisms by heptanol.

Effect of heptanol on myogenic contractions

Suppression of neurogenic contractions by heptanol can conceivably be explained on the basis of purely neuroinhibitory effects of this agent. To determine whether heptanol indeed affects muscle function independent of neuronal elements, we investigated its effect on myogenic contractions,

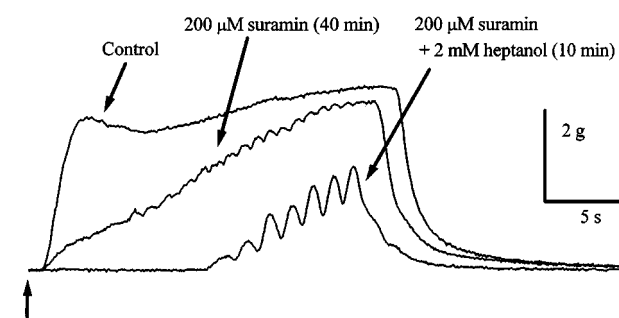


Figure 3 Persistence of heptanol-induced contractile oscillations in the second phase in the presence of suramin (200 μ M). Exposure to suramin for 40 min inhibits profoundly the first (purinergic) phase of the neurogenic contraction, while leaving force developed in the second phase almost unaffected (middle trace). Vertical arrow denotes start of stimulation. Stimulation at 8 Hz (10 V, 5 ms pulses).

which originate in the muscle without neuronal contribution. Neurogenic contractions were first eliminated by applying a combination of the P_{2x} purinoceptor antagonist suramin ($200\text{ }\mu\text{M}$) together with α_1 adrenoceptor antagonist prazosin ($1\text{ }\mu\text{M}$). Figure 4A shows that this combination completely abolished neurogenic transmitter-mediated contractions.

To elicit myogenic contractions, stimulation was switched to the muscle stimulation electrodes using appropriate pulse

parameters (see Methods), which elicited contractions of the type illustrated in Figure 4B, a. These contractions, since they arise despite the presence of suramin and prazosin, are therefore likely to be of purely myogenic origin. To confirm this, the same pair of electrodes was used to deliver stimulation selective for nerve stimulation (10 V, 5-ms pulses at 8 Hz). Figure 4B, b shows that in the presence of the antagonists, this stimulation failed to generate any detectable force, indicating that neurogenic contributions were entirely eliminated. The latency of the myogenic response ($0.32 \pm 0.02\text{ s}$, $n=6$) was about 60% less than that of the neurogenic response ($0.83 \pm 0.11\text{ s}$, $n=9$) (see Figure 4B, a). This is expected from the elimination of neuronal conduction, synaptic delay and time taken for the summation of EJPs to reach threshold for generation of muscle action potential.

Following the application of 2.0 mM heptanol for 20–30 min, peak myogenic force was almost entirely suppressed in all trials, by $98 \pm 1\%$ ($n=5$) (Figure 5A–C). The reduction of force observed when using the long duration pulses required for myogenic contraction could possibly occur as a result of electrode polarization. However, the suppression of force persisted when stimulation was delivered after reversing electrode polarity (Figure 5D). This indicates that the suppression of the myogenic force was unrelated to electrode polarization and was indeed a heptanol mediated effect. The suppression was reversible on washout of heptanol (Figure 5E,F).

Discussion

The role of gap junctional communication in the generation and maintenance of smooth muscle contraction has received strong support in previous studies from the effects of heptanol on the contractions (Christ *et al.*, 1993; Christ, 1995). Heptanol at concentrations between 1.0 and 5.6 mM has been shown to selectively block gap junctions in various tissues including smooth muscle (Christ *et al.*, 1991; Bastide *et al.*, 1995; Blennerhassett & Garfield, 1991; Bukauskas *et al.*, 1992; Peracchia, 1991; Christ, 1995), and to inhibit agonist evoked contractions in smooth muscle such as rat aorta and corpus cavernosum (Christ *et al.*, 1991; 1993). However, neurogenic contractions have not yet been similarly investigated. In the present inquiry, heptanol has been used at a concentration of 2.0 mM. This concentration falls within the range mentioned above for specific action on gap junctional communication. Furthermore, in electrical recordings, heptanol at concentrations of 2.0 mM completely and reversibly abolished the EJP of the *vas deferens* (Manchanda & Venkateswarlu, 1997). Reversible suppression of EJPs was observed at concentrations greater than 2.0 mM (up to 10 mM) as well, while at concentrations below 2.0 mM there was a dose-dependent suppression, but not abolition, of the EJP (Manchanda & Venkateswarlu, 1997; unpublished observations). Since 2.0 mM was the lowest concentration required for complete, reversible abolition of the EJP in the absence of non-specific actions (Manchanda & Venkateswarlu, 1997), we used this concentration in these studies.

The results presented here show that 1-heptanol inhibits, profoundly and reversibly, the neurogenic contractions of the guinea-pig *vas deferens*. These findings may, therefore, throw additional light on the significance of intercellular coupling in the modulation of smooth muscle tone *in vivo*, to which neuronal input makes significant contributions. However the interpretation of the effects of heptanol on neurogenic contractions is complicated by the contribution to these

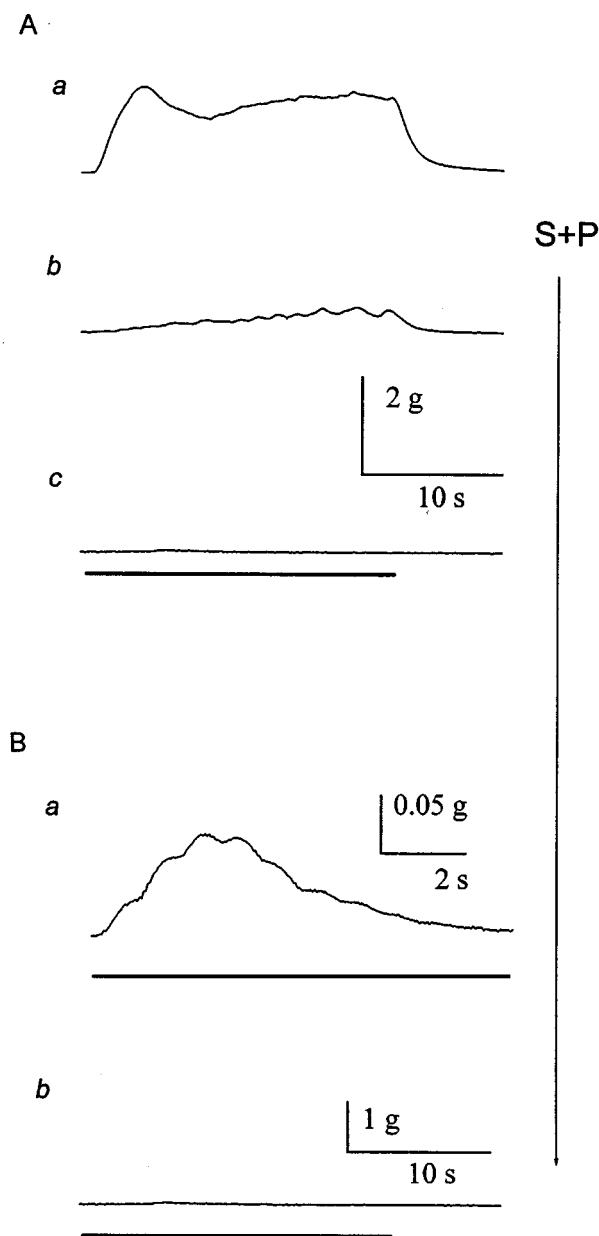


Figure 4 Generation of myogenic contractions of the guinea-pig *vas deferens*. (A) Suppression of neurogenic force, generated by hypogastric nerve stimulation at 8 Hz in the presence of suramin and prazosin (S+P). a: Control; b,c: 40 and 70 min respectively after the application of a mixture of S ($200\text{ }\mu\text{M}$) and P ($1\text{ }\mu\text{M}$). The calibration bar applies to all the traces. (B) a: Myogenic force generated by direct muscle stimulation after abolition of the neurogenic force by S+P, using 3-V, 200-ms pulses at 1 Hz with field stimulation electrodes. b: Absence of force generation in the presence of S+P by stimulation with 10-V, 5-ms pulses at 8 Hz (suitable for intramural nerve stimulation) with the field stimulation electrodes. Duration of stimulation indicated by thick line (common for (A)). The vertical arrow shows that traces (A)b,c and (B)a,b were all obtained in the presence of S+P.

contractions by prejunctional processes. This raises the possibility that the observed effects of heptanol arise from interference with neuronal mechanisms rather than with postjunctional cell-to-cell coupling.

Postjunctional site of action of heptanol

Previous work has already shown that the site of action of heptanol is probably located postjunctionally. For instance, Christ *et al.* (1993, 1996) have shown from various lines of evidence that heptanol blocks gap junctions, including its inhibition of α_1 -agonist induced contractions coupled with the

lack of effect on KCl-induced contractions in vascular smooth muscle. Furthermore, presynaptic effects on neuronal conduction have been ruled out by the observation that the *vas deferens* nerve action potential elicited by preganglionic stimulation remains unaffected in the presence of heptanol (Manchanda & Venkateswarlu, 1997).

Perhaps the most direct confirmation that heptanol affects selectively postjunctional mechanisms in the *vas deferens* stems from our observation that it abolishes the myogenic contractions, which do not include neuronal contribution. To place confidence in these results it is essential to be confident that the method used here for direct muscle stimulation excites only postjunctional smooth muscle, without involving prejunctional elements. This has been ensured in the present work by the choice of stimulation parameters for muscle excitation (Geddes & Baker, 1975; Holman *et al.*, 1977), and the concomitant use of suramin and prazosin at concentrations that completely abolished the neurogenic response (Figure 4).

The suppression of myogenic contractions by heptanol indicates strongly that this agent has a postjunctional, post-receptor site of action in the guinea-pig *vas deferens*. Such a site of action could be located in any of the processes leading from receptor activation to contraction, including receptor-mediated second messenger generation, activation of the contractile machinery by cytosolic Ca^{2+} (Somlyo & Somlyo, 1960; Somlyo *et al.*, 1985), and spread and co-ordination of contractile activity in smooth muscle through intercellular pathways. A large body of evidence gathered in studies on several smooth muscle organs indicates, however, that the mechanism lies probably in the specific inhibition of cell-to-cell coupling by this agent (Christ *et al.*, 1991; 1993; 1996).

Such an action would result in inhibition of contractions as follows. Electrically evoked smooth muscle contraction results from influx of Ca^{2+} into the cells through voltage gated calcium channels (Somlyo & Somlyo, 1960; Mekata, 1981). In the present experiments, action potentials during muscle stimulation would be directly evoked in only a narrow band of cells directly encircled by the cathode. A much larger number of cells is presumably recruited for contraction by the spread of the action potentials, or of elevated cytosolic $[\text{Ca}^{2+}]$, through gap junctions. If heptanol blocks gap junctional communication, the recruitment of cells for contraction would be reduced, resulting in inhibition of the myogenic response.

In view of the arguments presented above, we restrict attention below to blockade of gap junctional communication by heptanol as the mechanism underlying its effects on neurogenic contractions.

Inhibition of neurogenic contractions

The purinergic phase of contraction in the *vas deferens* depends upon the generation of regenerative action potentials in the smooth muscle cells, which in turn are triggered by ATP-mediated EJPs (Blakeley *et al.*, 1981). The sparseness of direct innervation (Merrillees, 1968; Bennett, 1973) and the low probability of release from the axonal varicosities (Cunnane & Stjärne, 1984) of the guinea-pig *vas deferens* suggest that only a small proportion of cells are directly acted upon by neurotransmitter, and hence develop EJPs, following each stimulus delivered to the nerve. In the uninnervated cells, EJPs are passively transmitted from the directly activated cells. Since this transmission most likely takes place through gap junctions, block of these junctions would suppress activation of uninnervated cells in the following ways. (1) EJPs would not be relayed to the uninnervated cells (Manchanda & Venkateswarlu, 1977), precluding the development of action

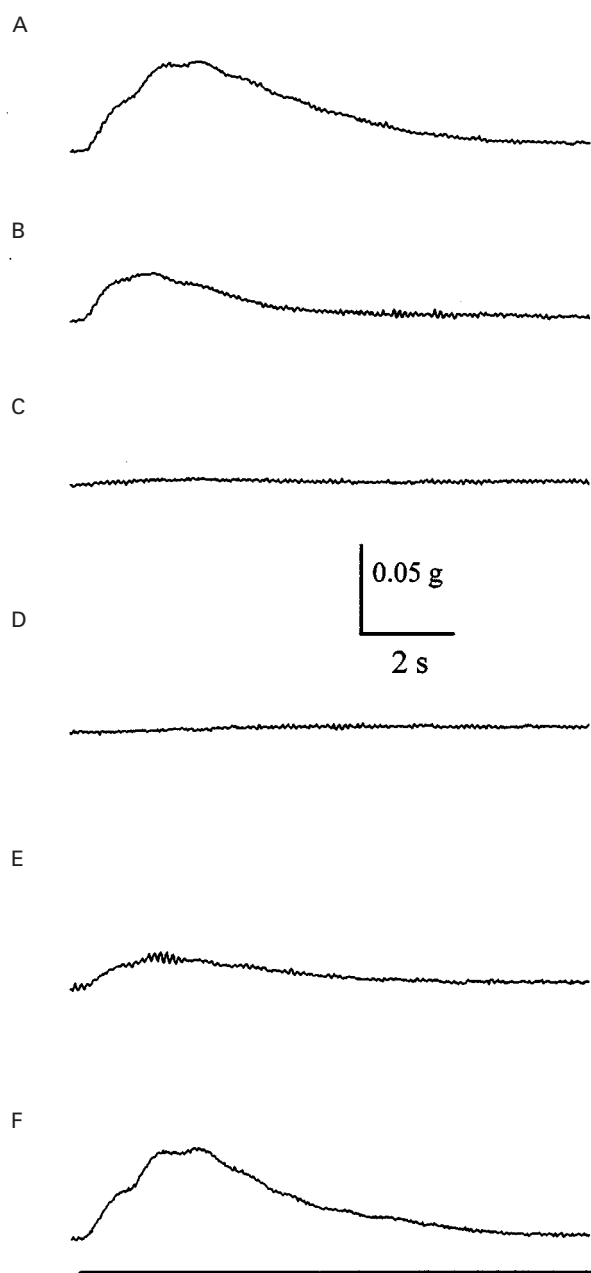


Figure 5 Reversible suppression of myogenic force by 2.0 mM heptanol. (A) Control; (B,C) 20 and 30 min respectively after application of heptanol. (D) absence of force generation following reversal of stimulation polarity using the same parameters as in (C). (E,F) 30 and 50 min respectively after start of heptanol washout. The thick line represents the duration of stimulation for each trace.

potentials in them. (2) Since cell-to-cell coupling is inhibited, relay of action potentials into these cells from the directly activated cells would also not occur. (3) Finally, any elevation of cytosolic $[Ca^{2+}]$ in the directly activated cells, associated either with the EJP or the action potential, would not be relayed to these cells. All these factors would contribute to the reduction in recruitment of cells activated for contraction, and resultant suppression of contractile force in the presence of heptanol.

The suppression of the noradrenergic phase by heptanol can be explained along lines similar to those mentioned for the purinergic phase above, and for its inhibition of agonist-induced contraction of vascular smooth muscle (Christ *et al.*, 1991, 1993; Christ, 1995). In many smooth muscle organs, including the *vas deferens*, noradrenaline evokes contraction not *via* direct electrical activation involving EJPs but *via* biochemical pathways involving second messengers such as IP_3 and Ca^{2+} (Somlyo *et al.*, 1985; Han *et al.*, 1988). Because of the features of neuronal activation mentioned above, NA, like ATP also activates directly a small fraction of smooth muscle cells. Contractile activation of the noninnervated cells would then depend upon the cell-to-cell diffusion of Ca^{2+} presumably through gap junctions (Christ *et al.*, 1992). Hence heptanol, if it blocks gap junctional communication, would profoundly reduce the number of cells recruited for contraction, inhibiting the noradrenergic phase.

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Improvement by nefiracetam of β -amyloid-(1-42)-induced learning and memory impairments in rats

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1 We have previously demonstrated that continuous i.c.v. infusion of amyloid β -peptide ($A\beta$), the major constituent of senile plaques in the brains of patients with Alzheimer's disease, results in learning and memory deficits in rats.

2 In the present study, we investigated the effects of nefiracetam [N-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl) acetamide, DM-9384] on $A\beta$ -(1-42)-induced learning and memory deficits in rats.

3 In the $A\beta$ -(1-42)-infused rats, spontaneous alternation behaviour in a Y-maze task, spatial reference and working memory in a water maze task, and retention of passive avoidance learning were significantly impaired as compared with $A\beta$ -(40-1)-infused control rats.

4 Nefiracetam, at a dose range of 1–10 mg kg⁻¹, improved learning and memory deficits in the $A\beta$ -(1-42)-infused rats when it was administered p.o. 1 h before the behavioural tests.

5 Nefiracetam at a dose of 3 mg kg⁻¹ p.o. increased the activity of choline acetyltransferase in the hippocampus of $A\beta$ -(1-42)-infused rats.

6 Nefiracetam increased dopamine turnover in the cerebral cortex and striatum of $A\beta$ -(1-42)-infused rats, but failed to affect the noradrenaline, serotonin and 5-hydroxyindoleacetic acid content.

7 These results suggest that nefiracetam may be useful for the treatment of patients with Alzheimer's disease.

Keywords: Alzheimer's disease; amyloid β -peptide; nefiracetam (DM-9384); reference memory; voltage-sensitive calcium channels; working memory

Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; $A\beta$, amyloid β -peptide; ACh, acetylcholine; AD, Alzheimer's disease; ANOVA, one-way analysis of variance; APP, β -amyloid precursor protein; ChAT, choline acetyltransferase; DOPAC, 3,4-dihydroxyphenylacetic acid; GABA, γ -aminobutyric acid; HVA, homovanillic acid; VSCC, voltage-sensitive calcium channels

Introduction

Alzheimer's disease (AD) is the most common cause of progressive decline of cognitive function in aged humans. The disease is characterized neuropathologically by the presence of numerous senile plaques and neurofibrillary tangles accompanied by neuronal loss. The senile plaques are composed of amyloid β -peptide ($A\beta$), a 40–42 amino acid peptide fragment of the β -amyloid precursor protein (APP) (Kang *et al.*, 1987). Transgenic mice, which overexpress human APP containing the mutations associated with familial AD, develop many of the pathological characterizations associated with AD (Games *et al.*, 1995; Johnson-Wood *et al.*, 1997; Sturchler-Pierrat *et al.*, 1997). Furthermore, $A\beta$ is cytotoxic to neurons (Yankner *et al.*, 1990) and renders neurons vulnerable to various insults including excitotoxicity (Koh *et al.*, 1990; Mattson *et al.*, 1992). These previous findings suggest that $A\beta$ has a central role in the pathogenesis of AD.

We have previously demonstrated that continuous infusion of $A\beta$ -(1-40) into the cerebral ventricle of rats results in learning and memory deficits, suggesting that accumulation of $A\beta$ in the brain is related to cognitive impairments in AD (Nitta *et al.*, 1994; Nabeshima & Nitta, 1994). Continuous $A\beta$ -(1-40) infusion causes a decrease in choline acetyltransferase

(ChAT) activity in the cerebral cortex and hippocampus (Nitta *et al.*, 1994), changes in ciliary neurotrophic factor levels (Yamada *et al.*, 1995a), activation of glial cells as shown by an increase in glial fibrillary acidic protein immunoreactivity (Nitta *et al.*, 1997), and a deficiency of long-term potentiation in the CA1 field of the hippocampus (Nabeshima & Itoh, 1997). Repeated administration of propentofylline, which stimulates nerve growth factor synthesis/secretion in quiescent astroglial cells (Shinoda *et al.*, 1990), improves $A\beta$ -(1-40)-induced learning and memory deficits (Yamada *et al.*, 1998). We have also demonstrated that NC-1900, an active fragment analogue of arginine vasopressin, improves cognitive dysfunction in this non-transgenic animal model of AD (Tanaka *et al.*, 1998). Although the mechanisms of the brain dysfunction caused by accumulation of $A\beta$ in the brain are obscure, dysfunction of cholinergic and dopaminergic neuronal systems may be responsible, since *in vivo* brain microdialysis revealed a marked reduction of nicotine- and/or KCl-induced stimulation of acetylcholine (ACh) release in the hippocampus/cerebral cortex and 3,4-dihydroxyphenylethylamine (dopamine) release occurred in the striatum without affecting the basal levels of these neurotransmitters (Itoh *et al.*, 1996).

Nefiracetam, a pyrrolidone derivative, is a novel compound developed for drug therapy against cognitive disorders in aged humans (for review see Nabeshima, 1994; Yamada & Nabeshima, 1996). This compound shows remarkable effects

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in animals on cognitive impairments caused by various drug treatments (Nabeshima *et al.*, 1990; 1991b; 1994), basal forebrain lesion (Nabeshima *et al.*, 1991a), cerebral ischemia (Tanaka *et al.*, 1992), and carbon monoxide exposure (Hiramatsu *et al.*, 1992). Electrophysiological studies have demonstrated that nefiracetam modulates the currents through L-type voltage-sensitive calcium channels (VSCC) (Yoshii & Watabe, 1994), γ -aminobutyric acid (GABA)_A receptors (Huang *et al.*, 1996), and nicotinic ACh receptors (Nishizaki *et al.*, 1998), by interacting with a cyclic AMP-dependent protein kinase and/or a Ca^{2+} -dependent protein kinase C pathway. Since the ameliorating effect of nefiracetam on scopolamine-induced memory impairments is antagonized by treatment of VSCC antagonists, it is suggested that activation of VSCC is involved in the improving effect of nefiracetam on cognitive deficits (Yamada *et al.*, 1994).

In the present study, we investigated the effects of nefiracetam on $\text{A}\beta$ -induced impairments of spontaneous alternation behaviour in a Y-maze task, spatial reference and working memory in a water maze task, and retention of passive avoidance learning. Furthermore, the effect of this compound on ChAT activity and monoamine metabolism were examined in the $\text{A}\beta$ -treated rats. Since recent evidence suggests that $\text{A}\beta$ -(1-42) plays a more important role than $\text{A}\beta$ -(1-40) in the pathology of AD (Jarrett & Lansbury, 1993; Iwatsubo *et al.*, 1994), rats were continuously infused with $\text{A}\beta$ -(1-42) into the cerebral ventricle. Control rats were infused with non-toxic reverse fragment $\text{A}\beta$ -(40-1). We confirmed that the vehicle itself failed to induce any behavioural and neurochemical changes at this flow rate (Nitta *et al.*, 1994).

Methods

Animals

The rats used in the present study were males of the Wistar strain (7 weeks old; Charles River Japan Inc., Yokohama, Japan) weighing 250 ± 20 g at the beginning of experiments. They were housed in groups of two or three in a temperature- and light-controlled room (23°C; 12-h light cycle starting at 09 h 00 min) and had free access to food and water, except

during the behavioural experiments. All experiments were performed in accordance with the Guidelines for Animal Experiments of the Nagoya University School of Medicine, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgery

Rats were anaesthetized with pentobarbital (50 mg kg^{-1}) i.p., and the infusion cannula for $\text{A}\beta$ was implanted into the right ventricle (A: -0.3 , L: 1.2 , V: 4.5), according to the atlas of Paxinos & Watson (1986). Continuous infusion of $\text{A}\beta$ -(1-42) ($300 \text{ pmol day}^{-1}$) was maintained for at least 2 weeks by attaching an infusion cannula to a mini-osmotic pump (Alzet 2002; Alza, Palo Alto, CA, U.S.A.) (Nitta *et al.*, 1994). The control rats were infused with $\text{A}\beta$ -(40-1). We have confirmed that the vehicle by itself has no effect on learning behaviour at this flow rate (Nitta *et al.*, 1994; 1997).

Drug administration and experimental design

The experimental schedule is shown in Figure 1. The behavioural tests and the administration of nefiracetam were started on day 7 after the start of $\text{A}\beta$ -(1-42) infusion. The behavioural tests were carried out sequentially. Nefiracetam at doses of 1, 3 and 10 mg kg^{-1} or saline was administered orally to $\text{A}\beta$ -(1-42)-infused rats for 14 consecutive days, 1 h before the behavioural test throughout the experimental period. $\text{A}\beta$ -(40-1)-infused control rats were administered with saline. Each group consisted of eight to nine rats. On day 20 after the start of $\text{A}\beta$ infusion, rats were killed 1 h after the last administration of nefiracetam for the measurement of ChAT activity and the contents of monoamines and their metabolites in the brain.

Measurement of locomotor activity

Locomotor activity was measured on day 7 after the start of $\text{A}\beta$ infusion. The experimental apparatus consisted of a locomotor cage ($25 \times 42 \times 20 \text{ cm}$), with photobeams placed 2 cm above the floor at 1-inch intervals along two sides of the

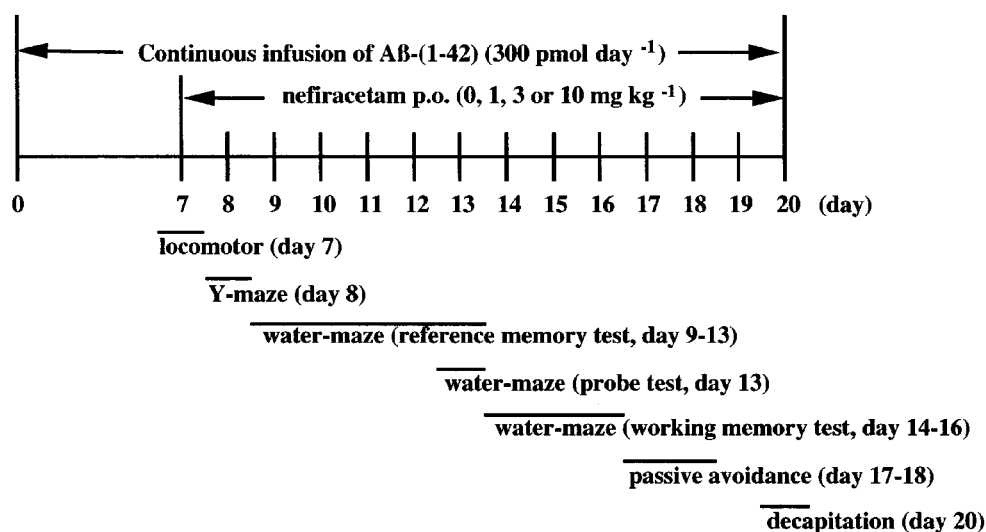


Figure 1 Experimental schedule.

cage (Colombus Instruments, U.S.A.). Locomotor activity was measured during a 10-min period (Fuji *et al.*, 1993).

Y-maze task

The Y-maze task in rats was carried out on day 8 after the start of A β infusion, as described previously (Maurice *et al.*, 1994). The experimental apparatus consisted of a black-painted Y-maze made of plywood. Each arm of the Y-maze was 35 cm long, 25 cm high and 10 cm wide and positioned at an equal angle. Each rat was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The sequence of arm entries was recorded manually. A spontaneous alternation behaviour, which is regarded as a measure of spatial memory (Maurice *et al.*, 1994; Yamada *et al.*, 1996), was defined as entry into all three arms on consecutive choices in overlapping triplet sets. The per cent spontaneous alternation behaviour was calculated as the ratio of actual to possible alternations (defined as the total number of arm entries $- 2$) $\times 100$.

Water maze task

The water maze task (Morris, 1984), with some modification (Tanaka *et al.*, 1998), was carried out from day 9 to 16 after the start of A β infusion. The experimental apparatus consisted of a circular water tank (140 cm in diameter and 45 cm high). A transparent platform (10 cm in diameter and 25 cm high) was set inside the tank, which was filled, to a height of 27 cm, with water of temperature approximately 23°C; the surface of the platform was 2 cm below the surface of the water. The pool was located in a large test room, in which there were many cues external to the maze (e.g. pictures, lamps, etc.). The position of the cues remained unchanged throughout the water maze task.

Reference memory test: For each training trial, the rat was put into the pool at one of five starting positions, the sequence of the positions being selected randomly. The platform was located in a constant position throughout the test period in the middle of one quadrant, equidistant from the centre and edge of the pool. In each training session, the latency to escape onto the hidden platform was recorded. If the rat found the platform, it was allowed to remain there for 15 s and was then returned to its home cage. If the rat was unable to find the platform within 90 s, the training was terminated and a maximum score of 90 s was assigned. Training was conducted for 5 consecutive days, twice a day, from day 9 to day 13 after the start of A β infusion.

Probe test: Immediately after the tenth training trial on day 13 after the start of A β infusion, the platform was removed from the pool and animals were tested on a 30 s spatial probe trial. The time spent in the platform-quadrant where the platform had been located during training was measured.

Working memory (repeated acquisition) test: Working memory test was conducted for three consecutive days from day 14 to day 16 after the start of A β infusion, and consisted of five trials (one session) per day. The working memory test was procedurally similar to standard water maze test training, except that the platform location was changed for each session. Since the platform position was changed daily, this task evaluated working memory (Frick *et al.*, 1995). For each trial, the rat was put into the pool at one of five starting positions, the sequence of the positions being selected randomly. The first trial of each session was an informative sample trial in which

the rat was allowed to swim to the platform in its new location and to remain there for 15 s. The rat was then placed in a home cage for an intertrial interval of 1 min. The platform remained in the same location throughout the remaining four trials of the day. Spatial working memory was regarded as the mean escape latency of the second to fifth trials. The ability of working memory in each rat was assessed by the mean performance for three consecutive days from day 14 to day 16.

Multiple-trial passive avoidance task

The multiple-trial passive avoidance task was carried out on day 17 and 18 after the start of A β infusion, as described previously (Yamada *et al.*, 1996). The experimental apparatus consisted of two compartments (25 $\times$ 15 $\times$ 15 cm high), one illuminated and one dark, both equipped with a grid floor. The two compartments were separated by a guillotine door. In the acquisition trial, each rat was placed in the illuminated compartment; when the animal entered the dark compartment, the door was closed and an inescapable footshock (0.3 mA, 5 s) was delivered through the grid floor. The rat was removed after receiving the footshock and was placed back into the light compartment by the experimenter. The door was again opened 30 s later to start the next trial. Training continued in this manner until the rat stayed in the light compartment for 120 s on a single trial. In the retention trial, given 24 h after the acquisition test, the rat was again placed in the illuminated compartment and the time taken to enter the dark compartment was measured as step-through latency. When the rat did not enter for at least 300 s, a score of 300 s was assigned.

Measurement of ChAT activity and monoamine and metabolite content

Rats were killed 1 h after the last administration of nefiracetam on day 20 after the start of A β -(1-42) infusion. The brains were dissected into four regions, the frontal cortex, parietal cortex, striatum and the hippocampus, after which they were rapidly frozen and stored in a deep freezer at -80°C until assayed. Measurement of ChAT activity was carried out as reported previously (Kaneda & Nagatsu, 1985). The content of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), and noradrenaline was determined by a high-performance liquid chromatography system with an electrochemical detector (Eicom, Kyoto, Japan), as described by Yamada *et al.* (1995b).

Drugs

A β -(1-42) and A β -(40-1) were obtained from Bachem (Torrance, CA, U.S.A.), and dissolved in 35% acetonitrile containing 0.1% trifluoroacetic acid at a concentration of 25 μM . Nefiracetam [N-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidiny)l acetamide, DM-9384] was kindly provided by Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan), and dissolved in saline.

Statistical analysis

Results were expressed as means $\pm$ s.e.mean. Statistical significance between A β -(40-1)-treated control and A β -(1-42)-treated groups was determined by Student's *t*-test for locomotor activity, number of arm entries in the Y-maze task, number of training trials in the passive avoidance task, and the

neurochemical data. Mann-Whitney *U*-test was also used for the two group comparison in spontaneous alternation behaviour in Y-maze, performance in water maze task and step-through latency in the passive avoidance task. Statistical significance in the behavioural and neurochemical effects of nefiracetam was determined by one-way analysis of variance (ANOVA) or the Kruskal-Wallis test, followed by Bonferroni's test. Two-way ANOVA was also conducted for analysing data of the training trials of the water maze.

Results

Effects of nefiracetam on locomotor activity in the $A\beta$ -(1-42)-treated rats

There was no difference in locomotor activity counts on day 7 after the start of $A\beta$ infusion between $A\beta$ -(40-1)-infused control (1734 ± 21 counts/10 min, $n=8$) and the $A\beta$ -(1-42)-infused group (1708 ± 38 counts/10 min, $n=9$) ($P=0.9112$). The activity counts in the $A\beta$ -(1-42)-infused rats that received an oral administration of nefiracetam at doses of 1, 3 and 10 mg kg⁻¹ prior to the measurement of locomotor activity, were 1842 ± 21 , 2089 ± 19 , and 1817 ± 37 counts/10 min, respectively. One-way ANOVA revealed that there was no statistical difference in locomotor activity counts between vehicle-treated and nefiracetam-treated $A\beta$ -(1-42)-infused rats [$F(3,30)=0.835$, $P=0.4853$].

Effects of nefiracetam on spontaneous alternation behaviour in the $A\beta$ -(1-42)-treated rats

As shown in Figure 2A, the spontaneous alternation behaviour in the $A\beta$ -(1-42)-infused group was significantly lower than that in the $A\beta$ -(40-1)-infused control group ($P<0.01$). There was a statistical difference in spontaneous alternation behaviour between vehicle-treated and nefiracetam-treated $A\beta$ -(1-42)-infused rats [KW=8.395, $P=0.0385$]. *Post-hoc* analysis revealed that nefiracetam at a dose of 10 mg kg⁻¹ significantly attenuated the impairment of this behaviour induced by $A\beta$ -(1-42) ($P<0.05$). The number of arm entries did not differ between the five different treatment groups of rats (Figure 2B), indicating that changes in alternation behaviour were not due to generalized exploratory, locomotor or motivational effects.

Effects of nefiracetam on performance of the water maze task in the $A\beta$ -(1-42)-treated rats

Changes in escape latency time taken to find the hidden platform produced by training trials in each group of rats are shown in Figure 3A. Two-way ANOVA with all treatment groups revealed significant main effects of group [$F(4,370)=3.437$, $P=0.0089$] and training [$F(9,370)=86.354$, $P<0.0001$], but not group by trial interactions [$F(36,370)=0.9808$, $P>0.05$]. *Post hoc* analysis indicated that performance in the $A\beta$ -(1-42)-infused group was significantly impaired, compared to that in the $A\beta$ -(1-40)-infused control group ($P<0.0188$). Repeated daily administration of nefiracetam at a dose of 3 mg kg⁻¹ significantly ameliorated the impairment of performance caused by $A\beta$ -(1-42) ($P<0.001$) (Figure 3A). Nefiracetam at 10 mg kg⁻¹ also improved performance in the $A\beta$ -(1-42)-treated rats, but the effect was not significant ($P=0.0514$).

A 30-s spatial probe trial was carried out on day 13 after the start of $A\beta$ -(1-42) infusion, following the tenth training trial

(Figure 3B). The $A\beta$ -(1-42)-infused group, compared with the $A\beta$ -(40-1)-treated control group, showed a significant decrease in the time spent in the quadrant in which the platform had been located (platform-quadrant) during training (control group: $53.8 \pm 3.9\%$, $A\beta$ -(1-42)-infused group: $33.0 \pm 2.4\%$) (Figure 3B). There was a significant group effect on the time spent in the platform-quadrant between the vehicle- and nefiracetam-treated $A\beta$ -(1-42)-infused groups [KW=11.098, $P<0.0112$]. *Post-hoc* analysis revealed that all doses of nefiracetam examined in the present study significantly increased the time spent in the platform-quadrant ($P<0.01$), relative to the $A\beta$ -(40-1)-treated control group.

The escape latencies in the first trial (sample trial) and in the second to fifth trials (test trials) of the working memory test are shown in Figure 4A and B, respectively. Although there was no difference in the escape latency in the sample trial (Figure 4A), a marked difference in the test trials was observed between $A\beta$ -(40-1)-infused control and $A\beta$ -(1-42)-treated groups

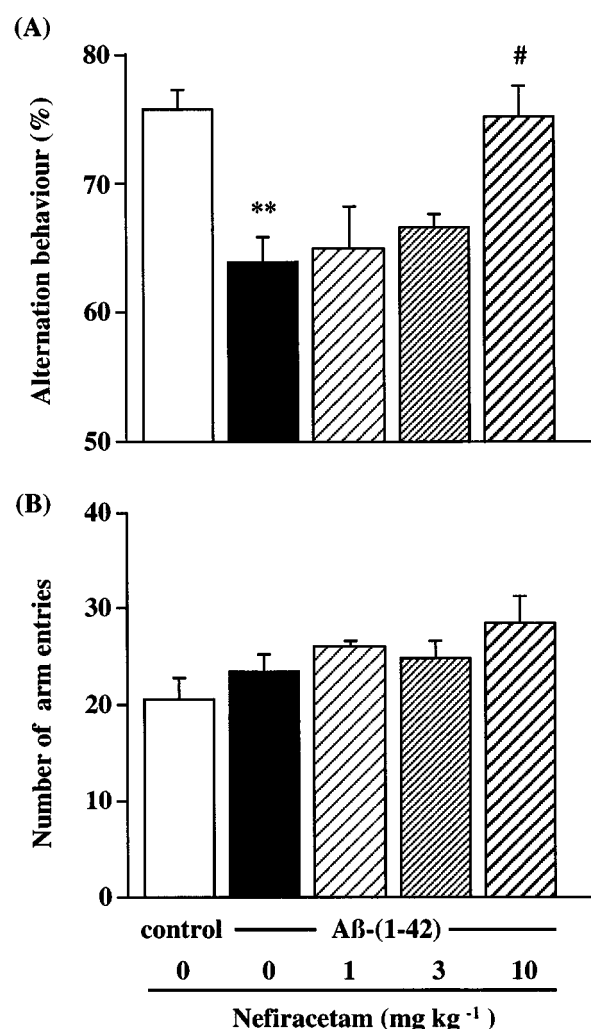


Figure 2 Effects of nefiracetam on spontaneous alternation behaviour in the $A\beta$ -(1-42)-treated rats. Spontaneous alternation behaviour (A) and the number of arm entries (B) during an 8-min session in the Y-maze task were measured on day 8 after the start of $A\beta$ infusion. Daily oral administration of nefiracetam was started on day 7, and spontaneous alternation behaviour was measured 1 h after the administration. Columns indicate means $\pm$ s.e. mean ($n=8-9$). ** $P<0.01$ vs $A\beta$ -(40-1)-treated control rats. # $P<0.05$ vs $A\beta$ -(1-42)-treated rats.

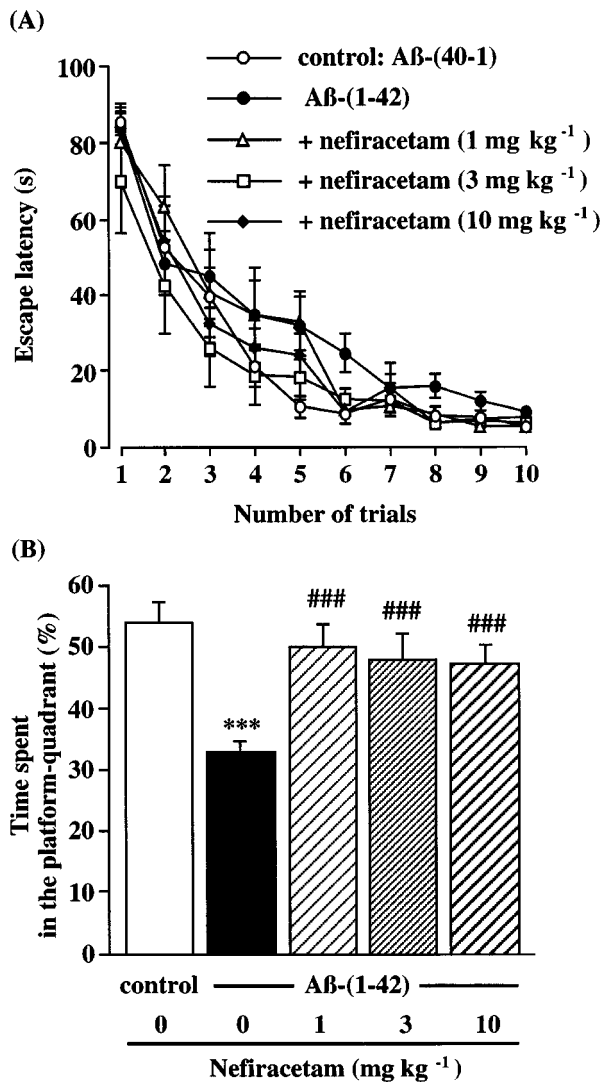


Figure 3 Effects of nefiracetam on performance in the training trials (A) and in the probe trial (B) of the water maze task in the A β -(1-42)-treated rats. The training trials were carried out on days 9–13 after the start of A β infusion. The probe trial was carried out on day 13 after the start of A β infusion, immediately after the tenth training trial. Daily oral administration of nefiracetam was started on day 7, and the training was carried out 1 h after the administration. Symbols indicate the means $\pm$ s.e.mean ($n=8-9$). *** $P<0.001$ vs A β -(40-1)-treated control rats. ### $P<0.001$ vs A β -(1-42)-treated rats.

($P<0.001$) (Figure 4B). Nefiracetam significantly and dose-dependently ameliorated the impairment of performance in the test trials in the A β -(1-42)-infused rats ($P<0.001$) (Figure 4B), without affecting the escape latency in the sample trial (Figure 4A).

Effects of nefiracetam on performance of the multi-trial passive avoidance task in the A β -(1-42)-treated rats

In the acquisition trial, there was no difference in the step-through latency (Figure 5A) or the number of training trials (Figure 5B) among the five treatment groups. Furthermore, there was no apparent difference in vocalization of rats in each treatment group when they received a footshock.

In the retention trial, however, a significant reduction of the step-through latency was observed in the A β -(1-42)-treated

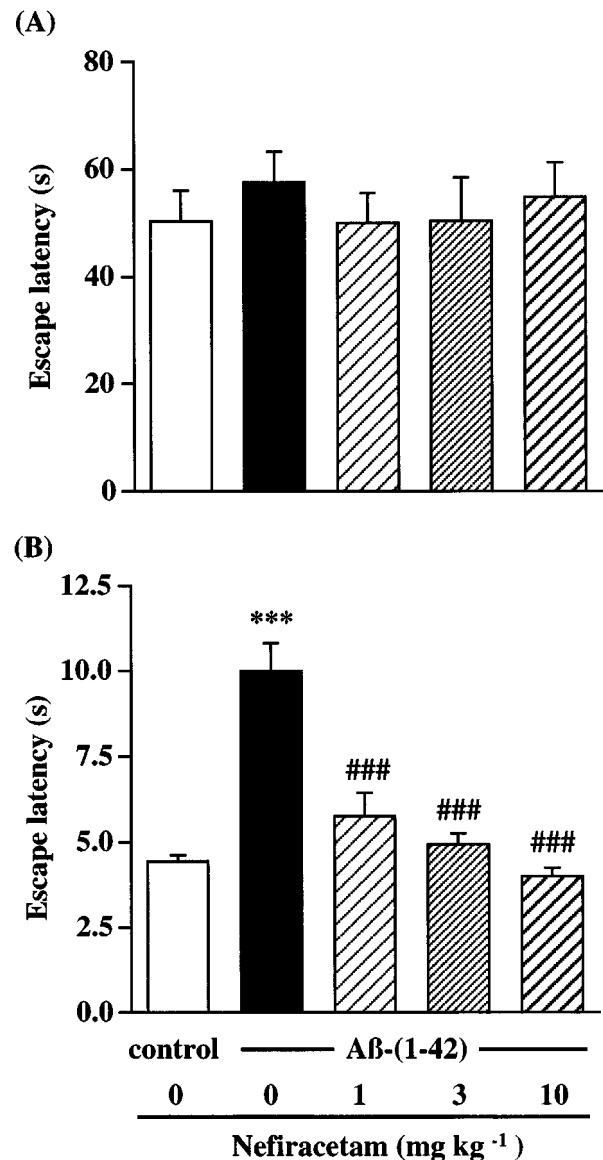


Figure 4 Effects of nefiracetam on performance in the sample trial (A) and in the test trials (B) of the working memory test of the water maze task in the A β -(1-42)-treated rats. The working memory test (five trials per day) was carried out on days 14–16 after the start of A β infusion. Daily oral administration of nefiracetam was started on day 7, and the test was carried out 1 h after the administration. Values are the means $\pm$ s.e.mean ($n=8-9$). *** $P<0.001$ vs A β -(40-1)-treated control rats. ### $P<0.001$ vs A β -(1-42)-treated rats.

groups compared with the A β -(40-1)-treated control group ($P<0.01$). The deficits of retention of the passive avoidance learning in the A β -(1-42)-treated rats were improved by the treatment with nefiracetam at doses of 1, 3, and 10 mg kg⁻¹ [KW = 10.110, $P=0.0177$].

Effects of nefiracetam on choline acetyltransferase activity in the A β -(1-42)-treated rats

Table 1 shows that continuous infusion of A β -(1-42) had no significant effect on ChAT activity in the hippocampus, frontal cortex or the striatum. Repeated oral administration of nefiracetam at a dose of 3 mg kg⁻¹ for 14 days caused a significant increase (25%) in ChAT activity in the hippocampus, but not in other areas of the brain such as the frontal

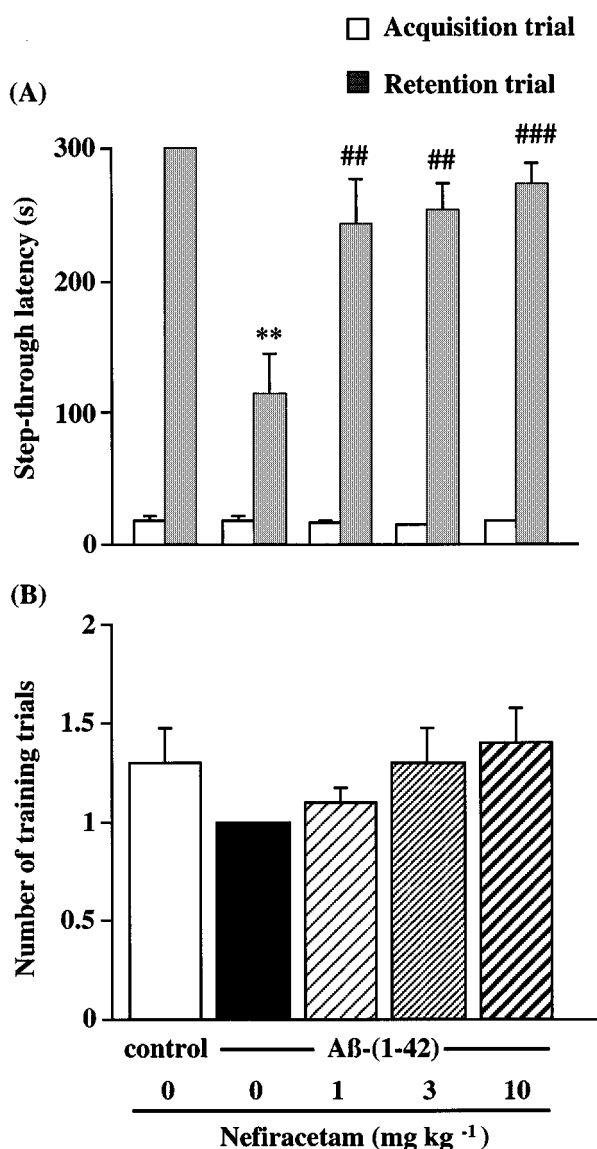


Figure 5 Effects of nefiracetam on step-through latency (A) and the number of training trials (B) in the multiple-trial passive avoidance task in the A β -(1-42)-treated rats. The task was carried out on days 17–18 after the start of A β infusion. Daily oral administration of nefiracetam was started on day 7, and the passive avoidance task was carried out 1 h after the administration. Values are the means $\pm$ s.e.mean ($n=8-9$). ** $P<0.01$ vs A β -(40-1)-treated control rats. ### $P<0.01$, #### $P<0.001$ vs A β -(1-42)-treated rats.

cortex and striatum, of the A β -(1-42)-treated rats. Nefiracetam, at doses of 1 and 10 mg kg⁻¹, failed to affect ChAT activity in any of the brain areas examined.

Effects of nefiracetam on monoamine and metabolite content in the A β -(1-42)-treated rats

Table 2 shows the effects of nefiracetam on dopamine metabolism in the brains of rats continuously infused with A β -(1-42) into the cerebral ventricle. It is evident that continuous infusion of A β -(1-42) had no significant effect on dopamine, DOPAC and HVA levels in the frontal and parietal cortex, striatum and hippocampus (Table 2). There were also no differences in 5-HT, 5-HIAA and noradrenaline contents in

Table 1 Effects of nefiracetam on the ChAT activity in the brains of rats continuously infused with A β -(1-42) into the cerebral ventricle

Treatment	Dose (mg kg ⁻¹)	ChAT activity (pmol min ⁻¹ mg ⁻¹ protein)		
		Hippocampus	Frontal cortex	Striatum
Control	0	1617 $\pm$ 145	1299 $\pm$ 96	4649 $\pm$ 280
A β -(1-42)	0	1571 $\pm$ 88	1137 $\pm$ 62	4556 $\pm$ 254
+Nefiracetam	1	1556 $\pm$ 123	1175 $\pm$ 48	4955 $\pm$ 200
	3	1978 $\pm$ 97**	1150 $\pm$ 36	4755 $\pm$ 177
	10	1517 $\pm$ 98	1229 $\pm$ 43	4726 $\pm$ 162

Rats were killed 1 h after the last administration of nefiracetam on day 20 after the start of the A β -(1-42) infusion. Control rats were infused with A β -(40-1). Each value represents the means $\pm$ s.e.mean ($n=8-9$). ** $P<0.01$ vs A β -(1-42)-treated rats.

any of these areas of the brain between A β -(40-1)-infused control and A β -(1-42)-treated animals (data not shown).

Nefiracetam significantly increased dopamine content in the frontal and parietal cortex, and striatum, but not hippocampus, in the A β -(1-42)-treated rats. The effect was most significant at the lowest dose (1 mg kg⁻¹). Nefiracetam also increased DOPAC levels in the parietal cortex, and DOPAC and HVA levels in the striatum at doses of 1 and 3 mg kg⁻¹. Furthermore, this compound increased the DOPAC:dopamine ratio in the parietal cortex, by more than 2 fold, at the dose range of 1–10 mg kg⁻¹. Nefiracetam did not affect the content of 5-HT, 5-HIAA or noradrenaline in any areas of the brain examined in the A β -(1-42)-treated animals (data not shown).

Discussion

Previous studies, including those from our laboratory, have demonstrated that intracerebral injection of A β causes brain dysfunction as evidenced by neurodegeneration and an impairment of learning and memory (Kowall *et al.*, 1991; Flood *et al.*, 1994; Nitta *et al.*, 1994, 1997; Giovannelli *et al.*, 1995; Maurice *et al.*, 1996; Yamada *et al.*, 1998) although neurotoxic effects of A β *in vivo* have been somewhat controversial (Clemens & Stephenson, 1992; Games *et al.*, 1992; Winkler *et al.*, 1994). In the present study, therefore, we extensively investigated behavioural changes in A β -(1-42)-treated rats.

We observed that continuous infusion of A β -(1-42) into the cerebral ventricle caused an impairment of spontaneous alternation behaviour in a Y-maze and performance in a water maze task, both of which are considered to involve spatial memory. Retention of long-term passive avoidance learning was also impaired by the continuous infusion of A β -(1-42). The feature of learning and memory impairments in rats induced by continuous infusion of A β -(1-42) is, in general, similar to that found in rats infused with A β -(1-40) (Nitta *et al.*, 1994, 1997; Yamada *et al.*, 1998; Tanaka *et al.*, 1998). However, ChAT activities in the brains of A β -(1-42)-infused rats did not differ from those in the A β -(40-1)-infused control rats whereas continuous infusion of A β -(1-40) decreased the hippocampal ChAT activity (Nitta *et al.*, 1994, 1997; Yamada *et al.*, 1998). Therefore, there may be some difference in the neurotoxicity *in vivo* caused by A β -(1-40) and A β -(1-42).

It is unlikely that the impairment of performance of the A β -(1-42)-treated rats in learning and memory tasks is due to changes in motivation or sensorimotor function since different

Table 2 Effects of nefiracetam on dopamine metabolism in the brains of rats continuously infused with A β -(1-42) into the cerebral ventricle

<i>Treatment</i>	<i>Dose</i> (mg kg ⁻¹)	<i>Dopamine</i>	<i>DOPAC</i> (ng g ⁻¹ tissue)	<i>HVA</i>	<i>DOPAC/Dopamine</i>	<i>HVA/Dopamine</i>
<i>Frontal cortex</i>						
Control	0	28.6 ± 6.7	20.7 ± 4.6	7.3 ± 2.0	0.741 ± 0.062	0.247 ± 0.020
A β -(1-42)	0	22.2 ± 1.4	15.2 ± 1.3	5.5 ± 0.5	0.704 ± 0.077	0.252 ± 0.023
+ Nefiracetam	1	29.7 ± 2.5*	18.1 ± 1.5	7.9 ± 1.4	0.617 ± 0.038	0.267 ± 0.040
	3	29.4 ± 1.3*	19.6 ± 1.9	5.6 ± 0.6	0.690 ± 0.091	0.195 ± 0.019
	10	23.8 ± 1.4	16.0 ± 1.1	5.1 ± 0.5	0.674 ± 0.036	0.213 ± 0.017
<i>Parietal cortex</i>						
Control	0	12.8 ± 1.1	9.4 ± 0.8	2.3 ± 0.1	0.747 ± 0.059	0.183 ± 0.011
A β -(1-42)	0	10.0 ± 0.9	9.3 ± 2.0	1.9 ± 0.1	0.915 ± 0.156	0.193 ± 0.008
+ Nefiracetam	1	15.9 ± 1.4**	8.6 ± 0.5	7.9 ± 1.7**	0.563 ± 0.044	0.474 ± 0.071**
	3	13.0 ± 1.1	10.7 ± 1.0	5.8 ± 1.0*	0.906 ± 0.153	0.435 ± 0.077*
	10	10.1 ± 0.6	9.5 ± 1.3	4.7 ± 0.3	0.998 ± 0.185	0.476 ± 0.042**
<i>Striatum</i>						
Control	0	19189 ± 1435	2035 ± 112	2508 ± 130	0.109 ± 0.006	0.135 ± 0.009
A β -(1-42)	0	18794 ± 905	1952 ± 112	2605 ± 163	0.104 ± 0.004	0.140 ± 0.008
+ Nefiracetam	1	24791 ± 941**	2571 ± 166*	3186 ± 196	0.104 ± 0.007	0.130 ± 0.009
	3	22875 ± 2010	2949 ± 192**	3432 ± 218**	0.140 ± 0.023	0.164 ± 0.029
	10	19901 ± 758	1891 ± 82	2606 ± 71	0.095 ± 0.003	0.132 ± 0.005
<i>Hippocampus</i>						
Control	0	10.2 ± 0.5	13.9 ± 1.0	4.4 ± 0.4	1.386 ± 0.120	0.428 ± 0.033
A β -(1-42)	0	9.6 ± 0.8	15.4 ± 1.5	4.3 ± 0.4	1.586 ± 0.145	0.468 ± 0.051
+ Nefiracetam	1	9.7 ± 0.6	12.6 ± 1.5	3.7 ± 0.2	1.277 ± 0.132	0.387 ± 0.028
	3	9.0 ± 1.5	12.4 ± 3.0	3.7 ± 0.5	1.330 ± 0.208	0.420 ± 0.021
	10	9.2 ± 0.7	13.2 ± 1.5	4.1 ± 0.3	1.410 ± 0.090	0.458 ± 0.036

Rats were killed 1 h after the last administration of nefiracetam on day 20 after the start of the A β -(1-42) infusion. Control rats were infused with A β -(40-1). Each value represents the mean $\pm$ s.e. mean ($n = 8-9$). * $P < 0.05$, ** $P < 0.01$ vs A β -(1-42)-treated rats.

motivation is involved in these behavioural tasks, and different skill is required for better performance in each task. Actually, the locomotor activity and the number of total arm entries in the Y-maze task in the A β -(1-42)-treated rats did not differ from those in the A β -(40-1)-treated control rats, indicating no changes in motor function and exploratory activity. There were also no differences between the A β -(40-1)-treated control and A β -(1-42)-treated rats in the sensitivity to electric footshock in the acquisition trial of the passive avoidance task and the escape latency onto the submerged platform in the first training trial and the sample trial of working memory test in the water maze task. These results indicate no changes in shock sensitivity and swimming ability. Therefore, it is likely that impairment of performance in the A β -(1-42)-treated rats is due to learning and memory deficits.

Nefiracetam, at a dose range of 1–10 mg kg⁻¹, significantly ameliorated impairment of spontaneous alternation behaviour in the Y-maze task, performance in the water maze task, and shortened step-through latency in the retention trial of passive avoidance task in the A β -(1-42)-treated rats. This compound did not affect locomotor activity, sensitivity to electric footshock in the acquisition trial of the passive avoidance task or the escape latency onto the submerged platform in the first training trial and the sample trial of working memory test in the water maze task in the A β -(1-42)-treated rats. Therefore, it is unlikely that the observed improvement by nefiracetam of performance in various cognitive tasks is due to changes in sensorimotor function and/or motivation in the A β -(1-42)-treated rats. Rather, it is plausible that nefiracetam ameliorates learning and memory deficits caused by the continuous infusion of A β -(1-42) into the cerebral ventricle in rats.

The mechanism of learning and memory dysfunction in the A β -(1-42)-treated rats is unknown at present. We have previously demonstrated, by using *in vivo* brain dialysis, that high potassium- and nicotine-induced increase in ACh and dopamine release in the hippocampus/cerebral cortex and the striatum, respectively, is markedly impaired by the continuous infusion of A β -(1-40) although the basal levels of these neurotransmitters in the A β -(1-40)-infused rats did not differ from those in vehicle-infused control animals (Itoh *et al.*, 1996). Harkany *et al.* (1998) showed that bilateral injection of β -amyloid(Phe(SO₃H)²⁴)25-35 peptide, a metabolically stable analogue of A β -(25-35), into the rat nucleus basalis magnocellularis caused a reduction of cortical acetylcholinesterase-positive projections. Maurice *et al.* (1996) demonstrated that tacrine, a cholinesterase inhibitor, and (–)-nicotine improved the A β -(25-35)-induced impairment of spontaneous alternation behaviour in a Y-maze, passive avoidance learning, and water maze learning in mice. An *in vitro* experiment has shown that A β -(25-35) activates tau protein kinase 1/glycogen synthase kinase 3 β , leading to an inactivation of pyruvate dehydrogenase, and causes an impairment of ACh synthesis without affecting ChAT activity (Hoshi *et al.*, 1996). In light of these previous findings, we consider that dysfunctions of cholinergic and dopaminergic neuronal systems are responsible, at least in part for the A β -induced learning and memory deficits.

Nefiracetam activates VSCC *in vitro* (Yoshii & Watabe, 1994) and the effect is involved in the amelioration by nefiracetam of scopolamine-induced memory impairment (Yamada *et al.*, 1994). Accordingly, it is plausible that nefiracetam ameliorates the A β -(1-42)-induced learning and

memory deficits by activating VSCC, and thereby stimulates release of ACh and dopamine in the brain. Indeed, it has been shown that nefiracetam stimulates the turnover of ACh (Abe *et al.*, 1994; Kawajiri *et al.*, 1994), dopamine (Abe *et al.*, 1992; 1994) and GABA (Watabe *et al.*, 1993), as well as ACh release in the frontal cortex (Sakurai *et al.*, 1998). In the present study, we have demonstrated that nefiracetam can increase dopamine turnover in the cerebral cortex and striatum in the $A\beta$ -treated rats.

One might argue against our hypothesis that the amelioration by nefiracetam of $A\beta$ -(1-42)-induced learning and memory deficits is due to the activation of VSCC, since accumulating evidence suggests that high concentration of $A\beta$ destabilizes intracellular calcium mobilization (Fraser *et al.*, 1997). Some studies have suggested that calcium influx through VSCC contributes to $A\beta$ -induced neurotoxicity *in vitro* and that VSCC antagonists, by reducing such influx, are able to attenuate $A\beta$ -induced neurotoxicity (Weiss *et al.*, 1994; Abe & Kimura, 1996).

We do not argue against the calcium hypothesis of the $A\beta$ -induced neurotoxicity. However, ChAT activities in the brains of $A\beta$ -(1-42)-treated rats did not differ from those in $A\beta$ -(40-1)-treated control rats, suggesting that memory deficits in the $A\beta$ -(1-42)-treated rats are not directly associated with cholinergic neurotoxicity. Furthermore, nefiracetam did not improve nor potentiate the $A\beta$ -(1-42)-induced learning and memory impairments if daily administration of this compound began 3 days before the start of $A\beta$ -(1-42) infusion (unpublished observation). In such experiments, nefiracetam was administered p.o. 1 h after the behavioural test. Therefore, it is unlikely that the ameliorating effects of nefiracetam observed in the present study are due to its neuroprotective effect against $A\beta$ -(1-42). Rather it is possible that amelioration by nefiracetam of $A\beta$ -(1-42)-induced learning and memory impairments is associated with its acute memory-enhancing effect (Nabeshima, 1994).

It has been suggested that low concentrations of soluble $A\beta$ can potentially induce cholinergic and dopaminergic hypoactivity (Itoh *et al.*, 1996) that is not dependent on concurrent neurotoxicity (for review see Auld *et al.*, 1998). Accordingly, we speculate that learning and memory deficits observed in the $A\beta$ -(1-42)-treated rats are accompanied by hypoactivity of cholinergic and dopaminergic neurons, which is not directly related to the neurodegeneration and that nefiracetam can ameliorate learning and memory impairments by stimulating ACh (Sakurai *et al.*, 1998) and dopamine release through the activation of VSCC.

In addition, nefiracetam modulates GABA_A (Huang *et al.*, 1996) or nicotinic ACh receptor currents (Nishizaki *et al.*, 1998), stimulates ChAT and glutamate decarboxylase activities (Shiotani *et al.*, 1992; Tanaka *et al.*, 1992; Watabe *et al.*, 1993), and muscarinic ACh receptor binding (Nabeshima *et al.*, 1991b). Therefore, these effects may also contribute to the amelioration by nefiracetam of $A\beta$ -(1-42)-induced learning and memory deficits. In fact, we observed an increase in the hippocampal ChAT activity in the $A\beta$ -(1-42)-treated rats which had received repeated oral administration of nefiracetam at a dose of 3 mg kg⁻¹ for 14 days although the effect was not dose-related.

In conclusion, we have demonstrated in the present study that a novel cognition enhancing drug, nefiracetam, ameliorates learning and memory deficits in rats which had previously received a continuous infusion of $A\beta$ -(1-42) into the cerebral ventricle. Our findings suggest that nefiracetam may be useful for the treatment of patients with AD and that clinical trials of nefiracetam for the treatment of AD are warranted.

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Mibefradil (Ro 40-5967) inhibits several Ca^{2+} and K^{+} currents in human fusion-competent myoblasts

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1 The effect of mibefradil (Ro 40-5967), an inhibitor of T-type Ca^{2+} current ($I_{\text{Ca(T)}}$), on myoblast fusion and on several voltage-gated currents expressed by fusion-competent myoblasts was examined.

2 At a concentration of 5 μM , mibefradil decreases myoblast fusion by 57%. At this concentration, the peak amplitudes of $I_{\text{Ca(T)}}$ and L-type Ca^{2+} current ($I_{\text{Ca(L)}}$) measured in fusion-competent myoblasts are reduced by 95 and 80%, respectively. The IC_{50} of mibefradil for $I_{\text{Ca(T)}}$ and $I_{\text{Ca(L)}}$ are 0.7 and 2 μM , respectively.

3 At low concentrations, mibefradil increased the amplitude of $I_{\text{Ca(L)}}$ with respect to control.

4 Mibefradil blocked three voltage-gated K^{+} currents expressed by human fusion-competent myoblasts: a delayed rectifier K^{+} current, an *ether-à-go-go* K^{+} current, and an inward rectifier K^{+} current, with a respective IC_{50} of 0.3, 0.7 and 5.6 μM .

5 It is concluded that mibefradil can interfere with myoblast fusion, a mechanism fundamental to muscle growth and repair, and that the interpretation of the effect of mibefradil in a given system should take into account the action of this drug on ionic currents other than Ca^{2+} currents.

Keywords: Myoblast; potassium current; mibefradil; myoblast fusion

Abbreviations: $I_{\text{Ca(L)}}$, L-type Ca^{2+} current; $I_{\text{Ca(T)}}$, T-type Ca^{2+} current; $I_{\text{h-eag}}$, Human *ether-à-go-go* K^{+} current; $I_{\text{K(DR)}}$, Delayed rectifier K^{+} current; $I_{\text{K(IR)}}$, Inward rectifier K^{+} current; $I_{\text{K(NI)}}$, Non-inactivating K^{+} current

Introduction

Mibefradil is a pharmacological agent accepted as a rather selective inhibitor of Ca_{T} channels (Ertel & Ertel, 1997). This agent has recently been used in the therapy of some cardiovascular diseases (Clozel *et al.*, 1997) before being withdrawn from the market in June 1998 due to its interactions with other drugs (Po & Zhang, 1998).

Recently, we have proposed that a low-threshold transient Ca^{2+} current ($I_{\text{Ca(T)}}$) of fusion-competent myoblasts might contribute to an inward flux of Ca^{2+} ions (Liu *et al.*, 1998) and thereby play a role in myoblast fusion, a process essential to skeletal muscle development and repair. For this reason, we examined the effect of mibefradil on myoblast fusion, expecting it to be reduced if $I_{\text{Ca(T)}}$ was involved. We found that mibefradil markedly reduces myoblast fusion. However, we also found that, in addition to inhibiting $I_{\text{Ca(T)}}$, mibefradil very efficiently blocks several types of voltage-gated K^{+} channels, as shall be described here.

Methods

Dissociation and cell cultures

Samples of human skeletal muscle were obtained during corrective orthopaedic surgery of young patients (9 months to 17 years old) without any known neuromuscular disease in accordance with the guidelines of the ethical committee of the University Hospital of Geneva (written informed consent was

obtained from patients or their legal guardians). Myoblasts (Baroffio *et al.*, 1993) and fusion-competent myoblasts (Krause *et al.*, 1995) were prepared as described.

Electrophysiology

Whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) was used as in Bernheim *et al.* (1996). Leak current subtraction procedures are discussed in the Figure legends. (i) Potassium currents. Extracellular solution (mM): NMG-Cl (100), KCl (5), MgCl_2 (3), HEPES (5), NaOH (50), acetic acid (50), and glucose (8). The pH was adjusted to 7.3 with NMG. Intracellular (pipette) solution (mM): KCl (110), NaCl (5), MgCl_2 (1), HEPES (5), BAPTA (20), and glucose (5). The pH was adjusted to 7.3 with KOH. (ii) Currents through calcium channels. Extracellular solution (mM): BaCl_2 (10), TEA-Cl (90), tetrodotoxin (10 μM), NMG-Cl (50), KCl (5), MgCl_2 (2), HEPES (5), and glucose (8). The pH was adjusted to 7.3 with NMG. Intracellular (pipette) solution (mM): KCl (145), MgCl_2 (2), HEPES (10), BAPTA (1), Mg-ATP (3), and glucose (5). The pH was adjusted to 7.3 with KOH.

Fusion index

The fusion index is defined as the number of nuclei in myotubes divided by the total number of nuclei counted. Cultures were fixed 5 min with ice-cold 100% methanol, and stained with haematoxylin. Nuclei were counted in 20 randomly chosen microscope fields in two separate cultures. One microscope field usually contains between 100 and 150 nuclei. In *t*-tests, *n* refers to the number of microscope fields counted.

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Statistics

All data are expressed as means $\pm$ s.e.mean.

Results

Effect of mibefradil on myoblast fusion

Human myoblasts can be maintained in an undifferentiated state for several weeks, as long as they are exposed to a growth medium (Baroffio *et al.*, 1993; Ham *et al.*, 1989). Alternatively, myoblast fusion can be rapidly induced when the proliferation medium is replaced with differentiation medium (StClair *et al.*, 1992), and maximum fusion is usually reached within 48 h.

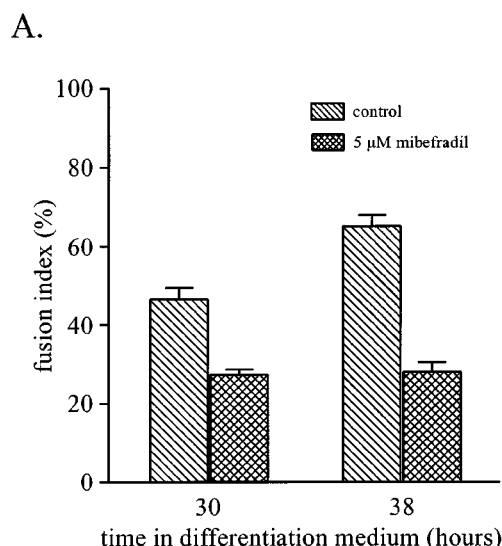
In preliminary experiments, we examined the effect on fusion of a concentration of mibefradil known to block a large fraction of $\text{I}_{\text{Ca(T)}}$ ($5 \mu\text{M}$; Clozel *et al.*, 1997). Figure 1 illustrates the dramatic effect $5 \mu\text{M}$ mibefradil has on myoblast fusion. In Figure 1A, it can be seen that after 30 h of exposure to the differentiation medium containing the drug, the fusion index was reduced by 43% with respect to control. Eight hours later, when fusion was nearly maximal in the control cultures (Baroffio *et al.*, 1996), the fusion index in sister cultures exposed to mibefradil was still at the same low level and was reduced by 57% with respect to control. The photomicrographs in Figure 1B illustrate the difference between a control culture (top) and its sister culture exposed to mibefradil (bottom). In the presence of the drug, although the cells look perfectly healthy, there are very few multinucleated myotubes, with the vast majority of the cells remaining as mononucleated myoblasts.

Effect of mibefradil on Ca^{2+} currents

The sensitivity of voltage-gated Ca^{2+} channels to mibefradil was examined in fusion-competent myoblasts. Fusion-competent

myoblasts are obtained by plating myoblasts at a very low density in differentiation medium. Under these conditions, the cells are induced to differentiate and thus begin to express Ca^{2+} and K^{+} currents (Bernheim *et al.*, 1996; Liu *et al.*, 1998), but they are prevented from fusing due to lack of neighbouring cells (Krause *et al.*, 1995). In the differentiation medium, not all myoblasts necessarily express the same currents at a given time. This is due to the fact that (1) only about 70% of the myoblasts are going to fuse (Baroffio *et al.*, 1993), (2) cells are not strictly synchronized, and (3) the currents appear in a sequential order with some currents being probably expressed just before fusion. Regarding Ca^{2+} currents, Figure 2A (inset) shows the inward current activated by a depolarization in a myoblast expressing exclusively $\text{I}_{\text{Ca(T)}}$. This current was recorded in the presence of 10 mM Ba^{2+} in the extracellular solution. Also, in order to eliminate any contribution of a voltage-gated sodium current (Hamann *et al.*, 1994), tetrodotoxin ($10 \mu\text{M}$) was added to the bath solution which contained no sodium ions. It can be seen that the inward current activates rapidly and then quickly inactivates within a few tens of ms. The addition of mibefradil ($2 \mu\text{M}$) markedly decreased the inward current (the effect was reversible; data not shown). Figure 2A shows the current-voltage relationship of $\text{I}_{\text{Ca(T)}}$, together with that recorded in the presence of $2 \mu\text{M}$ mibefradil. The current suppressed by mibefradil activates at potentials more depolarized than -60 mV . This current is also blocked efficiently by amiloride and Ni^{2+} (data not shown). These data all consistently indicate that the inward current is $\text{I}_{\text{Ca(T)}}$.

In Figure 2B, similar data are shown for a myoblast expressing $\text{I}_{\text{Ca(L)}}$ in addition to $\text{I}_{\text{Ca(T)}}$. Inset, it can be seen that the same concentration of mibefradil ($2 \mu\text{M}$) was less efficient in blocking $\text{I}_{\text{Ca(L)}}$ than $\text{I}_{\text{Ca(T)}}$. The addition of $3 \mu\text{M}$ nifedipine, a blocker of $\text{I}_{\text{Ca(L)}}$, markedly reduced the current remaining in the presence of mibefradil, indicating that this steady inward current was indeed mainly $\text{I}_{\text{Ca(L)}}$. Comparison of the current-voltage relationship of Figure 2B in the absence and in the



B.

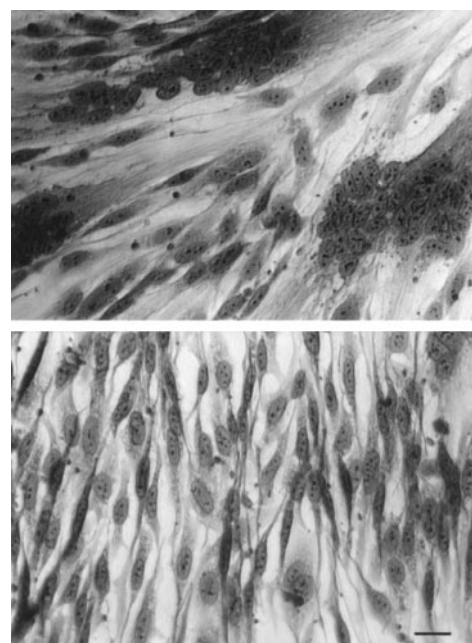


Figure 1 Mibefradil reduces myoblast fusion in primary culture. (A) Myoblast fusion was induced by exposing myoblasts to differentiation medium and the fusion index was evaluated after 30 and 38 h, in control conditions and in sister cultures exposed to $5 \mu\text{M}$ mibefradil. Mibefradil decreased myoblast fusion significantly ($P < 0.0001$) at the two time points evaluated. (B) Upper photograph: culture fixed after 38 h in differentiation medium (control condition) and stained with haematoxylin. Lower photograph: sister culture after 38 h in differentiation medium containing $5 \mu\text{M}$ mibefradil. Scale bar is $25 \mu\text{m}$.

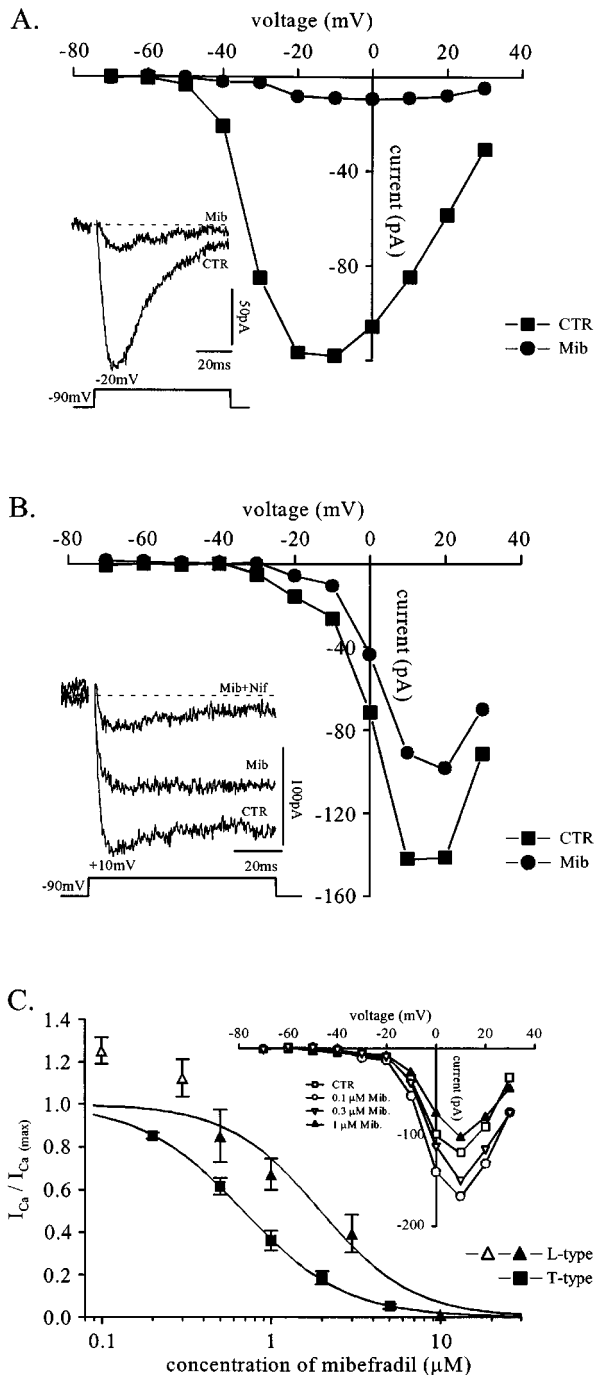


Figure 2 Effect of mibefradil on Ca^{2+} currents of fusion-competent myoblasts. (A) Recording from a fusion-competent myoblast (membrane capacitance 42 pF) exposed 3 days to differentiation medium. The cell, superfused with a solution containing 10 mM Ba^{2+} (see Methods), was held at -90 mV and stepped for 80 ms to a series of potentials between -70 and $+30$ mV. Leak current was estimated by adding 1 mM cadmium at the end of the experiment and was subtracted. Peak Ba^{2+} currents (current through T-type Ca^{2+} channels) were plotted as a function of the voltage during a step in the absence and in the presence of $2 \mu\text{M}$ mibefradil. Inset: Ba^{2+} current recorded during a step to -20 mV with and without $2 \mu\text{M}$ mibefradil. (B) Fusion-competent myoblast (membrane capacitance 10 pF) exposed 3 days to differentiation medium. Steady-state Ba^{2+} currents (current through mainly L-type Ca^{2+} channels) measured at the end of a 80 ms step and plotted as a function of the voltage during the step, in the absence and in the presence of $2 \mu\text{M}$ mibefradil. Leak current was estimated as in (A) and subtracted. Inset: Ba^{2+} current recorded during a step to $+10$ mV in control, in $2 \mu\text{M}$ mibefradil, and $3 \mu\text{M}$ nifedipine in addition to mibefradil. (C) Peak Ba^{2+} currents (current through T-type Ca^{2+} channels) recorded during a step to -20 mV and normalized to their amplitude

presence of mibefradil with that of Figure 2A confirms that $I_{\text{Ca(L)}}$ is less efficiently inhibited by mibefradil than $I_{\text{Ca(T)}}$ at all voltages.

Figure 2C illustrates the degree of inhibition of $I_{\text{Ca(T)}}$ and $I_{\text{Ca(L)}}$ at different mibefradil concentrations. The IC_{50} of mibefradil for $I_{\text{Ca(T)}}$ is $0.7 \mu\text{M}$. For $I_{\text{Ca(L)}}$, as few fusion-competent myoblasts express this current, the inhibition by mibefradil was tested both on fusion-competent myoblasts ($n=3$) and in freshly formed myotubes ($n=5$). The two sets of data were pooled as there was no difference between them. Interestingly, the effect of mibefradil on $I_{\text{Ca(L)}}$ of fusion-competent myoblasts and freshly-formed myotubes is bimodal. At $0.1 \mu\text{M}$, there is an increase in the amplitude of the inward current by 25% with respect to the control level. At higher concentrations, however, inhibition takes place. The IC_{50} for $I_{\text{Ca(L)}}$, taking into account exclusively the filled triangles, is near $2 \mu\text{M}$.

Figure 2C indicates that the concentration of mibefradil ($5 \mu\text{M}$) used to inhibit fusion in Figure 1 would have blocked 95% of $I_{\text{Ca(T)}}$ and 80% of $I_{\text{Ca(L)}}$. As we observed previously that $I_{\text{Ca(L)}}$ is not required for fusion of primary cultures of human myoblasts (Bernheim *et al.*, 1996), the effect of mibefradil on myoblast fusion would suggest that a functional $I_{\text{Ca(T)}}$ is important in the fusion process. This, however, only applies if the effect of mibefradil is restricted to Ca^{2+} currents. Indeed, we recently demonstrated that a block of an inward rectifier K^{+} current ($I_{\text{K(IR)}}$), which contributes to the final hyperpolarization of myoblasts to -65 mV, could nearly totally inhibit myoblast fusion (Liu *et al.*, 1998). Therefore, it was important to demonstrate that mibefradil is not affecting $I_{\text{K(IR)}}$, or any of the other K^{+} currents, which also contribute to the hyperpolarization of myoblasts, i.e. $I_{\text{K(NI)}}$, a non-inactivating K^{+} current (Bernheim *et al.*, 1996), which we know now is the human *ether-à-go-go* K^{+} channel ($I_{\text{h-eag}}$, Bijlenga *et al.*, 1998; Occhiodoro *et al.*, 1998), and $I_{\text{K(DR)}}$, a delayed rectifier K^{+} current (Widmer *et al.*, 1995).

Effect of mibefradil on K^{+} currents

Figure 3A illustrates the effect of mibefradil on $I_{\text{K(DR)}}$. The cell represented had no $I_{\text{h-eag}}$, which occurs in only a small fraction of fusion-competent myoblasts (Bernheim *et al.*, 1996), so that $I_{\text{K(DR)}}$ could be studied in isolation. The inset shows the marked reduction of the outward current when the myoblast was exposed to $3 \mu\text{M}$ mibefradil. The blocking effect occurred at all voltages, as attested by the current-voltage relationship in Figure 3A.

In Figure 3B, it can be seen that mibefradil is also very efficient at inhibiting another outward K^{+} current, $I_{\text{h-eag}}$. As $I_{\text{h-eag}}$ does not inactivate with time, the inset illustrates the steady current recorded at $+40$ mV in the absence and in the

measured in control solution are plotted as a function of mibefradil concentration. The line is a Hill equation ($\text{IC}_{50}=0.68 \mu\text{M}$, Hill coefficient=1.4, $n=5$). All recordings were from fusion-competent myoblasts cultured 3 days in differentiation medium. A similar experiment was performed with the current through L-type calcium channels. Normalized steady-state Ba^{2+} currents measured at the end of the 80 ms voltage step to $+10$ mV were plotted as a function of mibefradil concentration and fitted with a Hill equation ($\text{IC}_{50}=2 \mu\text{M}$, Hill coefficient=1.6, $n=4$ or more); the open triangles represent Ba^{2+} currents potentiated by low concentrations of mibefradil ($n=8$). Inset: Effect of low concentrations of mibefradil on current through L-type calcium channels. Steady state Ba^{2+} currents (leak subtracted) were measured at the end of the 80 ms step and plotted as a function of the voltage during the step for different mibefradil concentrations.

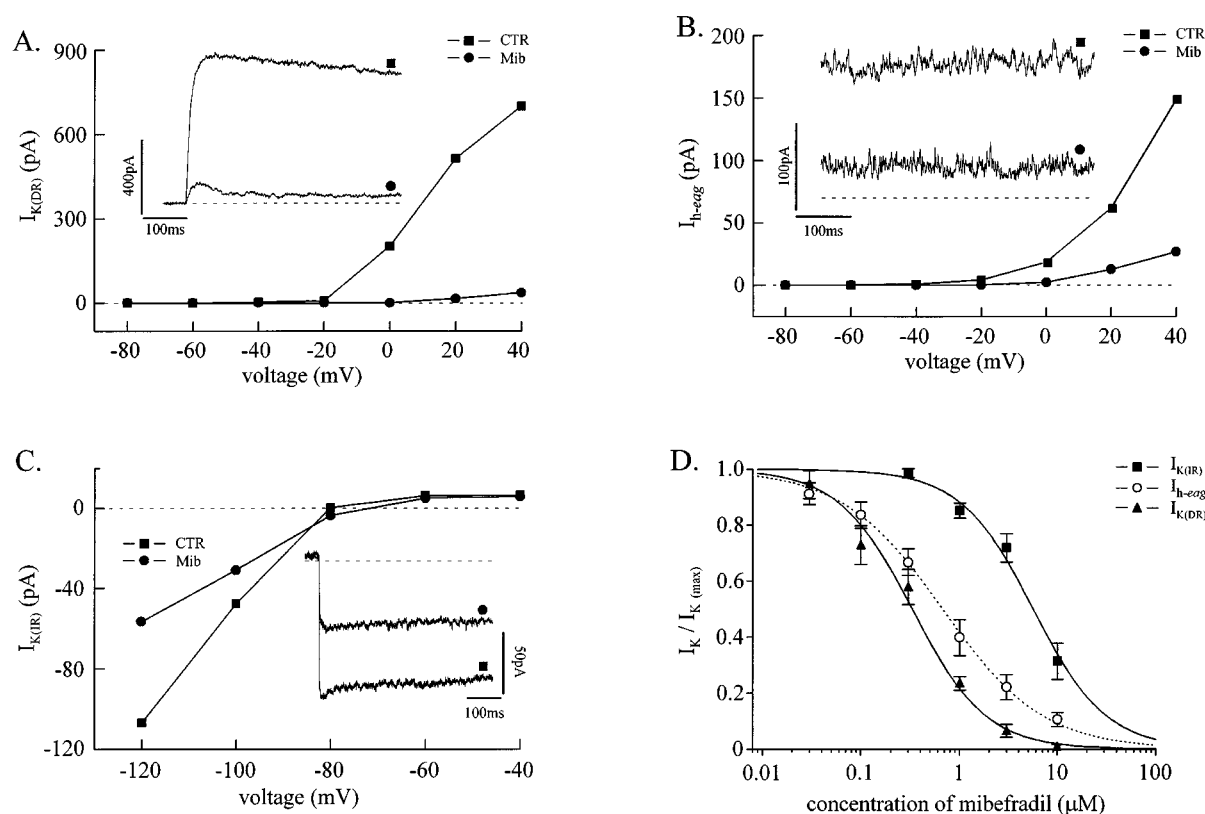


Figure 3 Mibefradil inhibits different types of K^{+} currents of fusion-competent myoblasts. All experiments were done with fusion-competent myoblasts exposed to differentiation medium for 3 days. (A) A myoblast (membrane capacitance 18 pF) was held steadily at -80 mV and stepped for 500 ms to a series of voltages between -80 and $+40$ mV. The outward current obtained at the end of the step was plotted as a function of the voltage during a step in the absence and in the presence of $3 \mu\text{M}$ mibefradil. Leak current was obtained by linear extrapolation from the current-voltage relationship between -80 and -40 mV and subtracted. This cell had no I_{h-eag} , so that the outward current represents exclusively $I_{K(DR)}$. Currents recorded from the same cell (during a step to $+40$ mV) without and with $3 \mu\text{M}$ mibefradil are shown. (B) Another fusion-competent myoblast (membrane capacitance 17 pF) was held steadily at $+40$ mV for 3 min, and stepped for 500 ms to a series of voltages between -80 and $+40$ mV. Symbols represent the amplitude of I_{h-eag} recorded in the absence and in the presence of $3 \mu\text{M}$ mibefradil. Leak current was obtained as in (A) and subtracted. Steady-state currents at $+40$ mV in the absence and in the presence of $3 \mu\text{M}$ mibefradil are shown (same cell). The dotted line indicates the leak at $+40$ mV. (C) A fusion-competent myoblast (membrane capacitance 26 pF) expressing $I_{K(IR)}$ was held steadily at -60 mV and stepped to a series of voltages between -120 and -40 mV for 500 ms. Leak subtracted $I_{K(IR)}$ currents measured in the absence and in the presence of $10 \mu\text{M}$ mibefradil were plotted against the voltage during the step. Leak current was obtained by adding $500 \mu\text{M}$ Ba^{2+} to the bath solution. Inward K^{+} currents recorded from the same cell at -120 mV in the absence and in the presence of $10 \mu\text{M}$ mibefradil are shown. (D) $I_{K(DR)}$, I_{h-eag} , and $I_{K(IR)}$ normalized to their respective control values are plotted as a function of mibefradil concentration. The three data sets were fitted with a Hill equation.

presence of $3 \mu\text{M}$ mibefradil. As for $I_{K(DR)}$, the block of I_{h-eag} by mibefradil occurred at all voltages.

At a higher concentration range, mibefradil can also block $I_{K(IR)}$. Figure 3C shows that $I_{K(IR)}$ is reduced by 50% by mibefradil at a concentration of $10 \mu\text{M}$. The complete dose-response curve for five cells is illustrated in Figure 3D. The IC_{50} of mibefradil for $I_{K(IR)}$ is $5.6 \mu\text{M}$.

Figure 3D also illustrates the effect of various mibefradil concentrations on $I_{K(DR)}$ and I_{h-eag} . The IC_{50} of mibefradil for $I_{K(DR)}$ and I_{h-eag} are 0.3 and $0.7 \mu\text{M}$, respectively, i.e. very similar to that of $\text{ICa}_{(T)}$ in human fusion-competent myoblasts.

Discussion

This study on the effect of mibefradil on human myoblasts was initiated in order to evaluate the role of $\text{ICa}_{(T)}$ in myoblast fusion. Unfortunately, despite its strong inhibitory effect on fusion, mibefradil did not allow us to assess the importance of $\text{ICa}_{(T)}$ in the fusion process. However, an interesting observation emerged from this study: mibefradil inhibits several types of voltage-gated K^{+} currents including an inward rectifier, and

is as efficient an inhibitor of the outward K^{+} currents as of $\text{ICa}_{(T)}$.

Selectivity of mibefradil on T-type Ca^{2+} current

In venous smooth muscle myocytes and heart cells, mibefradil has a selectivity for $\text{ICa}_{(T)}$ over $\text{ICa}_{(L)}$ in an excess of 10 (Ertel & Ertel, 1997; Mishra & Hermsmeyer, 1994). In neurons, however, the situation is different. In embryonic rat spinal cord motoneurons, all inward Ca^{2+} currents were blocked in the same concentration range with an IC_{50} of $1.4 \mu\text{M}$ (Viana *et al.*, 1997). In myoblasts, the IC_{50} of mibefradil for $\text{ICa}_{(L)}$ is in the same range ($2 \mu\text{M}$) as the IC_{50} for rat motoneurons, although, the IC_{50} for $\text{ICa}_{(T)}$ is three times smaller ($0.7 \mu\text{M}$). Hence, in skeletal muscle myoblasts, the selectivity of mibefradil for $\text{ICa}_{(T)}$ is halfway between that of neurons and smooth muscle cells. It is possible that the differences in results on various cell types with respect to selectivity for $\text{ICa}_{(T)}$ reflects the existence of different types of $\text{Ca}_{(T)}$ channels in these cells. A $\text{Ca}_{(T)}$ channel has recently been cloned ($\alpha 1\text{G}$; Perez-Reyes *et al.*, 1998), and it appears that there exists more than one type of $\text{Ca}_{(T)}$ channel ($\alpha 1\text{H}$; Cribbs *et al.*, 1998). In any case, the

difference in mibefradil IC_{50} for low- and high-voltage activated Ca^{2+} channels in skeletal muscle myoblasts is not sufficiently large to allow a very specific inhibition of $\text{I}_{\text{Ca}(\text{T})}$ versus $\text{I}_{\text{Ca}(\text{L})}$.

Enhancement of $\text{I}_{\text{Ca}(\text{L})}$ by low concentrations of mibefradil

Low concentrations of mibefradil significantly and very reproducibly enhanced $\text{I}_{\text{Ca}(\text{L})}$ in human fusion-competent myoblasts. An increase of $\text{I}_{\text{Ca}(\text{L})}$ by low concentrations of mibefradil has also been described in Chinese hamster ovary (CHO) cells co-transfected with the smooth muscle L-type $\alpha 1_{\text{C}_b}$ subunit and an L-type β subunit. This enhancement of $\text{I}_{\text{Ca}(\text{L})}$ by mibefradil was, however, only observed in a subpopulation of transfected CHO cells, and was not present when CHO cells were transfected with the $\alpha 1_{\text{C}_b}$ subunit alone (Welling *et al.*, 1995). At higher concentrations, an inhibitory effect of mibefradil was, on the other hand, always observed. It has been hypothesized that this dual action could be caused by the existence of two separate binding sites for mibefradil (Welling *et al.*, 1995). We did not further explore the underlying mechanism but if this should occur in cell types that are the pharmacological target of this type of agent, an increase in Ca^{2+} influx through depolarized membranes containing Ca_{L} channels could occur at certain mibefradil concentrations.

Effect of mibefradil on K^+ currents

There are several indications that mibefradil may act on channels other than Ca^{2+} channels. For example, mibefradil inhibits Ca^{2+} -activated Cl^- currents and volume-sensitive Cl^- currents in endothelial cells with an IC_{50} in the range of $5 \mu\text{M}$ (Nilius *et al.*, 1997). However, in these cells, mibefradil at concentrations up to $30 \mu\text{M}$ did not affect an inwardly rectifying K^+ channel (Kir1.0 or Kir4.0 families; Voets *et al.*, 1996; Nilius *et al.*, 1997), and we have not noticed any report of an effect of this drug on outward K^+ currents. Therefore, it was unexpected to observe that, when compared to $\text{I}_{\text{Ca}(\text{T})}$,

mibefradil could block outward K^+ currents with equal efficiency ($\text{I}_{\text{h-eag}}$) or even more efficiently ($\text{I}_{\text{K}(\text{DR})}$). Interestingly, these two outward currents belong to different superfamilies, as $\text{I}_{\text{K}(\text{DR})}$ is a member of the *Shaker* superfamily, and $\text{I}_{\text{h-eag}}$ is the human *ether-à-go-go* K^+ channel (Bijlenga *et al.*, 1998; Occhiodoro *et al.*, 1998). If this effect of mibefradil on K^+ channels occurs in other cell types, effects on action potential duration, resting membrane potential or even rhythmic activity should be expected. Regarding $\text{I}_{\text{K}(\text{IR})}$ in human myoblasts (Kir2.0 family; Liu *et al.*, 1998), the sensitivity to mibefradil is clearly present but is more comparable to that of $\text{I}_{\text{Ca}(\text{L})}$, than to that of $\text{I}_{\text{Ca}(\text{T})}$ or of the outward K^+ currents of the same preparation.

Effect of mibefradil on fusion

There is a marked effect of mibefradil on myoblast fusion. However, at the concentration used in the experiment illustrated in Figure 1, more than 90% of $\text{I}_{\text{Ca}(\text{T})}$, $\text{I}_{\text{K}(\text{DR})}$ and $\text{I}_{\text{h-eag}}$ and approximately 50% of $\text{I}_{\text{K}(\text{IR})}$ would have been blocked. Our experiences with caesium ions on myoblast fusion indicated that blocking 50% of $\text{I}_{\text{K}(\text{IR})}$ reduced fusion by approximately 25%. Therefore, the block of $\text{I}_{\text{K}(\text{IR})}$ by mibefradil cannot alone explain the 57% inhibition of fusion seen with $5 \mu\text{M}$ mibefradil. At present, we cannot distinguish whether there is an isolated or combined contribution of $\text{I}_{\text{Ca}(\text{T})}$, $\text{I}_{\text{K}(\text{DR})}$ or $\text{I}_{\text{h-eag}}$ to the residual 32% inhibition in fusion by mibefradil. It is worth noting however that a high concentration of this drug interferes with a mechanism essential to muscle growth and repair.

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Effects of phrixotoxins on the Kv4 family of potassium channels and implications for the role of I_{to1} in cardiac electrogenesis

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1 In the present study, two new peptides, phrixotoxins PaTx1 and PaTx2 (29–31 amino acids), which potentially block A-type potassium currents, have been purified from the venom of the tarantula *Phrixotrichus auratus*.

2 Phrixotoxins specifically block Kv4.3 and Kv4.2 currents that underlie I_{to1} , with an $5 < IC_{50} < 70$ nM, by altering the gating properties of these channels.

3 Neither are the *Shaker* (Kv1), *Shab* (Kv2) and *Shaw* (Kv3) subfamilies of currents, nor HERG, KvLQT1/IsK, inhibited by phrixotoxins which appear specific of the *Shal* (Kv4) subfamily of currents and also block I_{to1} in isolated murine cardiomyocytes.

4 In order to evaluate the physiological consequences of the I_{to1} inhibition, mice were injected intravenously with PaTx1, which resulted in numerous transient cardiac adverse reactions including the occurrence of premature ventricular beats, ventricular tachycardia and different degrees of atrio-ventricular block.

5 The analysis of the mouse electrocardiogram showed a dose-dependent prolongation of the QT interval, chosen as a surrogate marker for their ventricular repolarization, from 249 ± 11 to 265 ± 8 ms ($P < 0.05$).

6 It was concluded that phrixotoxins, are new and specific blockers of Kv4.3 and Kv4.2 potassium currents, and hence of I_{to1} that will enable further studies of Kv4.2 and Kv4.3 channel and/or I_{to1} expression.

Keywords: Spider toxins; voltage-gated potassium channels; I_{to} ; Kv4 channels; A-type current; electrocardiogram

Abbreviations: AV, atrio-ventricular; ECG, electrocardiogram; EIMS, electrospray ionization mass spectrometry; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid

Introduction

Toxins isolated from animal venoms have been invaluable tools for probing the structure and function of potassium (K^+) channels from the Kv family (Aiyar *et al.*, 1995; Bidard *et al.*, 1987; Harvey & Anderson, 1991; Miller, 1995; Mockzydowski *et al.*, 1988; Pongs, 1992a; Rehm & Lazdunski, 1988; Strong, 1990; Swartz & McKinnon, 1997). Snake, bee, sea-anemone and scorpion toxins specifically target *Shaker* (Kv1) and *Shaw* (Kv3) related K^+ channel subfamilies (Bidard *et al.*, 1987; Diochot *et al.*, 1998; Garcia *et al.*, 1994; Grissmer *et al.*, 1994; Pongs, 1992b; Rehm *et al.*, 1988; Schweitz *et al.*, 1995) whereas newly isolated spider toxins are useful tools to analyse the *Shab* (Kv2) and *Shal* (Kv4) related K^+ channels, which are widely spread in mammalian cells (Sanguinetti *et al.*, 1997; Swartz & McKinnon, 1997).

The *Shal* subfamily comprises three genes, Kv4.1, Kv4.2 and Kv4.3 that all express transient K^+ currents in heterologous systems (Baldwin *et al.*, 1991; Pak *et al.*, 1991; Serodio & Rudy, 1998). These genes are abundantly expressed in several types of central and peripheral neurons as well as in the heart, with differential topographic expression (Kaczmarek & Strumwasser, 1984; Segal *et al.*, 1984; Serodio *et al.*, 1994, 1996; Serodio & Rudy, 1998). Thus, while Kv4.1 has only been characterized in the central nervous system (Pak *et al.*, 1991), Kv4.3 and Kv4.2 have also been located in the heart where their differential distribution, pharmacology and kinetics suggest that they are the molecular constituents of the transient K^+ current I_{to1} (Barry & Nerbonne, 1996; Dixon *et al.*, 1996;

Yeola & Snyders, 1997). I_{to1} modulates the action potential duration and/or shape (Barry *et al.*, 1995; Barry & Nerbonne, 1996; Dixon & McKinnon, 1994; Dixon *et al.*, 1996) and has a prominent role in mammalian heart cells by counterbalancing the fast inward sodium (Na^+) current in phase 1 of the action potential. Furthermore, the myocardial regional gradient of I_{to1} participates in the fact that epicardial layers are the fastest to repolarize (Litovsky & Antzelevitch, 1987; Wettwer *et al.*, 1994).

This paper describes the purification, structure and properties of two new toxins, phrixotoxins PaTx1 and PaTx2, that have been isolated from the venom of the Chile fire tarantula *Phrixotrichus auratus*. These toxins proved to be specific and potent blockers of the Kv4.3 and the Kv4.2 channels, as well as of the I_{to1} current in rat isolated ventricular myocytes. They have been used to analyse the exact contribution of the I_{to1} current in the cardiac function of mice.

Methods

Purification of phrixotoxins

The venom from *Phrixotrichus auratus* (Araneae, Theraphosidae) was purchased from Spider Farm (Feasterville, PA, U.S.A.). In a first screening attempt, we identified inhibitory activities of diluted venom (1 : 1000) for Kv4.3 currents among several representatives of K^+ channels tested, and purified the responsible peptides.

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Active components were purified on a C18 reverse phase high performance liquid chromatography (HPLC) column using an aliquot of crude venom (12.5 mg). Lyophilized venom was diluted to 1.25 ml with 0.1% aqueous trifluoroacetic acid (TFA) and centrifuged at 4500 r.p.m. for 15 min. The supernatant was filtered through 0.22 μ m filters (Millex GV4) and fractionated on a Beckman ODS C18 reversed-phase HPLC column (10 $\times$ 250 mm) equilibrated in 85% solvent A (0.1% TFA in water) 15% solvent B (0.1% TFA in acetonitrile). Linear gradients of solvent B were used, at a flow rate of 1 ml min⁻¹. The UV absorbance of the effluent was monitored at 220 nm and 280 nm. The active fractions PaE and PaF were further purified on a TSK SP-5PW cation-exchange column (7.5 $\times$ 75 mm) equilibrated in 100% of solvent C (1% acetic acid in water). The final purification step for the fractions PaE5 and PaF4 was carried out on a Waters Symmetry C18 reversed phase column (4.6 $\times$ 250 mm) equilibrated in 10% of solvent B and 90% of solvent A.

Peptide sequencing and mass determination

Lyophilized E5 and F4 peptides were reduced with 2-mercaptoethanol and pyridylethylated with 4-vinylpyridine before sequencing. They were submitted to Edman degradation on an Applied Biosystems model 477 A microsequencer. Molecular weights were determined by electrospray ionization mass spectrometry (EIMS).

Cell cultures and transfection

Two days before transfection COS M6 cells were plated at a density of 20,000 on cover glasses and transfected by a modification of the DEAE-dextran/chloroquine method using 0.05 μ g of super coiled pCI-Kv4.n plasmid DNA. A CD8-expressing plasmid (0.02–0.1 μ g) was added in all transfection experiments to visualize transfected cells using anti-CD8 antibody-coated beads (Jurman *et al.*, 1994). Currents were recorded within 1–2 days following transfection.

Plasmid constructions

pBluescript-Kv4.1 (GenBank accession number M64226), Kv4.2 (GenBank accession number S64320) and Kv4.3 (GenBank accession number U75448) constructs were kindly provided by Drs L. Salkoff (Pak *et al.*, 1991), L. Jan (Baldwin *et al.*, 1991) and D. McKinnon (Dixon *et al.*, 1996) respectively. The *Sall*/*NotI* fragment excised from pBS-Kv4.1 and the *XhoI*/*XbaI* fragment excised from pBS-Kv4.3 were subcloned into the mammalian expression vector pCI (Promega Madison, Wisconsin, U.S.A.) to give pCI-Kv4.1 and pCI-Kv4.3. The entire coding sequence of Kv4.2 was amplified by PCR using a low error rate DNA polymerase (Pwo DNA polymerase, Boehringer Mannheim, Meylan, France) and subcloned into pCI vector to give pCI-Kv4.2. All constructs were characterized by restriction analysis and by a partial or complete sequencing on both strands by the dideoxy nucleotide chain termination method using an automatic sequencer (Applied Biosystems, model 373A).

Myocytes isolation

The cell isolation was performed according to Mitra (Mitra & Morad, 1986). Briefly, the rat heart was excised and rapidly mounted on a modified Langendorff apparatus and subsequently perfused with Ca-free Tyrode solution containing 1.5 mg ml⁻¹ collagenase (type II, Sigma, 370 u mg⁻¹) and

50 μ M Ca²⁺ Tyrode. Pieces of ventricle were gently shaken and single myocytes were collected after filtration and stored at 4°C until use.

Electrophysiological measurements

Electrophysiological measurements on COS transfected cells were performed 48 h after transfection using the whole-cell configuration of the patch-clamp technique. The pipette solution used for COS cells was (in mM): KCl 150, MgCl₂ 3, EGTA 5, HEPES-KOH 10 at pH 7.4. The pipette solution used for myocytes was (in mM): NaCl 10, KCl 136, CaCl₂ 0.5, EGTA 5, Mg-ATP 5, HEPES-KOH 5, at pH 7.4. The extra cellular solution composition for K⁺ currents recordings in COS cells was (in mM): KCl 5, NaCl 150, CaCl₂ 1, MgCl₂ 3, HEPES-NaOH 1, pH 7.4. The extra cellular solution used for myocytes was (in mM): Choline Cl 135, KCl 5.4, CaCl₂ 2, MgCl₂ 1, Glucose 10, HEPES-KOH 10 at pH 7.4. Cd²⁺ (0.5 mM) was added to block Ca²⁺ currents. Bovine serum albumin 0.1% was added in toxin-containing solutions in order to prevent their adsorption.

The Kv4.2 and Kv4.3 current-voltage curves were determined by applying 10 mV incremental depolarizing pulses from a holding potential of -80 mV. Peak transient currents elicited by depolarizations, were measured after digital leak subtraction calculated at the end of depolarization (at 300 ms). All averaged data are presented as means $\pm$ s.e.mean.

Specificity of PaTx1 and PaTx2 for the Kv4.2 and Kv4.3 channels

For screening purposes, *Xenopus* oocytes were injected with Kv1.1; Kv1.2; Kv1.3; Kv1.4; Kv2.1; Kv2.2; Kv3.1; Kv3.2; Kv3.4; Kv4.2; Kv4.3, and IRK1 complementary RNAs (cRNA) as described previously (Duprat *et al.*, 1995). Currents were recorded 2–4 days following the injection using the two microelectrode voltage-clamp protocol. In order to determine the specificity of PaTx1 and PaTx2, increasing concentrations of toxins (100 nM to 1 μ M), were applied on Kv1.n, Kv3.n, IRK1 currents expressed in *Xenopus* oocytes as well as on Kv1.5, Kv2.n, the background K⁺ channel TREK-1, and the two components of the delayed rectifier, HERG and KvLQT1/Isk expressed in COS cells (Barhanin *et al.*, 1996; Fink *et al.*, 1996).

Animal electrophysiology

We analysed the physiological effects associated with intravenous injections of increasing doses (0.1–40 nmol) of PaTx1 by measuring standard electrocardiographic parameters on anaesthetized Balb C mice, that were maintained on sterile regular rodent chow (R03, UAR, Epinay-France) and allowed free access to food and water in a facility at 21 $\pm$ 1°C with 12 h light/dark cycles.

Animal preparation for electrocardiography

Twelve-week-old mice of both sexes, weighing 23–29 g, were studied. Mice were anaesthetized with sodium pentobarbital (SANOFI-France, 45 mg kg⁻¹ (i.p.) for the female adults and 55 mg kg⁻¹ (i.p.) for the male adults). Surface three-lead electrocardiograms (ECG) (bipolar lead DI, DII and DIII) were obtained by placement of dry electrodes carefully wrapped around each of the mouse four limbs. Respiratory and heart rates were continuously monitored during the procedure. A warning light was used to maintain body temperature within a range of 36 $\pm$ 1°C for prevention of hypothermia.

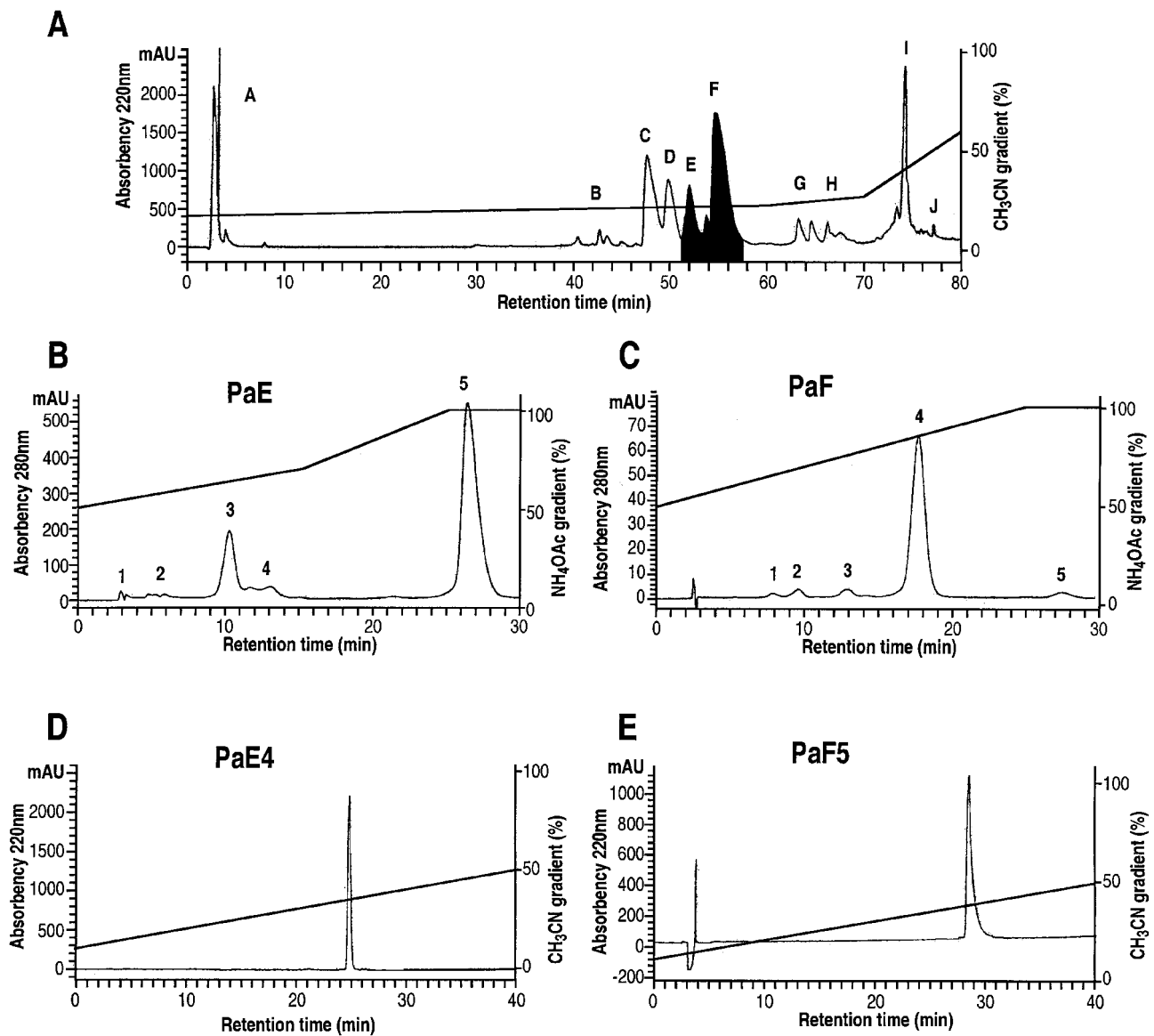


Figure 1 Purification of phrixotoxins from *Phrixotricus auratus* venom. (A) Reverse phase HPLC chromatogram monitored at 220 nm illustrating the separation of 10 fractions – A – J – after loading 500 μ l of diluted venom in a Beckman ODS C18 column. The stepwise solvent B (0.1% TFA in acetonitrile) gradient (from 15–30% over 60 min, from 30–35% over 10 min, then from 35–60% over 10 min) is indicated. Collected fractions E and F, active on Kv4.3 channels, are shaded. (B,C) Ion exchange chromatograms illustrating the second purification step for fractions PaE and PaF as described in ‘Methods’. A stepwise gradient for fraction PaE, from 50–70% over 15 min, then from 70–100% over 10 min of solvent D (ammonium acetate 1 M) was used and allowed the separation of five fractions. Activity against Kv4.3 channels was recovered in PaE5. The other active fraction PaF4 was purified from the fraction PaF with a linear gradient from 50–100% solvent D over 25 min. The effluent was monitored at 280 nm. (D, E) The fractions PaE5 and PaF4 were further purified on C18 reverse phase column with a linear solvent B gradient from 10–50% over 40 min.

PaTx1	1	-YCQKMMWTCDS	SA	-RKCCGLVCRL	--	-WCKKII	--	29				
PaTx2	1	-YCQKMMWTCDEE	EE	-RKCCGLVCRL	--	-WCKRII	NM	31				
HpTx1	1	-DC	CGTIWHYC	GT	DQSE	CCEGWK	CSR	--Q	LCKYVI	DW	33	
HpTx2	1	DD	CGKLFSGC	DT	N-AD	CCEGYV	VCRL	--	-WCKL	DW	--	30
HpTx3	1	-EC	GTLFSGC	ST	H-AD	CCEGFIC	KL	--	-WCRY	ERTW	31	
HaTx1	1	-E	CRYLFGGC	KTT	-SD	CC	KHLG	CK	FRDKY	CAWDF	TFS	35
HaTx2	1	-E	CRYLFGGC	KTT	-AD	CC	KHLG	CK	FRDKY	CAWDF	TFS	35

Figure 2 PaTx1 and PaTx2 sequences. Homologies of phrixotoxins PaTx1 and PaTx2 with heteropodotoxins (HpTx1, HpTx2, and HpTx3) (Sanguinetti et al., 1997) from *Heteropoda venatoria* and with hanatoxins (HaTx1 and HaTx2) (Swartz & MacKinnon, 1997) from *Grammostola spatulata*. Black boxes indicate sequence identities, and grey boxes indicate sequence homologies.

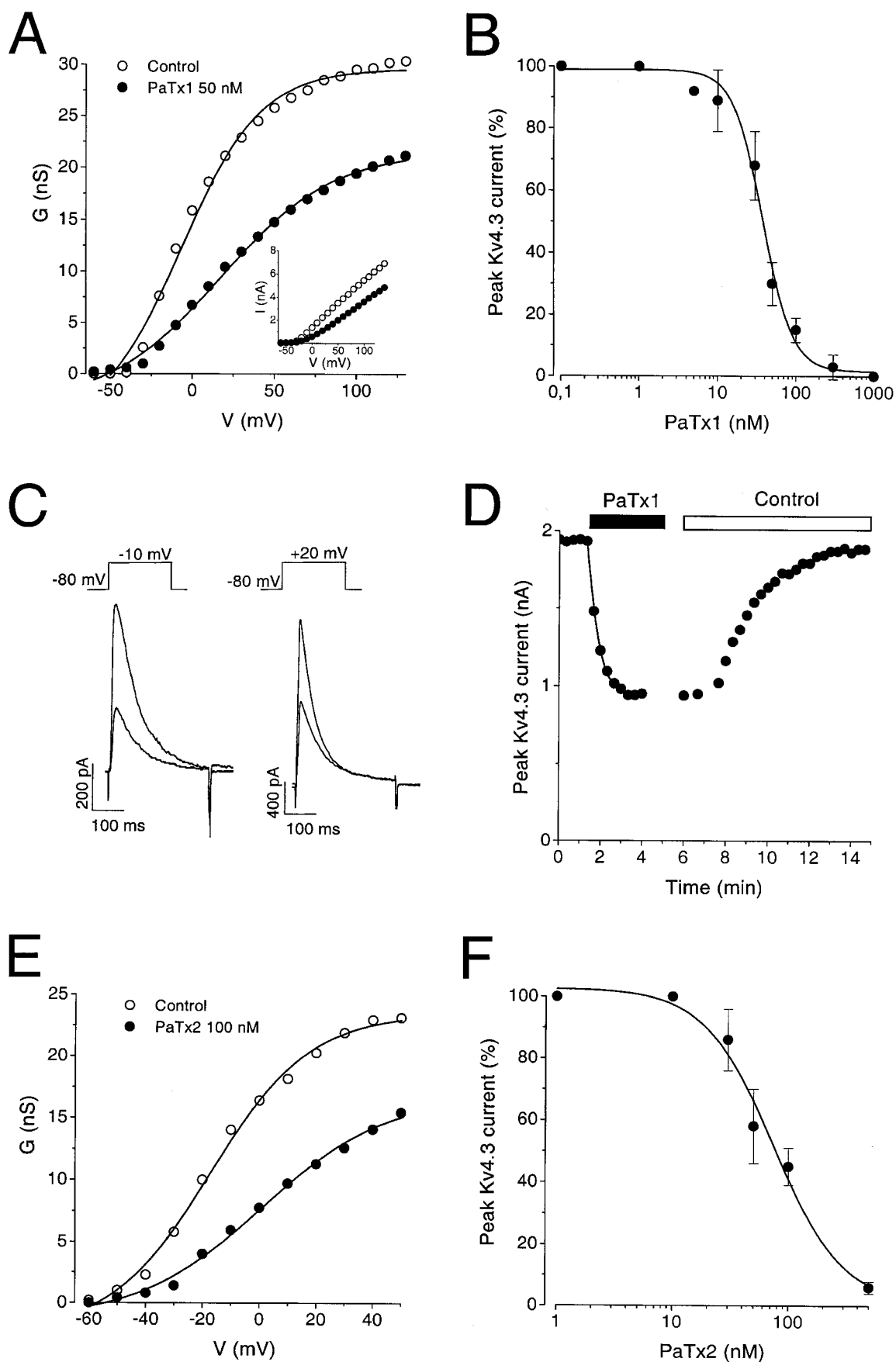


Figure 3 Effect of PaTx1 and PaTx2 on Kv4.3 currents. Kv4.3 currents were recorded in COS transfected cells in the whole-cell configuration. Holding potential: -80 mV. (A) Conductance-voltage relationship for Kv4.3 peak current measured before and during application of 50 nM PaTx1. Conductances were calculated with equation (1): $G = I/(V - E_K)$ where G is the conductance, I the amplitude of the peak current, V the test potential, E_K the reversal potential for K^+ (-85 mV). The G_{max} value was evaluated from equation (2): $G = G_{max}/(1 + \exp((V_{0.5} - V)/k))$ where $V_{0.5}$ is the midpoint potential and k is the curve slope. In this example, the midpoint potential ($V_{0.5}$) values in control conditions and after inhibition by 50 nM PaTx1 were -7 and $+17$ mV respectively. The G_{max} values were 30 and 21 nS before and after PaTx1 inhibition. Inset: Current-voltage curve. Currents were induced by a set

Table 1 Effects of PaTx1 on electrophysiological parameters of Kv4.2 and Kv4.3 channels

Parameters	Kv4.3		Kv4.2	
	Control (test pulse in mV)	PaTx1 (concentration in nM)	Control (test pulse in mV)	PaTx1 (concentration in nM)
Time to peak in ms $\pm$ s.e.mean 5 < n < 7	18 $\pm$ 2 (−10) 12 $\pm$ 1 (+20)	22 $\pm$ 5 (50) 25 $\pm$ 4 (100) 14 $\pm$ 2 (50) 19 $\pm$ 2 (100)	12 $\pm$ 1 (−10) 8 $\pm$ 1 (+20)	23 $\pm$ 6 (30) 32 $\pm$ 4 (100) 14 $\pm$ 2 (30) 16 $\pm$ 2 (100)
V _{0.5} act. in ms $\pm$ s.e.mean 2 < n < 9	−5.6 $\pm$ 3	+21 $\pm$ 3 (50) +27 $\pm$ 3 (100)	−13 $\pm$ 2	+11 $\pm$ 2 (50) +25 $\pm$ 4 (100)
V _{0.5} inact. in ms $\pm$ s.e.mean 2 < n < 10	−61 $\pm$ 3	−45 $\pm$ 5 (30)	−65 $\pm$ 2	−52 $\pm$ 4 (30)
τ_i in ms $\pm$ s.e.mean 5 < n < 11	47 $\pm$ 6 (−10) 30 $\pm$ 5 (+20)	75 $\pm$ 10 (100) 52 $\pm$ 9 (100)	24 $\pm$ 5 (−10) 16 $\pm$ 3 (+20)	55 $\pm$ 16 (30) 26 $\pm$ 6 (30)
τ_r in ms $\pm$ s.e.mean 3 < n < 8	194 $\pm$ 29	200 $\pm$ 45 (30)	165 $\pm$ 32	165 $\pm$ 29 (10)

Mean values $\pm$ s.e.mean are indicated for the corresponding parameters: V_{0.5} act. is the midpoint potential calculated from equation (2) for the conductance-voltage relation. V_{0.5} inact. is the midpoint potential given by the steady-state inactivation curve. τ_i is the time constant of inactivation of the Kv4 current evaluated from equation (4). τ_r is the time constant of recovery from inactivation for Kv4 currents calculated from equation (5).

Measurements of QT interval

Bipolar electrodes were connected to an adjustable bandpass differential amplifier (ORTEC Inc., U.S.A.). The ECG channels were amplified and filtered between 1 and 100 Hz, and a stable signal was reliably obtained before we proceeded. Signals were collected, stored and analysed on a PC computer using PCLAMP software. The PR interval was measured from the beginning of the surface P wave to the beginning of the R wave complex. The QRS was measured from the beginning of the Q wave, when it was present, or from the base of the R to the bottom of the S wave. The QT interval is calculated from the beginning of the Q wave (or from the base of the R wave if not possible) to the end of the T wave, defined as the point at which it returns to the isoelectric baseline. Due to the early activation and fast inactivation of *I_{to1}* *in vitro*, and in order to get a more specific measurement of the early phase of cardiac repolarization in mice, measurements were also made from the beginning of the S wave to the highest deflection of the rapid component of the T wave (ST_{max}) and to 50% of the steep descending slope of the rapid T wave (called ST_r for 'ST rapid'). QT intervals were corrected with Bazett's formula (QT_c(ms) = QT/RR(s)^{1/2}) which allows comparisons of QT durations at different heart rates. For each mouse, a set of 10 cardiac cycle length-QT interval pairs was obtained from their ECG recordings.

Injection of PaTx1

After recording of control parameters, 0.1–40 nmol of PaTx1 dissolved in 100 μ l of saline were injected (<3 s) into a tail

vein. ECGs were continuously recorded throughout the experiments up to 30 min after the injection.

Statistical analysis

Results are shown as means $\pm$ s.e.mean. Continuous variables, as QT_c and ST_r values and their increase from baseline were analysed with a one way analysis of variance (ANOVA, Statview 4.5, and SuperAnova 1.11, Abacus Corp) or with a Mann-Whitney-Wilcoxon rank-sum test. *P* < 0.05 was considered statistically significant.

Results

Purification of two peptides active on Kv4 channels

P. auratus whole venom fractionation on C18 reverse phase HPLC column yielded 10 fractions (noted A–J, see Figure 1A). The peaks PaE and PaF were selected for their blocking effects on Kv4.3 currents expressed in *Xenopus* oocytes and were further purified by an ion exchange purification step followed by a C18 reverse phase step (Figure 1B–E). Pure PaE5 and PaF4 peptides were eluted at 34 and 38% of acetonitrile respectively. The nomenclature that we propose for these toxins is phrixotoxin 1 (PaTx1) for PaE5 and phrixotoxin 2 (PaTx2) for PaF4.

PaTx1 has a molecular weight of 3547.60 Da and its sequence comprises 29 amino acids. It is 83.3% identical to PaTx2 (Figure 2). PaTx2, the most abundant peptide in the venom, has a

of depolarizing pulses at −60 to +120 mV in 10 mV increments. (B) Concentration-response relationship for PaTx1 block of the Kv4.3 current at −10 mV. The IC₅₀ value is 28 nM. The effects of 0.1 nM to 1 μ M phrixotoxin on Kv4.3 currents were tested at −10 mV from a holding potential of −80 mV. The dose response curve was fitted by equation (3): $I = I_{\max} + (I_{\min} - I_{\max}) / (1 + \exp((C - IC_{50})/k))$ where *I* is the amplitude of the peak current, *I*_{max} and *I*_{min} are the amplitudes of the current corresponding to control and saturating concentrations of phrixotoxins respectively, *C* is the concentration of the toxin, IC₅₀ is the concentration of toxin which correspond to 50% *I*_{max} and *k* is the curve slope. Each point is the mean $\pm$ s.e.mean of data from 3–6 cells. (C) Effect of 50 nM PaTx1 on Kv4.3 currents, recorded at a test pulse of −10 and +20 mV respectively, on the same cell. More blockade of Kv4.3 current by PaTx1 occurred at −10 mV (62%) than at 20 mV (51%). (D) Time course for Kv4.3 peak-current block with 50 nM PaTx1 at +20 mV and reversibility. The kinetics of current inhibition were fitted with equation (4): $I = I_{\text{res}} + I_{\max} \exp(-t/\tau_i)$ where *I* is the amplitude of the peak current relative to time (*t*), *I*_{max} is the initial peak current, *I*_{res} is the non inactivating component and τ_i is the time constant of inhibition. τ_i value is 0.57 ms. (E) Conductance-voltage relationship for Kv4.3 peak current measured before and during application of 100 nM PaTx2. (F) Concentration-response relationship for PaTx2 block of the Kv4.3 current at −10 mV. Each point is the mean $\pm$ s.e.mean of data from 3–5 cells. The IC₅₀ value determined from equation (3) is 71 nM.

molecular weight of 3921.77 Da as revealed by EIMS and is composed of 31 amino acids with six cysteine residues. The molecular weights deduced from sequence determination for C-terminal amidated peptides are comparable to the EIMS values: 3548.41 Da for PaTx1 and 3921.67 Da for PaTx2.

PaTx1 and PaTx2 share 50 and 47% sequence identities with HpTx2, a spider toxin isolated from *Heteropoda venatoria* venom which also has a blocking activity on Kv4.2 channels (Sanguinetti et al., 1997). Only 20% sequence identities were found, however, with hanatoxins isolated from the spider *Grammostola spatulata*, and which are active on Kv2.1 and Kv4.2 channels (Swartz & McKinnon, 1997) (Figure 2). No sequence homologies were found with other known K⁺ channel blockers.

Effects of PaTx1 and PaTx2 on Kv4.3 currents

PaTx1 at a concentration of 50 nM significantly blocked the Kv4.3 currents elicited in COS cells ($70 \pm 7\%$ at -10 mV and $58 \pm 7\%$ at $+20$ mV, $n=5$ cells), (Figure 3A inset and C). Conductance-voltage relations showed that this inhibition results from a shift of the activation curve (Figure 3A). The dose-response curve of Kv4.3 current inhibition by PaTx1 at -10 mV yielded an IC_{50} value of 28 nM (Figure 3B). The time course of the inhibition at $+20$ mV was best fitted, by a single exponential function with a time constant of 0.57 ms (Figure 3D). PaTx2 blocked Kv4.3 currents similarly but with a lower affinity ($IC_{50}=71$ nM, Figure 3E,F). Inhibition of Kv4.3 channels by PaTx1 or PaTx2 almost totally reversed

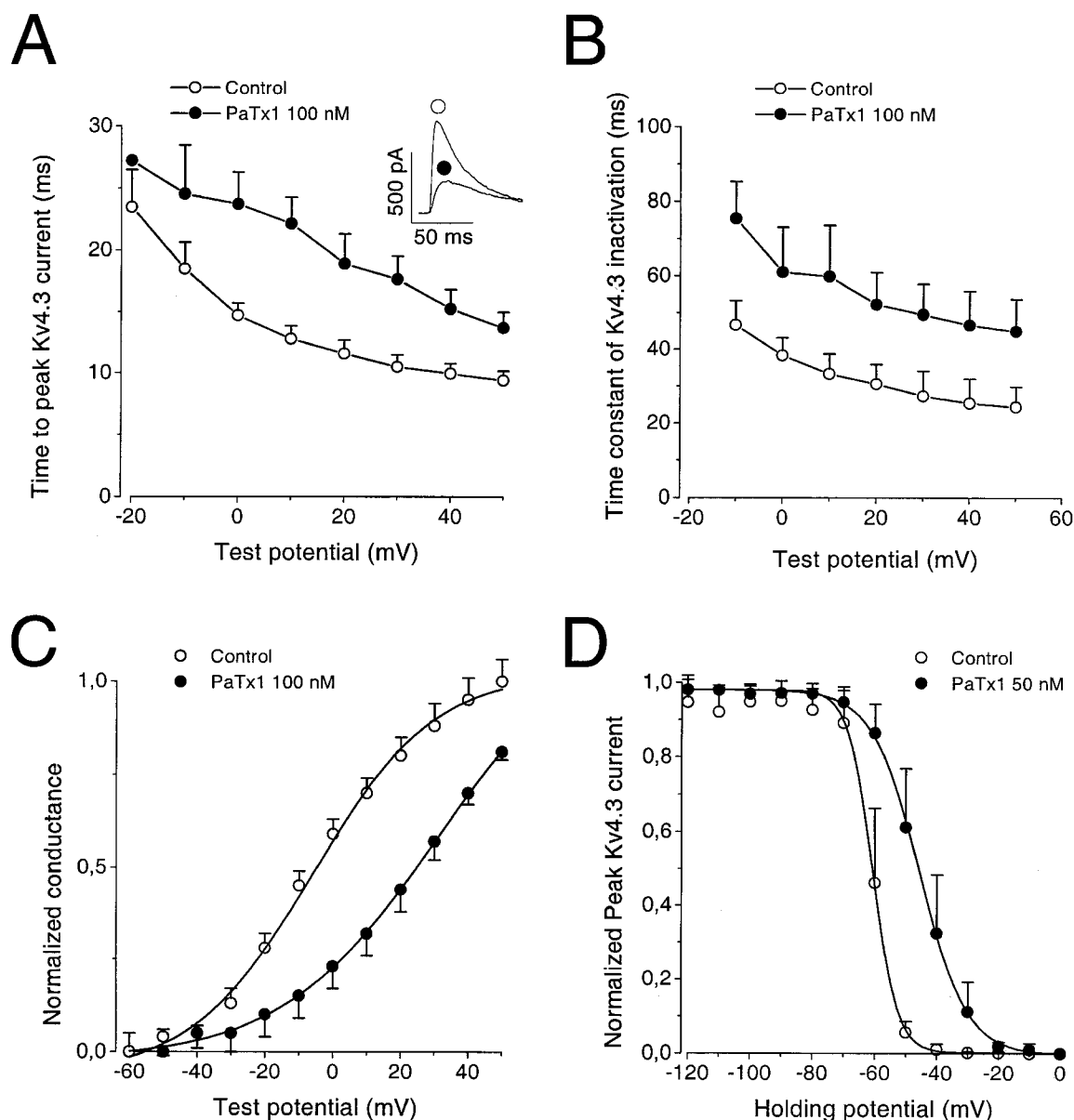


Figure 4 Effects of PaTx1 on the kinetics of activation and inactivation of Kv4.3 currents. Kv4.3 currents were all recorded in COS transfected cells in the whole cell configuration. The holding potential is -80 mV. (A) The time-to-peak current is represented against the test potential for Kv4.3 channel before and after inhibition of the current by 100 nM PaTx1. The inset shows the effect of PaTx1 at $+20$ mV. (B) The time constant of current inactivation is plotted against the test potential for Kv4.3 channel before and after inhibition of the current by 100 nM PaTx1. The decay of current inactivation was fitted according to equation (4). (C) Normalized conductance-voltage relationships for Kv4.3 currents in control and after inhibition by 100 nM PaTx1. Relative conductances were obtained after normalization with the G_{max} value. Points are means $\pm$ s.e. mean of data from four cells. (D) Steady-state inactivation curves for Kv4.3 currents evoked at $+30$ mV before and after their inhibition by 50 nM PaTx1. 10 s conditioning prepulses from -120 to $+10$ mV were applied prior to 200 ms depolarizing pulses to $+30$ mV. The same protocol was used after inhibition of Kv4.3 currents by PaTx1 at a concentration of 50 nM. The steady-state inactivation curves were fitted according to equation (3). Each point is the mean after normalization of Kv4.3 peak current amplitude from six cells.

upon washout ($91 \pm 9\%$ recovery after 5 min; $n = 13$, Figure 3D).

PaTx1 changes Kv4.3 kinetics of activation and inactivation

Mean values resulting from analysis of kinetic parameters are given in Table 1. The inhibition of Kv4.3 currents by PaTx1 (100 nM) is characterized by a concentration-dependent increase of the time-to-peak current (Figure 4A, Table 1). The Kv4.3 current inactivation is well fitted to a single exponential characterized by a time constant (τ_i) which significantly increases in the presence of 100 nM PaTx1 (Figure 4B, Table 1). Inhibition of Kv4.3 currents by PaTx1 also induced a shift of the normalized conductance-voltage relation and of the voltage-dependence of steady-state inactivation to more depolarized potentials (Figure 4C,D, Table 1).

Recovery of Kv4.3 channel from inactivation

At least 700 ms were required for Kv4.3 channels to recover from inactivation at -80 mV (Figure 5A,B). In control conditions, the Kv4.3 channel time constant for recovery (τ_r) was not significantly modified by 30 nM of PaTx1 (Figure 5B, Table 1).

Voltage dependence of inhibition of Kv4.3 channels by PaTx1 toxin

In order to determine the voltage dependence of Kv4.3 inhibition by PaTx1, high depolarizations ($+130$ mV) and/or pulse frequencies (0.2 and 1 Hz) were applied.

In 5 out of 8 cells, the current measured at $+130$ mV was inhibited by PaTx1 ($29 \pm 2\%$) suggesting that the toxin was still bound to the channel, even at high depolarizations (Figure 5C,D).

The frequency of stimulation did not affect the affinity of PaTx1 for the Kv4.3 channels. After steady-state inhibition of the Kv4.3 currents at 0.2 Hz by PaTx1 (50 nM), the blockade was not modified by increasing the stimulation frequency to 1 Hz ($n = 4$, data not shown). The steady-state inhibition of Kv4.3 currents was reached within 1.5 min whether at 0.2 or 1 Hz ($n = 5$).

Preferential affinity of PaTx1 for the inactivated and resting closed states of Kv4.3 channels

Affinity to the resting or inactivated states of the channel were evaluated by applying 50 nM of PaTx1 for 2 min prior to the first depolarization from either -80 or -40 mV up to -10 mV. Kv4.3 currents were equally inhibited in each of these conditions (Figure 5E,F). Inhibition was of $88 \pm 12\%$ and $80 \pm 13\%$, $n = 5$ and 4 cells) for the resting closed state and the inactivated state respectively as compared to inhibition obtained when PaTx1 was applied during the depolarizing step.

Effect of PaTx1 and PaTx2 on Kv4.2 currents

PaTx1 (50 nM) potently blocked Kv4.2 currents ($76 \pm 6\%$ at -10 mV and $59 \pm 7\%$ at $+20$ mV, $n = 6$ cells), (Figure 6A). The toxin blocked Kv4.2 currents with more affinity ($IC_{50} = 5$ nM) than the Kv4.3 currents evoked at -10 mV ($IC_{50} = 28$ nM, Figure 6A). When fitted by a single exponential function, the time course of inhibition yielded a time constant of 0.53 ms (data not shown).

PaTx2 similarly blocked the Kv4.2 currents but with a lower affinity ($IC_{50} = 34$ nM, not shown). PaTx1 or PaTx2 inhibition of the Kv4.2 channels almost totally reversed upon washout ($89 \pm 9\%$ recovery after 5 min; $n = 10$ cells).

Characteristics of Kv4.2 channel inhibition by PaTx1

PaTx1-induced modifications of the Kv4.2 electrophysiological parameters were comparable to those observed on Kv4.3 channels (see Table 1). PaTx1 decreased dose-dependently the kinetics of activation of the Kv4.2 current (Figure 6C). The time constants of Kv4.2 current inactivation were increased by 30 nM PaTx1 (Figure 6D). A shift of the normalized conductance-voltage and of the steady-state inactivation curves to more depolarized potentials occurred during application of 30 nM PaTx1 (Figure 6E,F). The time constant for recovery of the Kv4.2 channels (τ_r) was not modified by 10 nM of PaTx1 (Table 1).

The change of the stimulation frequency had no effect on the affinity and the kinetics of inhibition of PaTx1 for the Kv4.2 channel. After reaching steady-state block at 0.2 Hz, a stimulation frequency of 1 Hz did not modify the inhibition exerted by 30 nM PaTx1 ($n = 4$). The time required to reach steady-state inhibition was 1.5 min in both cases (data not shown).

PaTx1 blocked Kv4.2 channels in the closed and inactivated states. Kv4.2 currents evoked by depolarizations to -10 mV were similarly blocked by a 2 min application of 30 nM of PaTx1, either at -80 or -40 mV, as compared to the blockade obtained by applying the toxin during the depolarizing pulses (relative inhibition: $88 \pm 9\%$ and $81 \pm 13\%$, respectively, data not shown).

Effect of PaTx1 on I_{to1} current in cardiomyocytes

In a preliminary study, PaTx1 (50 nM) significantly blocked the I_{to1} current (by $60 \pm 7\%$, $n = 5$) evoked from depolarizations to $+20$ mV from a holding potential of -80 mV in isolated rat cardiomyocytes.

Effect of PaTx1 and PaTx2 on Kv4.1 currents

Kv4.1 currents expressed in COS cells were resistant to PaTx1 and PaTx2 block: 250 nM PaTx1 only blocked $39 \pm 4\%$ ($n = 4$ cells) and 300 nM PaTx2 blocked $20 \pm 2\%$ of the Kv4.1 currents evoked at -10 mV.

Specificity of phrixotoxins on the Kv4 subfamily of channels

Specificity of PaTx1 and PaTx2 toxins for Kv4 channels was evaluated by applying the toxins on different cloned K^+ channels expressed either in the *Xenopus* oocytes or in COS transfected cells. None of the Kv1 channel family recorded in *Xenopus* oocytes (Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5) were sensitive to up to $5 \mu\text{M}$ of PaTx1 or PaTx2 (data not shown). Furthermore, neither HERG, that underlies the cardiac rapid component I_{Kr} of the delayed rectifier I_K (Attali, 1996; Sanguinetti et al., 1995), nor the human KvLQT1 channel coexpressed with Isk that composes its slow component I_{Ks} , were affected by up to $0.3 \mu\text{M}$ PaTx1 when recorded in transfected COS cells (Barhanin et al., 1996).

The 20% sequence identity that PaTx1 and PaTx2 share with hanatoxins which are active on Kv2.1 channels (Swartz & McKinnon, 1997) prompted us to test the phrixotoxins on

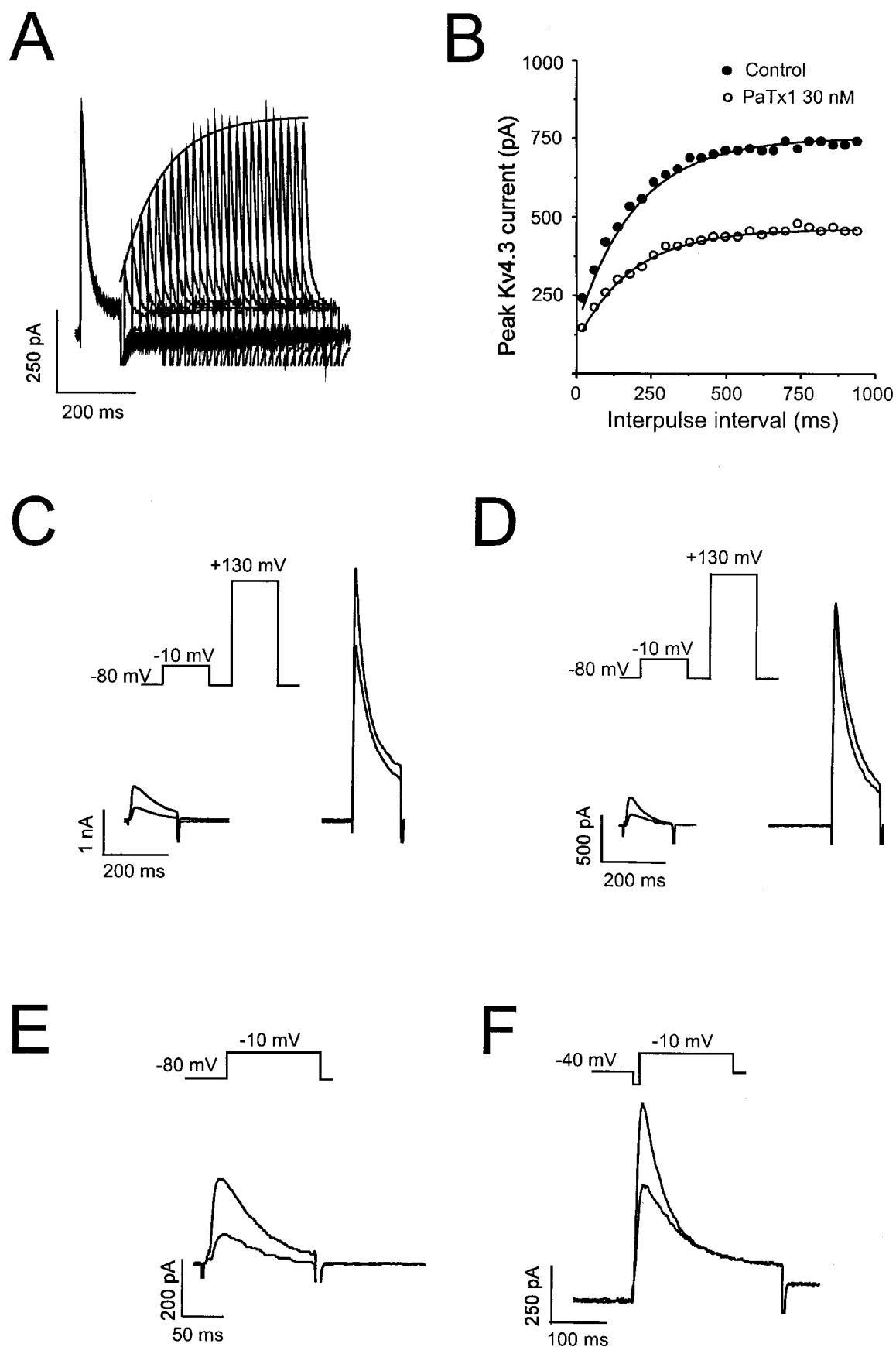


Figure 5 Voltage dependent inhibition of Kv4.3 channels by PaTx1. Kv4 currents were recorded in COS transfected cells in the whole-cell configuration. The holding potential was -80 mV. (A) Successive traces illustrating recovery from Kv4.3 channel inactivation in control conditions. The corresponding fit for the peak Kv4.3 current evoked at $+10$ mV is superimposed with the traces. (B) Peaks Kv4.3 currents were plotted against the interpulse interval in control and after inhibition by 30 nM of PaTx1. The time constant for recovery from channel inactivation was determined using a double pulse protocol where cells were depolarized from -80 mV to $+10$ mV for 200 ms with increasing interpulse intervals of time at -80 mV ($dt=20$ ms). Peak currents elicited by depolarizing pulses were plotted as a function of time and data points were fitted to equation (5): $I = I_{res} + I_{max}(1 - \exp(-t/\tau_r))$ where I is the peak current elicited by the second pulse, I_{max} is the amplitude of peak current elicited by the test pulse after recovery at

Kv2.1 and Kv2.2 currents. The Kv2.1 and Kv2.2 currents evoked at +20 mV from a holding potential of -80 mV were resistant to either 0.5 μ M PaTx1 (inhibition of Kv2.1: $6 \pm 3\%$, $n=5$ and Kv2.2: $16 \pm 10\%$, $n=8$) or 0.5 μ M PaTx2 (inhibition of Kv2.1: $8 \pm 5\%$, $n=4$ and Kv2.2: $11 \pm 6\%$, $n=5$).

None of the cloned channels from the Kv3 family recorded in injected *Xenopus* oocytes (Kv3.1, Kv3.2 and Kv3.4), were sensitive to phrixotoxins (up to 1 μ M, $n=3$ for each channel).

The inward rectifier channel IRK1 expressed in *Xenopus* oocytes was also insensitive to 1 μ M PaTx1 or PaTx2, as was the recently cloned two P domain background K⁺ channel TREK-1 (Fink et al., 1996; Patel et al., 1998), expressed in COS cells.

PaTx1 and PaTx2 toxins weakly blocked the neuronal TTX-sensitive Na⁺ currents of neuroblastoma cells but only at relatively high concentrations ($13 \pm 8\%$ inhibition by 0.1 μ M PaTx1, $n=5$ and $14 \pm 5\%$ inhibition by 0.5 μ M PaTx2, $n=2$).

These results indicate that PaTx1 and PaTx2 are good blockers of the Kv4 channel family with a marked specificity towards Kv4.2 and Kv4.3 channels.

Electrocardiographic modifications of cardiac repolarization induced by intravenous injections of PaTx1 in anaesthetized mice

Three bipolar lead electrocardiograms (ECGs, DI, DII, DIII) were recorded under pentobarbital anaesthesia before, during and after toxin injection with a good stability of the signal from all 11 mice studied (Figure 7). P wave, PR, QRS, ST_{max}, ST_r and QT intervals could be reliably measured in all animals. The average cycle length (RR interval) was 159 ± 14 ms. The P wave duration was 19 ± 2 ms, the PR interval was 36.5 ± 2.1 ms and the QRS interval was 12.1 ± 0.5 ms (Figure 7A). The T wave was biphasic with a rapid component, and a slower one either positive or negative to the isoelectric line. The duration of the ST_{max} interval was 4.20 ± 0.62 ms, and the ST_r interval 9.25 ± 0.89 ms. The average QT duration on the first recording of the experiment was 100 ± 9 ms, which corresponded to a QTc interval of 249 ± 11 ms. The injection of concentrations of PaTx1, ranging from 0.1–40 nmol, did not change significantly the RR interval, from 159 ± 14 ms in control conditions to 166 ± 10 ms after the injection ($P=0.15$, N.S.). The PR interval (39.2 ± 2.3 ms) did increase in some mice (Figure 8A) whereas the QRS interval duration (12.2 ± 0.4 ms) did not significantly change from control values, excluding therefore a relevant effect of the toxin on Na⁺ and/or Ca²⁺ currents. However, PaTx1 significantly increased the mean duration of the ventricular repolarization (QT_c) from 249 ± 11 to 265 ± 8 ms ($n=10$, $P<0.05$). The toxin

effects were mainly observed on the fast component of the T wave, which amplitude significantly decreased and which duration significantly increased after the toxin injection (Figure 7B). Thus, ST_{max} values increased from 4.20 ± 0.62 to 5.00 ± 0.72 ms, which represents an increase of $22 \pm 6\%$ ($P<0.05$, $n=10$), and ST_r from 9.25 ± 0.89 to 11.21 ± 0.99 ms, representing an increase of $22 \pm 4\%$ ($P<0.01$, $n=10$). There was a clear dose-effect relationship between the toxin injected and the resulting modifications of the fast T wave parameters (ST₅₀, Figure 8C). Besides those ECG effects, a generalized vasoconstriction was noticed after PaTx1 injection, followed by various transient cardiac rhythm and/or conduction problems including second and third degree atrio-ventricular (AV) block (4 out of 11 mice, Figure 8B), premature ventricular beats (Figure 7) and ventricular tachycardia (3 out of 11 mice).

Discussion

Voltage-gated potassium channels have a key role in controlling the rate of repolarization of neuronal and cardiac cells of excitable tissues. The members of the Kv4 subfamily are characterized by rapidly activated and inactivated currents. It is believed that *Shal* related Kv4 proteins are key components of the classical A-type K⁺ channels underlying the A-currents (I_A) found in many neuronal soma. A-type K⁺ channels are of particular interest because of their roles in regulating firing frequency, spike initiation and waveform. Three members have been identified in this K⁺ channel family, Kv4.1, Kv4.2 and Kv4.3. Kv4.1 is expressed at low levels in the brain, while Kv4.2 and Kv4.3 transcripts appear to be abundant, each with a unique pattern of expression, although there is an overlap expression of Kv4.2 and Kv4.3 transcripts in several neuronal regions (Serodio & Rudy, 1998; Serodio et al., 1996) suggesting the possible formation of heteropolymeric channels.

Further analyses of the precise physiological role of I_{to1} require specific and high affinity blockers. The two new toxins identified in this work, PaTx1 and PaTx2, are both specific and potent inhibitors of Kv4.2 and Kv4.3 channels. Concentrations of 100 nM of either PaTx1 or PaTx2 almost completely block both Kv4.3 and Kv4.2 currents while they are without effect on the Kv4.1 channel. The toxins alter the gating of the channels, by changing both their activation and inactivation properties. Phrixotoxins bind to Kv4.2 and Kv4.3 channels with a high affinity at both closed and inactivated states and display no use- or reverse-use-dependence. Both toxins induce a blockade that is maximal in a low depolarization range but

-80 mV for 20 s. I_{res} is the amplitude of the residual current remaining at the end of the first pulse, t the interval of time between the first and the second test pulses at +10 mV and τ_r the time constant of recovery. PaTx1 was tested at a concentration of 30 nM on the Kv4.3 current in order to block at least 50% of the current. Fit curves are shown and corresponding values for τ_r are: 199 ms in control and 185 ms after PaTx1 inhibition. (C) Effect of 50 nM PaTx1 on Kv4.3 current at high depolarizations to +130 mV. To test whether PaTx1 was still bound to the channel after large depolarizations, a test pulse to +130 mV was applied 700 ms after a 150 ms depolarization to -10 mV from a holding potential of -80 mV, in the absence (control) and in the presence of the toxin. In this example, the Kv4.3 current is inhibited by PaTx1 at -10 mV by 60% and at +130 mV by 18%. (D) In another example, using the same protocol as in (C), the Kv4.3 current is inhibited by PaTx1 at -10 mV by 63% and not at all at +130 mV. (E) PaTx1 is able to block the Kv4.3 channel in a closed state. The membrane potential was maintained at -80 mV during 2 min in the presence of a concentration of PaTx1 (50 nM), known to block 70% of the Kv4.3 current, before applying a depolarizing pulse to -10 mV. The inhibition of the current was measured after applying the toxin for 2 min to obtain the steady-state inhibition. Under a 2 min perfusion of 50 nM of PaTx1 at -80 mV, the Kv4.3 current is inhibited by 60% as early as the first stimulation to -10 mV. (F) PaTx1 can block the Kv4.3 channel in an inactivated state. The interaction of the toxin with the inactivated state of the channel was tested using a membrane potential maintained at -40 mV. After 2 min of superfusion with 50 nM PaTx1, the membrane potential was clamped to -80 mV during 700 ms for recovery from inactivation, followed by a depolarizing pulse to -10 mV. Comparison to control values assessed the degree of blockade. Under a 2 min perfusion of 50 nM of PaTx1 at -40 mV, the Kv4.3 current was inhibited by 70% as early as the first stimulation to -10 mV.

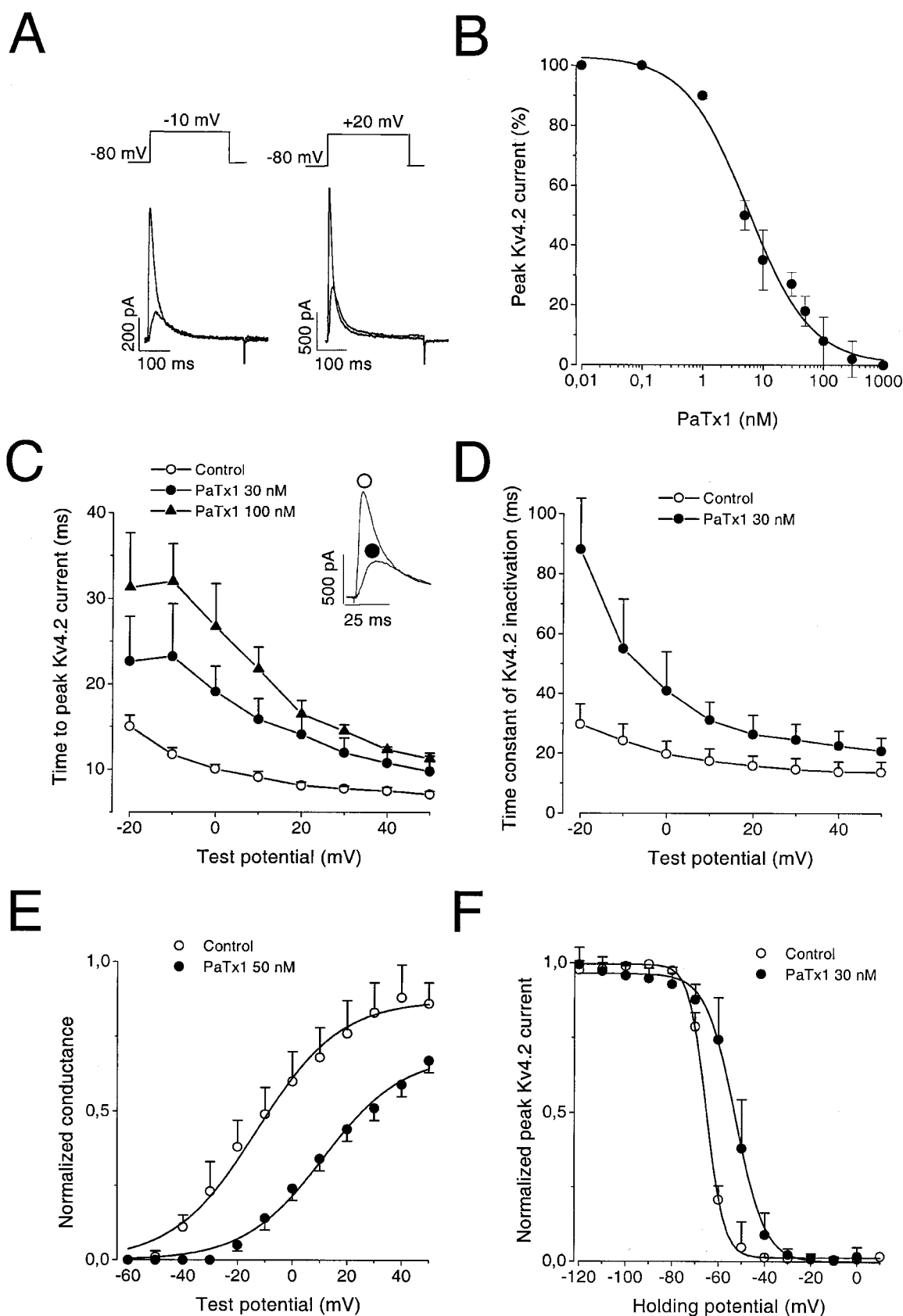


Figure 6 Effect of PaTx1 and PaTx2 on Kv4.2 currents. Kv4.2 currents were recorded in COS transfected cells in the whole-cell configuration. Holding potential: -80 mV. (A) Effect of 50 nM PaTx1 on Kv4.2 currents, recorded at a test pulse of -10 mV and +20 mV respectively, on the same cell. More blockade of Kv4.2 current by PaTx1 occurred at -10 mV (79%) than at +20 mV (67%). (B) Concentration-response relationship for PaTx1 block of the Kv4.2 current at -10 mV. Each point is the mean $\pm$ s.e. mean of data from 3–7 cells. The IC_{50} value determined from equation (3) is 5 nM. (C) The time-to-peak current is represented against the test potential for Kv4.2 channel before and after inhibition of the current by 30 and 100 nM PaTx1. The inset shows the effect of 30 nM PaTx1 at 20 mV. (D) The time constant of current inactivation is plotted against the test potential for Kv4.2 channel before and after inhibition of the current by 30 nM PaTx1. (E) Normalized conductance-voltage relationships for Kv4.2 currents in control and after inhibition by 50 nM PaTx1. Points are means $\pm$ s.e. mean of data from three cells. (F) Steady-state inactivation curves for Kv4.2 currents evoked at +30 mV before and after their inhibition by 30 nM PaTx1. Each point is the mean after normalization of Kv4.2 peak current amplitude from 7–9 cells.

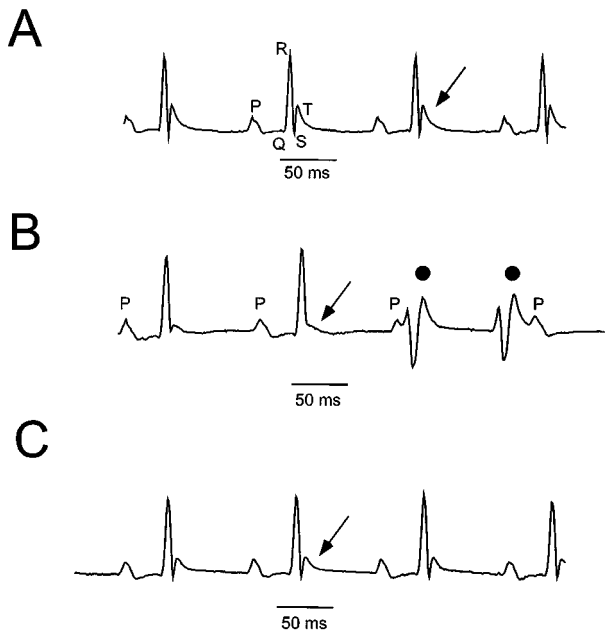


Figure 7 Electrocardiographic modifications induced by intravenous injections of PaTx1 in mice. (A) A representative lead I surface control ECG, recorded from a Balb C female by Pclamp Software (Axon). P, Q, R and T describe the P wave (atrial depolarization), the PR interval (atrio-ventricular conduction), QRS wave (ventricular depolarization) and the biphasic T wave (ventricular repolarization). The cycle length (RR) on the upper trace is 130 ms, with a PR at 37.5 ms, and QT at 62 ms. (B) ECG recorded 3 min after the intravenous injection of 12 nmol of PaTx1. The QT interval increased to 71 ms. A significant decrease of the fast component of the T wave occurred (arrow) and typical premature ventricular beats appeared (●). (C) The T wave modifications progressively reversed 20 min after the injection of PaTx1.

wanes and remains incomplete at high depolarizations. The fact that large depolarizations (+130 mV) can overcome the blocking effect is not uncommon for spider toxins. Such a phenomenon has already been described for the effects of hanatoxins on Kv2.1 channels (Swartz & McKinnon, 1997) and heteropodatoxins (HpTx) on Kv4.2 channels (Sanguinetti *et al.*, 1997). The ~50% identity that PaTx1 and PaTx2 share with HpTx2 and the fact that the location pattern of the cysteine residues is conserved among their subgroup, suggest a similar mode of action, even though these toxins are extracted from venoms from different spider families (Heteropodidae for HpTx and Theraphosidae for PaTx). However, besides blocking Kv4.2 channels, PaTx1 and PaTx2 also strongly inhibit the Kv4.3 channels, a property which had not been investigated for HpTx. On the other hand, it is not known whether HpTx inhibit K⁺ channels such as Kv1.1, Kv1.2, Kv1.3, Kv1.5, or of the Kv3 family, or of the inward rectifier and background K⁺ channel families.

Because of the abundant presence of Kv4 channels in the nervous system, it is not surprising that neurological effects were observed after intravenous injection of PaTx1 in mice. They were mainly characterized by a motor impairment prevailing on the posterior limbs and convulsions (data not shown). These effects are much less severe than those observed after intravenous injections of the MCD-peptide, a bee venom toxin, or of the dendrotoxins from the snake *Dendroaspis* venoms, which block the Kv1.1, Kv1.2 and Kv1.6 channels (Grissmer *et al.*, 1994; Halliwell *et al.*, 1986; Pongs, 1992b; Schweitz, 1984; Strong, 1990), inducing very severe epileptic seizures and convulsions (Bagetta *et al.*, 1992; Mourre *et al.*, 1997). The neurotoxic effects of PaTx1 were similar whether

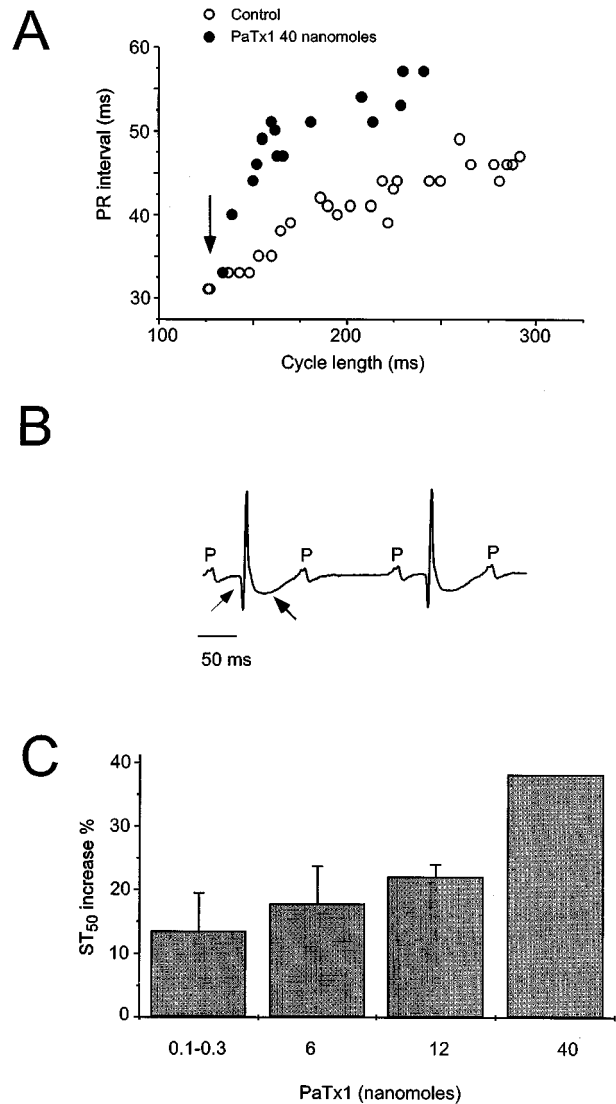


Figure 8 Electrocardiographic modifications induced by high concentrations of PaTx1 in mice. (A) Plot of the PR-RR relationships in a representative mouse, obtained during two successive anaesthesia, in control conditions and after injecting of 40 nmol of PaTx1. After anaesthesia induction, the heart rate progressively decrease, and the PR interval was prolonged accordingly, reflecting the AV conduction. Within seconds after the intravenous injection of PaTx1 (arrow), the PR interval abruptly increased. (B) A typical second degree atrio-ventricular block occurred 2 min after the injection of 40 nmol of PaTx1 with myocardial infarction. Note the Q wave and the huge negative T wave (arrows). (C), Dose-effect relationship of PaTx1 and the resulting increase in ST₅₀ measured 5 min after the injection (*n*=11).

the toxin injection was intravenous or intracisternal (data not shown), making it likely that PaTx1 crosses the blood brain barrier at least to some extent despite its peptidic nature.

There is an increasing body of evidence, including antisense and transgenic experiments (Johns *et al.*, 1997; Nakamura *et al.*, 1997) suggesting that Kv4.2 and Kv4.3 underlie the *I_{to1}* current in cardiac cells (Serodio *et al.*, 1996). First, Kv4.3 mRNA is expressed at significant levels in rat, canine and human cardiac myocytes eliciting rapidly inactivating A type potassium currents. Second, the kinetics and the current-voltage relationships of Kv4.2 and Kv4.3 channels closely resemble those of the native *I_{to1}* current. Third, both Kv4.3, Kv4.2 and *I_{to1}* share pharmacological similarities like insensitivity to the K⁺ channel blocker TEA and inhibition

by 4-aminopyridine (4-AP) or the antiarrhythmic drug flecainide (Grissmer *et al.*, 1994; Tseng *et al.*, 1996; Yeola & Snyders, 1997). In addition, differential expressions of Kv4.2 and Kv4.3 in different heart regions, like their predominance in atria or their characteristic transmural ventricular gradient clearly correspond to differential expression of the I_{to1} current explaining a faster repolarization in atria or in the subepicardial layers of the ventricle (Dixon & McKinnon, 1994; Dixon *et al.*, 1996; Serodio & Rudy, 1998; Serodio *et al.*, 1996; Yeola & Snyders, 1997).

The Kv1.5 gene which is highly expressed in the heart encodes I_{Kur} , the 4-AP sensitive sustained component of I_{to} (Guo *et al.*, 1997; Po *et al.*, 1993). The insensitivity of the Kv1.5 current to the phrixotoxins permits to discriminate the respective physiological roles of I_{to1} and I_{Kur} . This is important in mice given the role of the fast repolarizing currents that enable heart rates to approximate 600 beats min^{-1} .

The intravenous injection of PaTx1 in mice resulted in various transient heart rhythm and conduction problems. Within seconds after the injection, mice experienced different degrees of AV block (from a sharp augmentation of the PR interval to higher degrees of AV block). This points to the role that the Kv4 subfamily of channels might play in the conductive system, whether in the AV node or in the His bundle. The fact that toxin injections could induce ventricular arrhythmia indicates that events resulting in I_{to1} blockade (secondary effects of drugs, possible mutations in Kv4 channels) will generate these sorts of arrhythmia. I_{to1} plays an important role in shaping the early phase of the cardiac action potential repolarization. ECG parameters show that intravenous injections of PaTx1 induce a dose-dependent decrease of the rapid component of the T wave and a

prolongation of the QT interval. These effects on the T wave occur right after the QRS deflection, which corresponds to the rapid depolarization phase of the ventricular action potential where I_{to1} is activated.

In conclusion, we have isolated and purified from the venom of *Phrixotrichus auratus* two new toxins, PaTx1 and PaTx2, that are potent and specific blockers of Kv4.2 and Kv4.3 channels. The use of these pharmacological tools has confirmed the view that Kv4.2 and Kv4.3 channels are the essential components of I_{to1} current in cardiac cells. It has also contributed to the better understanding of the exact role of I_{to1} in cardiac electrogenesis in a mouse model.

Other types of Kv channels such as Kv1.4 or Kv3.4 also generate rapidly inactivating A-type K^+ channels (Rettig *et al.*, 1992; Tseng-Crank *et al.*, 1990). No specific toxin has been found yet for the Kv1.4 channels. However, because phrixotoxins block Kv4.2 and Kv4.3 channels without affecting Kv1.4 channels, A-type channels resulting from Kv1.4 expression or from Kv4.2/Kv4.3 expressions can now be easily discriminated. Easy discrimination can also be made between relative contributions of Kv3.4, Kv4.2 and Kv4.3 channels, since sea anemone BDS toxins (but not phrixotoxins) selectively block the Kv3.4 channels (Diochot *et al.*, 1998).

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***In vivo* demonstration of H₃-histaminergic inhibition of cardiac sympathetic stimulation by R- α -methyl-histamine and its prodrug BP 2.94 in the dog**

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1 The aim of this study was to investigate whether histamine H₃-receptor agonists could inhibit the effects of cardiac sympathetic nerve stimulation in the dog.

2 Catecholamine release by the heart and the associated variation of haemodynamic parameters were measured after electrical stimulation of the right cardiac sympathetic nerves (1–4 Hz, 10 V, 10 ms) in the anaesthetized dog treated with R- α -methyl-histamine (R-HA) and its prodrug BP 2.94 (BP).

3 Cardiac sympathetic stimulation induced a noradrenaline release into the coronary sinus along with a tachycardia and an increase in left ventricular pressure and contractility without changes in mean arterial pressure. Intravenous administration of H₃-receptor agonists significantly decreased noradrenaline release by the heart (R-HA at 2 μ mol kg⁻¹ h⁻¹: +77 $\pm$ 25 vs +405 $\pm$ 82; BP 2.94 at 1 mg kg⁻¹: +12 $\pm$ 11 vs +330 $\pm$ 100 pg ml⁻¹ in control conditions, $P \leq 0.05$), and increases in heart rate (R-HA at 2 μ mol kg⁻¹ h⁻¹: +26 $\pm$ 8 vs +65 $\pm$ 10 and BP 2.94 at 1 mg kg⁻¹: +30 $\pm$ 8 vs 75 $\pm$ 6 beats min⁻¹, in control conditions $P \leq 0.05$), left ventricular pressure, and contractility. Treatment with SC 359 (1 mg kg⁻¹) a selective H₃-antagonist, reversed the effects of H₃-receptor agonists. Treatment with R-HA at 2 μ mol kg⁻¹ h⁻¹ and BP 2.94 at 1 mg kg⁻¹ tended to decrease, while that with SC 359 significantly increased basal heart rate (from 111 $\pm$ 3 to 130 $\pm$ 5 beats min⁻¹, $P \leq 0.001$).

4 Functional H₃-receptors are present on sympathetic nerve endings in the dog heart. Their stimulation by R- α -methyl-histamine or BP 2.94 can inhibit noradrenaline release by the heart and its associated haemodynamic effects.

Keywords: H₃-receptors; R- α -methyl-histamine; BP 2.94; catecholamines; cardiac sympathetic nerves; dog

Abbreviations: BP, BP 2.94; CTL, Control; HR, Heart rate; R-HA, R- α -methyl-histamine; SC, SC 359

Introduction

H₃-receptors are presynaptic autoreceptors present on histaminergic nerve endings. Their stimulation is responsible for the inhibition of histamine release (Arrang *et al.*, 1983) and synthesis (Arrang *et al.*, 1987). H₃-receptors are also present on non histaminergic nerves such as serotonergic, cholinergic or adrenergic nerves in the central nervous system (Stark *et al.*, 1989) and on postganglionic sympathetic neurones (Ishikawa & Sperelakis, 1987; Imamura *et al.*, 1995) where they inhibit neuromediator release. R- α -methyl-histamine is a full agonist displaying a 15 fold higher potency than histamine at H₃-receptors and negligible potency at other receptor subtypes (Arrang *et al.*, 1987). In view of the actions of R- α -methyl-histamine, it was considered that H₃-receptor agonists display a potential therapeutic value in several fields such as migraine or inflammation (Göthert *et al.*, 1995). However, the efficacy of R- α -methyl-histamine in man is limited, as a consequence of its rapid metabolism (Rouleau *et al.*, 1997).

BP 2.94 ((R)-(-)-2-[N-[1-(1H-imidazol-4-yl)-2-propyl]-iminophenylmethyl]phenol) an azomethine derivative, is a stable prodrug of R- α -methyl-histamine, able to slowly release the bioactive compound, thus preventing its early metabolic degradation (Rouleau *et al.*, 1997). BP 2.94 has been shown to present antinociceptive and anti-inflammatory properties

attributable to an inhibition of tachykinin release (Rouleau *et al.*, 1997).

Since R- α -methyl-histamine attenuates the chronotropic and inotropic response to stimulation of sympathetic nerve endings in isolated guinea-pig atrial preparations by inhibiting noradrenaline release (Endou *et al.*, 1994; Imamura *et al.*, 1995), we questioned whether H₃-receptor agonists could modulate cardiac sympathetic activity *in vivo*. Therefore, the purpose of this study was to evaluate the effects of R- α -methyl-histamine and BP 2.94 on haemodynamic response and noradrenaline release induced by cardiac sympathetic nerve stimulation in the anaesthetized dog.

Methods

Animal preparation

This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (N.I.H. Publication No. 85-23, revised 1985).

Seventeen Beagle dogs (12–20 kg) were anaesthetized with sodium pentobarbital (30 mg kg⁻¹, i.v.) and α -chloralose (80 mg kg⁻¹ followed by 10 mg kg⁻¹ h⁻¹). Positive pressure respiration with room air was maintained by a pump (Roche-Kontron 3100S) connected to an endotracheal tube. The right

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femoral vein and artery were cannulated with polyethylene catheters for drug injection and arterial pressure measurement using pressure transducers (Baxter 33-260, Healthcare Corp. U.K.). A catheter was introduced into the jugular vein and positioned into the coronary sinus under fluoroscopic examination. Another catheter was introduced into the right carotid artery and placed in the left ventricle.

Lead II of the surface electrocardiogram, heart rate, arterial and left ventricular pressure and its first derivative (dP/dt) were continuously recorded on a computer (MacIntosh Power PC) using a data acquisition system (MacLab 8 channels, ADInstruments).

A thoracotomy was performed at the 3rd intercostal space. The right stellate ganglion was isolated by ligating the sympathetic chain upstream and downstream, and the nerve branches to the heart were stimulated (10 V, 10 ms) during 60 s with bipolar platinum electrodes. The stimulation frequency (1–4 Hz) was adapted in order to reach a HR increase ranging between 50 and 80 beats min⁻¹ in control conditions.

An heparin injection (Choay, 5000 UI) was performed at the beginning of the experimental protocol.

Catecholamine measurement

Noradrenaline and adrenaline concentrations in the arterial and coronary sinus blood were determined by the HPLC technique after isolation by the alumina adsorption technique (Eriksson, 1993). The chromatographic system consisted of a pump (model 501, Chromatofield), a C₁₈ reverse phase column (Lichrospher, 250 × 4 mm, Merck) and an electrochemical detector (Coulochem 5100A, ESA). Dihydroxybenzylamine (Sigma) was used as the internal standard.

Experimental groups

Five dogs were treated successively with intravenous injection of R- α -methyl-histamine (Bioprojet, France) at doses of 0.2 and 2 μ mol kg⁻¹ h⁻¹ and finally with R- α -methyl-histamine 2 μ mol kg⁻¹ h⁻¹ following injection of SC 359 (Ciproxifan, cyclopropyl 4-(3-(1H-imidazol-4-yl)propoxy)phenyl)ketone, 1 mg kg⁻¹ base, i.v.) (Bioprojet, France), a specific H₃-antagonist (Figure 1).

Twelve dogs were treated successively with intravenous injection of BP 2.94 (Bioprojet, France) at doses of 0.1 and 1 mg kg⁻¹. Seven dogs were then treated with SC 359 (1 mg kg⁻¹ base, i.v.) (Bioprojet, France) and the remaining five dogs served as controls and were treated with saline instead of SC 359 (Figure 1).

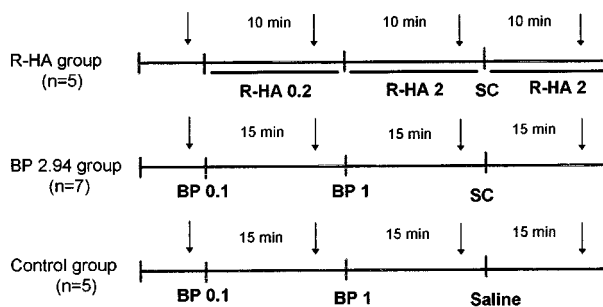


Figure 1 Experimental protocol. Arrows represent times for haemodynamic measurements and blood collection before and 30 s after the beginning of right cardiac sympathetic stimulation. R-HA 0.2: R- α -methyl-histamine at 0.2 μ mol kg⁻¹ h⁻¹ and R-HA 2 at 2 μ mol kg⁻¹ h⁻¹, BP 0.1: BP 2.94 at 0.1 mg kg⁻¹ and BP 1 at 1 mg kg⁻¹, SC: SC 359 at 1 mg kg⁻¹.

Experimental protocol

Haemodynamic measurements and blood collection (5 ml) into the coronary sinus and aorta were realized before and 30 s after the beginning of each nerve stimulation. Cardiac nerve stimulation was performed in control conditions, 15 min after treatment with BP 2.94 or SC 359 and 10 min after the beginning of each R- α -methyl-histamine infusion (Figure 1).

Statistical analysis of data

In order to compare control and agonist-treated groups, one- or two-way analyses of variance followed by *post hoc* multiple comparison Tukey tests were performed when normality was respected. When it was not, non parametric Kruskal–Wallis tests with multiple comparison Dunn's tests were used. To compare antagonist-treated groups to corresponding agonist-treated groups, paired Student *t*-test were used (SigmaStat statistical software v.2.0, Jandel Scientifics). Statistical significance was set at $P \leq 0.05$.

Results

Haemodynamic parameters

In control conditions, right cardiac sympathetic nerve stimulation induced an increase in heart rate (185 ± 6 vs

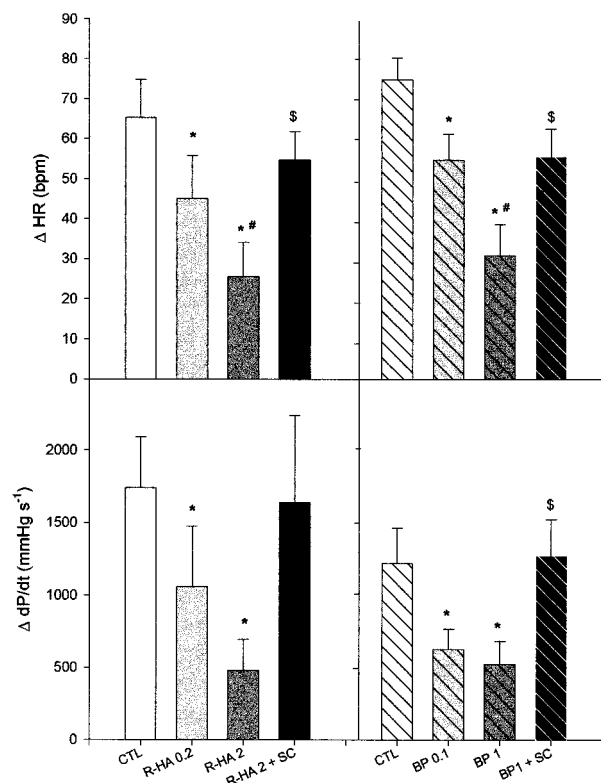


Figure 2 Increase in heart rate (HR) and left ventricular dP/dt upon right cardiac sympathetic nerve stimulation (means $\pm$ s.e.mean) in control conditions (CTL), and left panels: 10 min after treatment with R- α -methyl-histamine at 0.2 μ mol kg⁻¹ h⁻¹ (R-HA 0.2) and at 2 μ mol kg⁻¹ h⁻¹ before (R-HA 2) and after SC 359 at 1 mg kg⁻¹ base (R-HA 2 + SC), right panels: 15 min after treatment with BP 2.94 at 0.1 mg kg⁻¹ (BP 0.1) and 1 mg kg⁻¹ alone (BP 1) and 15 min after SC 359 at 1 mg kg⁻¹ base (BP 1 + SC). * $P \leq 0.05$ vs CTL, # $P < 0.05$ vs R-HA 0.2 or BP 0.1, \$ $P < 0.05$ vs R-HA 2 or BP 1.

113 ± 12 beats min⁻¹ before stimulation, $P \leq 0.001$), left ventricular pressure (145 ± 10 vs 112 ± 7 mmHg, $P \leq 0.001$) and dP/dt (3800 ± 350 vs 2400 ± 200 mmHg s⁻¹, $P \geq 0.001$) (Figure 2).

Treatment with R- α -methyl-histamine dose-dependently reduced the heart rate increase and significantly reduced the increase in contractility. Those effects were antagonized by concomitant administration of SC 359 (Figure 2). Treatment with BP 2.94 at 0.1 and 1 mg kg⁻¹ also dose-dependently and significantly reduced the increases in heart rate, left ventricular pressure and contractility (Figure 2). Treatment with SC 359 inhibited the effects of the BP 2.94 restoring the response to sympathetic stimulation (Figure 2). The response to BP 2.94 (1 mg kg⁻¹) was maintained for at least 30 min, since a similar response was obtained at 15 and 30 min post-dosing in the saline treatment group (Table 1). Figure 3 shows typical tracings of the heart rate and dP/dt response to sympathetic stimulation in presence of the H₃-receptor agonists and antagonist.

Administration of R- α -methyl-histamine or BP 2.94 tended to decrease or significantly decreased basal heart rate and dP/dt (Figure 4), mean arterial pressure and left ventricular pressure (data not shown). On the opposite, SC 359 significantly increased these parameters (Figure 4).

Catecholamine concentrations

Right cardiac sympathetic nerve stimulation induced an increase in coronary sinus noradrenaline concentration (461 ± 73 vs 115 ± 32 pg ml⁻¹ before stimulation, $P \leq 0.001$). Coronary sinus adrenaline and arterial noradrenaline and adrenaline concentrations were not modified by the stimulation (data not shown).

Treatment with R- α -methyl-histamine at 0.2 and 2 mg kg⁻¹ significantly reduced cardiac noradrenaline release upon cardiac sympathetic nerve stimulation (Figure 5). BP 2.94 at 0.1 mg kg⁻¹ and 1 mg kg⁻¹ also significantly reduced it (Figure 5). The effects of both drugs were reversed by SC 359 (Figure 5). Saline administration *in lieu* of SC 359 did not affect the effect of BP 2.94 (Table 1).

Discussion

Our study provides the first demonstration of the existence of prejunctional H₃-receptors on cardiac sympathetic nerve endings in the dog. Their stimulation by R- α -methyl-histamine or BP 2.94 can inhibit the effects of sympathetic stimulation by reducing cardiac noradrenaline release and its associated haemodynamic effects.

Table 1 Reversal by SC 359 but not by saline of the effects of BP 2.94 on the haemodynamic response to right cardiac sympathetic nerve stimulation

Treatment	HR (beats min ⁻¹)	Increase in dP/dt (mmHg s ⁻¹)	NA (pg ml ⁻¹)
BP 1	31 ± 8	500 ± 200	12 ± 11
BP 1 + SC	56 ± 7*	1300 ± 300*	123 ± 60*
BP 1 + saline	40 ± 4	500 ± 100	0 ± 1

Data are means ± s.e.mean. HR: heart rate, dP/dt: first derivative of the left ventricular pressure, NA: coronary sinus noradrenaline concentration, BP 1: BP 2.94 at 1 mg kg⁻¹; BP 1 + SC: BP 2.94 at 1 mg kg⁻¹ followed by SC 359 at 1 mg kg⁻¹ base and BP 1 + saline: BP 2.94 at 1 mg kg⁻¹ followed by saline. * $P \leq 0.05$ vs BP 1.

The model of cardiac sympathetic nerve stimulation used in this study was first described by Yamaguchi *et al.* (1975), who showed that it can be a useful tool for the *in vivo* study of the role of the sympathetic nervous system in the regulation of cardiovascular function. It has also been used to demonstrate the prejunctional modulatory effects of various agents (Uchida *et al.*, 1984; Yamaguchi *et al.*, 1986). This model permits a selective cardiac sympathetic stimulation since increases in heart rate and in left ventricular pressure and contractility are seen without significant changes in mean arterial pressure. There is a correlation between this haemodynamic response and noradrenaline release into the coronary sinus (Yamaguchi *et al.*, 1975). Our results are in accordance with this observation.

Prejunctional H₃-receptors have been identified on sympathetic nerve endings in many species such as the mouse (Schlicker & Marr, 1997), rat (Godlewski *et al.*, 1996), guinea-pig (Endou *et al.*, 1994), cat (Schlicker *et al.*, 1994) and man (Imamura *et al.*, 1995). All these studies report a reduction in noradrenaline release and/or sympathetic response by H₃-receptor stimulation. The signalling pathway of H₃-receptor

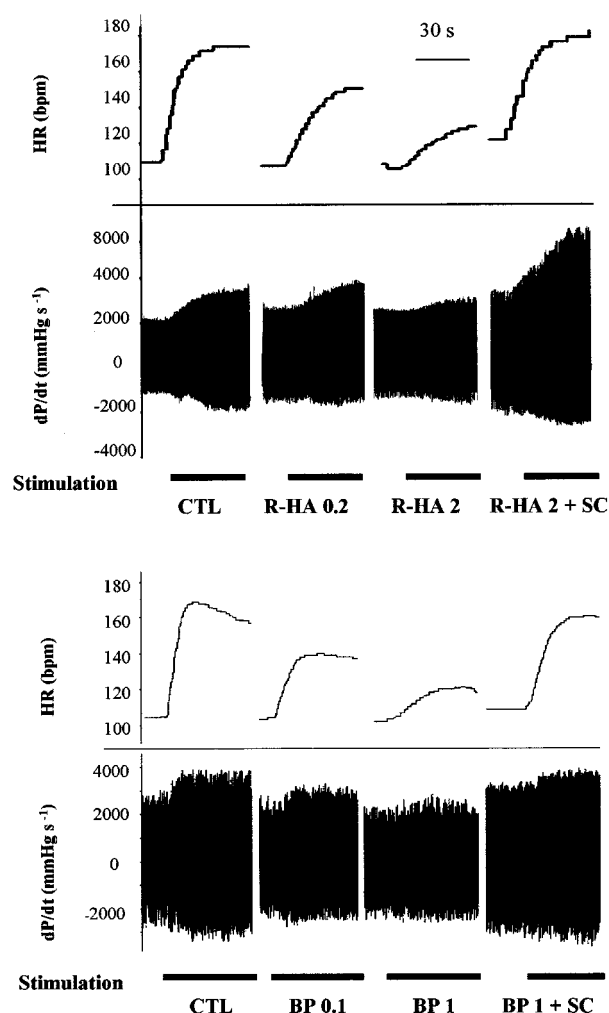


Figure 3 Tracings representing the changes in heart rate and left ventricular dP/dt upon right cardiac sympathetic nerve stimulation in control conditions (CTL) and upper panel: 10 min after treatment with R- α -methyl-histamine at 0.2 (R-HA 0.2) and at 2 μ mol kg⁻¹ h⁻¹ before (R-HA 2) and after SC 359 at 1 mg kg⁻¹ base (R-HA 2 + SC), lower panel: 15 min after treatment with BP 2.94 at 0.1 mg kg⁻¹ (BP 0.1) and 1 mg kg⁻¹ alone (BP 1) and 15 min after SC 359 at 1 mg kg⁻¹ base (BP 1 + SC).

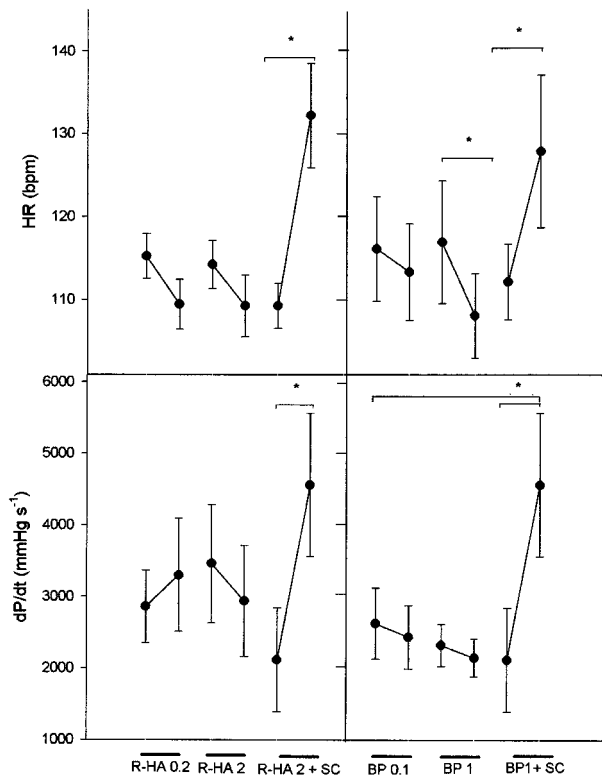


Figure 4 Heart rate (HR) and left ventricular dP/dt variation (means $\pm$ s.e. mean) induced by treatment with, left panels: R- α -methyl-histamine at $0.2 \mu\text{mol kg}^{-1} \text{h}^{-1}$ (R-HA 0.2) and at $2 \mu\text{mol kg}^{-1} \text{h}^{-1}$ before (R-HA 2) and after SC 359 at 1 mg kg^{-1} base (R-HA 2+SC), right panels: BP 2.94 at 0.1 mg kg^{-1} (BP 0.1) and 1 mg kg^{-1} alone (BP 1) and 15 min after SC 359 at 1 mg kg^{-1} base (BP 1+SC). * $P \leq 0.05$.

stimulation has not yet been elucidated but is known to depend on Gi/Go protein stimulation and N-type Ca²⁺ channel opening (Endou *et al.*, 1994) but not on AMPc synthesis (Schlicker *et al.*, 1994). Endou *et al.* (1994) on isolated guinea-pig atria and Imamura *et al.* (1994) on isolated guinea-pig hearts, have demonstrated that H₃-receptor stimulation by R- α -methyl-histamine reduces the inotropic and chronotropic response to sympathetic stimulation by inhibiting noradrenaline release. Our results are in accordance with these studies since R- α -methyl-histamine and BP 2.94 induced a dose-dependent decrease in electrically-evoked noradrenaline release and haemodynamic response. This effect was due to H₃-receptor stimulation since R- α -methyl-histamine (a selective H₃-receptor agonist) induced a clear inhibitory effect that was antagonized by SC 359. Indeed, SC 359 is a very potent (CI₅₀ for histamine release: 9 ± 2 vs 75 ± 19 nM for thioperamide) and selective (pK = 9.3 vs 5 for other histaminergic, adrenergic or serotonergic receptors) competitive H₃-receptor antagonist (Ligneau *et al.*, 1998). Our results confirm that BP 2.94 is a potent H₃-receptor agonist with a longer half-life than R- α -methyl-histamine since a single bolus was able to stimulate H₃-receptors for at least 30 min. Indeed, the recovery observed following SC 359 administration cannot be explained by a decreased activity of BP 2.94 since it was not observed when saline was injected *in lieu* of SC 359. In fact, BP 2.94 remained effective in inhibiting noradrenaline release 1 h after its administration.

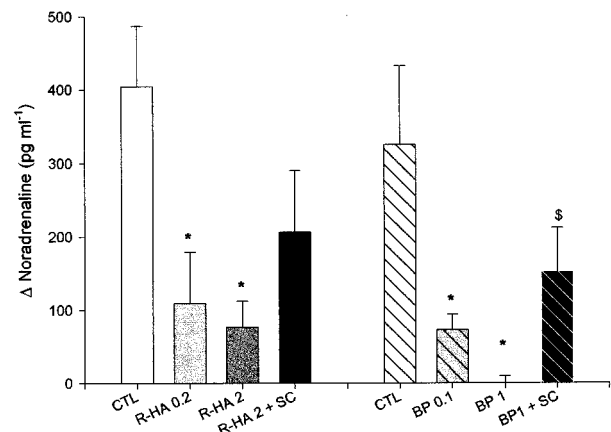


Figure 5 Increase in cardiac noradrenaline release (veno-arterial difference) upon right cardiac sympathetic nerve stimulation (means $\pm$ s.e. mean) in control conditions (CTL) and left panel: 10 min after treatment with R- α -methyl-histamine at $0.2 \mu\text{mol kg}^{-1} \text{h}^{-1}$ (R-HA 0.2) and at $2 \mu\text{mol kg}^{-1} \text{h}^{-1}$ before (R-HA 2) and after SC 359 at 1 mg kg^{-1} base (R-HA 2+SC), right panel: 15 min after treatment with BP 2.94 at 0.1 mg kg^{-1} (BP 0.1) and 1 mg kg^{-1} alone (BP 1) or 15 min after SC 359 at 1 mg kg^{-1} base (BP 1+SC). * $P \leq 0.05$ vs CTL, \$ $P \leq 0.05$ vs BP 1.

Basal cardiovascular effects of H₃ agonists vary according to the species. In the guinea-pig and in the rabbit, R- α -methyl-histamine induces bradycardia and hypotension (McLeod *et al.*, 1994) while in the rat, H₃ agonists have no effects (Corrucci *et al.*, 1995) at least not at the doses where they are selective for H₃-receptors. Our results show that, in the dog, H₃-receptor activation by BP 2.94 decreased heart rate and tended to reduce contractility and arterial pressure. H₃-antagonists fail to induce cardiovascular effects in the guinea-pig, rabbit and rat (Corrucci *et al.*, 1995). We observed that, in the dog, SC 359 induced an important vasopressive response and a tachycardia after R- α -methyl-histamine or BP 2.94. This can probably be explained by an antagonism of the effects of the H₃-agonists but also of the action of endogenous histamine on H₃-receptors. This suggests that in the dog, H₃-receptors are constitutionally functional and activated by endogenous histamine, thus exerting a basal retrocontrol on noradrenaline release. However, H₃-receptors remain responsive to exogenous agonists that can exert a more pronounced effect.

The important sympathoinhibitory action and good bioavailability of BP 2.94 suggests a potential clinical use in conditions associated with enhanced sympathetic tone such as myocardial ischaemia or heart failure. Indeed, H₃-agonists have been shown to reduce ischaemia-induced noradrenaline release in the isolated guinea-pig heart (Imamura *et al.*, 1996) and in isolated human atria (Hatta *et al.*, 1997). Also, H₃-receptor desensitization could contribute to the elevated plasma noradrenaline levels in cardiac failure (Seyedi *et al.*, 1997).

In conclusion, the present study shows that functional H₃-receptors inhibiting noradrenaline release are present on cardiac sympathetic nerve endings in the dog and can be activated by H₃-receptor agonists R- α -methyl-histamine and its prodrug BP 2.94.

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Modulation of chloride, potassium and bicarbonate transport by muscarinic receptors in a human adenocarcinoma cell line

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1 Short-circuit current (I_{SC}) responses to carbachol (CCh) were investigated in Colony 1 epithelia, a subpopulation of the HCA-7 adenocarcinoma cell line. In Krebs-Henseleit (KH) buffer, CCh responses consisted of three I_{SC} components: an unusual rapid decrease (the 10 s spike) followed by an upward spike at 30 s and a slower transient increase (the 2 min peak). This response was not potentiated by forskolin; rather, CCh inhibited cyclic AMP-stimulated I_{SC} .

2 In HCO_3^- free buffer, the decrease in forskolin-elevated I_{SC} after CCh was reduced, although the interactions between CCh and forskolin remained at best additive rather than synergistic. When Cl^- anions were replaced by gluconate, both Ca^{2+} - and cyclic AMP-mediated electrogenic responses were significantly inhibited.

3 Basolateral Ba^{2+} (1–10 mM) and 293B (10 μM) selectively inhibited forskolin stimulation of I_{SC} , without altering the effects of CCh. Under Ba^{2+} - or 293B-treated conditions, CCh responses were potentiated by pretreatment with forskolin.

4 Basolateral charybdotoxin (50 nM) significantly increased the size of the 10 s spike of CCh responses in both KH and HCO_3^- free medium, without affecting the 2 min peak. The enhanced 10 s spike was inhibited by prior addition of 5 mM apical Ba^{2+} . Charybdotoxin did not affect forskolin responses.

5 In epithelial layers prestimulated with forskolin, the muscarinic antagonists atropine and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP, both at 100 nM) abolished subsequent 10 μM CCh responses. Following addition of *p*-fluoro hexahydro-sila-difenidol (*p*F-HHSiD, 10 μM) or pirenzepine (1 μM), qualitative changes in the CCh response time-profile also indicated a rightward shift of the agonist concentration-response curve; however, 1 μM gallamine had no effect. These results suggest that a single M_3 -like receptor subtype mediates the secretory response to CCh.

6 It is concluded that CCh and forskolin activate discrete populations of basolateral K^+ channels gated by either Ca^{2+} or cyclic AMP, but that the Cl^- permeability of the apical membrane may limit their combined effects on electrogenic Cl^- secretion. In addition, CCh activates a Ba^{2+} -sensitive apical K^+ conductance leading to electrogenic K^+ transport. Both agents may also modulate HCO_3^- secretion through a mechanism at least partially dependent on carbonic anhydrase.

Keywords: Epithelial transport; carbachol; potassium channel

Introduction

Acetylcholine (ACh) is a major enteric neurotransmitter involved in the regulation of intestinal ion transport. Immunohistochemical studies have demonstrated the presence of choline acetyltransferase-like immunoreactivity in the submucous plexi of rat and guinea-pig small intestine (Bornstein & Furness, 1988) and in the majority of submucous neurones in both human small and large bowel (Potter *et al.*, 1996); a subpopulation of cholinergic neurones thus identified projects directly to the epithelium. ACh is released from nerve terminals in close proximity with the colonic mucosa in response to depolarizing stimuli (rat; Wu *et al.*, 1982), and after electric field stimulation (guinea-pig; Javed & Cooke, 1992). In the latter study, ACh release was closely correlated with an increase in short-circuit current (I_{SC}) attributable to the secretion of Cl^- anions. Secretory responses to ACh or metabolically stable cholinomimetics such as carbachol (CCh) have also been observed in *in vitro* preparations of small or large intestinal mucosae from a number of other species, including porcine distal jejunum (Chandan *et al.*, 1991a) and

colon (Traynor *et al.*, 1991), rat distal colon (Strabel & Diener, 1995) and human ileum (Isaacs *et al.*, 1976). The effects of CCh are mediated both indirectly, through stimulation of transmitter release from submucosal neurones, and *via* a direct action on epithelial muscarinic receptors (Chandan *et al.*, 1991b; O'Malley *et al.*, 1995).

The mechanism by which epithelial cells secrete Cl^- anions has been extensively studied in a number of human adenocarcinoma cell lines, in which the absence of modulation by neuronal or immune effects is assured (for a review, see Binder & Sandle, 1994). It is now generally accepted that Cl^- ions enter the cell through a basolateral $Na^+/K^+/2Cl^-$ cotransporter, inhibitable by loop diuretics such as piretanide, and leave through Cl^- channels in the apical membrane. This electrogenic transport requires the activation of basolateral K^+ channels, which serve to recycle K^+ ions across the basolateral membrane and to hyperpolarize the cell, creating a favourable electrochemical gradient for apical Cl^- exit. Regulation by second messengers occurs at two principal levels; the basolateral K^+ conductances are sensitive to intracellular Ca^{2+} and cyclic AMP concentrations, and the cystic fibrosis transmembrane conductance regulator (CFTR), an apical Cl^- channel critically involved in intestinal Cl^-

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secretion, is also activated indirectly by cyclic AMP (Morris *et al.*, 1994). Ca^{2+} -gated Cl^- channels have also been identified (Bajnath *et al.*, 1992), although the extent to which these are restricted to the apical domain is controversial (Morris *et al.*, 1994). In two human colonic cell lines, T84 and HT-29, CCh stimulates phosphoinositide hydrolysis and thereby increases intracellular Ca^{2+} , effects mediated by the M_3 muscarinic receptor (Dickinson *et al.*, 1992; Kopp *et al.*, 1989). Although such Ca^{2+} elevations are sustained in T84 epithelia, the associated Cl^- secretory response is short-lived (Dharmasathaphorn *et al.*, 1989) and may be limited by the release of additional inhibitory second messengers (e.g. arachidonic acid; Devor & Frizzell, 1998). On the other hand, the transient nature of Ca^{2+} increases measured in the HCA-7 human adenocarcinoma cell line may be sufficient to account for the timecourses of Cl^- secretion in response to either lysylbradykinin or histamine (MacVinish *et al.*, 1993).

The interaction between Ca^{2+} - and cyclic AMP-elevating agonists and the subsequent regulation of intestinal secretion is physiologically relevant for a number of reasons. In the guinea-pig small intestine, cholinergic secretomotor neurones also contain a number of co-released peptides which influence epithelial cyclic AMP levels *via* specific receptor populations, e.g. neuropeptide Y and calcitonin gene related peptide (Bornstein & Furness, 1988). Vasoactive intestinal peptide (VIP) is also a prominent submucous neurotransmitter, providing an extensive innervation of the epithelial layer, for example in rat jejunum (Ekblad *et al.*, 1987) and human colon (Domoto *et al.*, 1990). Its release results in a potent secretory response mediated by epithelial cyclic AMP accumulation (Schwartz *et al.*, 1974). Thus the epithelial actions of ACh *in vivo* are invariably dependent on a background of other agents which stimulate or inhibit cyclic AMP formation. Indeed the presence of endogenous neurotransmitters and eicosanoids is necessary for efficient induction of Cl^- secretion by CCh in the rat distal colon (Strabel & Diener, 1995). Cellular investigations have generally revealed a synergistic interaction between cyclic AMP- and Ca^{2+} -mediated secretory agonists, including those in T84 (Dharmasathaphorn & Pandol, 1986), HT-29 cl. 16E (Merlin *et al.*, 1995) and HCA-7 (MacVinish *et al.*, 1993) adenocarcinoma cell lines. In HT-29 and T84 cells this synergism relies on the cooperative activation of discrete populations of basolateral K^+ channels governed by Ca^{2+} and cyclic AMP. In contrast, preliminary studies with a sub-population of the HCA-7 cell line, called Colony 1, have shown some unusual features in electrogenic CCh responses (Holliday *et al.*, 1997); in particular, forskolin-stimulated increases in I_{SC} are inhibited by CCh, rather than potentiated. In this study we therefore pursue the mechanisms underlying the changes in I_{SC} associated with CCh and forskolin, using charybdotoxin, Ba^{2+} ions and the chromanol derivative 293B to discriminate between different Ca^{2+} - and cyclic AMP-gated K^+ channel populations. We show that the absence of potentiation arises from both a limitation in the rate of Cl^- secretion across the apical membrane and the additional involvement of Ca^{2+} -stimulated transepithelial K^+ and HCO_3^- transport.

Methods

Cell culture

Colony 1 epithelia were kindly provided by Dr S. Kirkland (Imperial College, London, U.K.). This cell line was initially isolated by seeding HCA-7 cells at low density and using

cloning cylinders to trypsinize the resulting individual colonies (Marsh *et al.*, 1993). They were maintained in 25 cm² flasks (Falcon, Beckton Dickinson, Oxford, U.K.) containing Dulbecco's Modified Eagle's medium (DMEM; Gibco, Paisley, U.K.) supplemented with glucose (25 mM), foetal calf serum (10%; ICN Biomedicals, Oxford, U.K.) and the antibiotics kanamycin (100 mg ml⁻¹; ICN) and amphotericin B (1.2 mg ml⁻¹; ICN). Cells were incubated at 37°C in a humidified atmosphere of 95% O₂ / 5% CO₂. Once confluent, epithelial layers (passages 17–22) were trypsinized (0.5% w/v in versene; Worthington, Lorne Laboratories, Reading, U.K.) and seeded onto collagen-coated filters (Millipore, Hertfordshire, U.K.) as previously described by Cuthbert *et al.* (1987). Growth was restricted to a central well of 0.2 cm² area by the use of a Sylgard washer.

Short-circuit current (I_{SC}) measurement

After 6–8 days, confluent Colony 1 cells on filters were mounted in Ussing chambers and bathed in one of the following solutions (composition in mM): Krebs-Henseleit (KH; NaCl 118, KCl 4.7, NaHCO₃ 25, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 2.5, glucose 11.1; pH 7.4), HCO₃⁻ free (for experiments requiring the use of Ba^{2+} ions; HEPES 10, NaCl 140, KCl 5.4, CaCl₂ 1.2, MgCl₂ 1, glucose 11.1; pH 7.4) or Cl⁻ free buffers (Na gluconate, 118, K gluconate 4.7, NaHCO₃ 25, KH₂PO₄ 1.2, MgSO₄ 1.2, CaSO₄ 2.5, glucose 11.1; pH 7.4). These solutions (15 ml in each reservoir) were aerated with 95% O₂ / 5% CO₂ (KH and Cl⁻ free buffers) or 100% O₂ (HCO₃⁻ free). Epithelial layers were voltage-clamped at zero potential (DVC-1000, World Precision Instruments, Florida, U.S.A.) and the resulting I_{SC} recorded continuously on chart recorders. Drugs were added to the basolateral reservoir (unless otherwise stated) and the peak changes in I_{SC} were measured and calculated as $\mu\text{A cm}^{-2}$. Concentration-response curves were constructed from single additions of the desired agent, to eliminate difficulties associated with the rapid desensitization of Colony 1 cells to many agonists, and EC₅₀ values were calculated (with 95% confidence limits) using the curve-fitting program Graphpad Prism (v. 2.01, Graphpad Software Inc., California, U.S.A.). Cells were pretreated with K^+ channel blockers for 10 min before stimulation with either forskolin or CCh; in experiments where charybdotoxin was used, 0.01% bovine serum albumin (BSA) was added to both basolateral and apical reservoirs prior to its addition. Results are expressed as the means $\pm$ 1 s.e.mean. of *n* observations. Statistical analysis of two data groups was carried out using the Student's unpaired *t*-test; for multiple comparisons one way analysis of variance was performed followed by Bonferroni's corrected *t*-test. A significance level of $P < 0.05$ was adopted throughout.

Materials

293B (*trans*-6-cyano-4-[N-ethylsulphonyl-N-methylamino]-3-hydroxy-2,2-dimethyl-chromane) was the kind gift of Prof R. Greger (Physiologisches Institut der Albert-Ludwigs-Universität, Freiburg, Germany). Piretanide was a gift from Hoechst Marion Roussel (Swindon, U.K.). Other drugs were purchased from the following companies: forskolin, CCh, acetazolamide and atropine (Sigma, Poole, U.K.), 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), *p*-fluoro hexahydro-sila-difenidol HCl (*p*F-HHSiD), gallamine and pirenzepine (RBI, St. Albans, U.K.) and charybdotoxin (Alamone Labs, Jerusalem, Israel). Agents were made up as aqueous stock solutions with the following exceptions: 293B (dimethyl

sulphoxide), forskolin and *pF*-HHSiD (95% ethanol) and charybdotoxin (prepared in 0.1% BSA, 100 mM NaCl, 10 mM Tris and 1 mM EDTA; pH 7.5). Non-aqueous solvents were diluted to a concentration of 0.1% v/v on addition of drugs to the reservoir. BaCl₂ was dissolved at the required concentration in HCO₃⁻ free solution.

Results

Responses to CCh in untreated and forskolin-stimulated cells

Krebs-Henseleit Colony 1 cells exhibited basal resistances of $39.3 \pm 0.6 \Omega \text{ cm}^2$ and basal I_{SC} levels of $10.8 \pm 0.4 \mu\text{A cm}^{-2}$ ($n = 520$). Addition of $10 \mu\text{M}$ CCh to untreated epithelial layers resulted in a rapid downward spike at 10 s, followed by a transient increase in I_{SC} (maximum at 2 min) which returned to basal levels within 15 min (Figure 1A and Table 1). EC₅₀ values for these two phases were $6.5 \mu\text{M}$ ($3.1\text{--}13.7 \mu\text{M}$) and $3.6 \mu\text{M}$ ($1.1\text{--}11.1 \mu\text{M}$; $n = 4\text{--}14$) respectively (Figure 1B). A shoulder in the I_{SC} increases after $10 \mu\text{M}$ CCh was distinguished as a separate spike component at 30 s in 4 out of 14 cases and became more clearly defined after higher agonist concentrations ($100 \mu\text{M}$ response: $+10.9 \pm 2.7 \mu\text{A cm}^{-2}$, $n = 4$; approximate EC₅₀ $19.9 \mu\text{M}$ ($0.8\text{--}49.0 \mu\text{M}$; $n = 4$); Figure 1A).

Forskolin, a direct activator of adenyl cyclase, produced sustained elevations in I_{SC} (EC₅₀ 517 nM ; Holliday *et al.*, 1997), the response after a maximal concentration ($10 \mu\text{M}$) being $+64.0 \pm 2.7 \mu\text{A cm}^{-2}$ ($n = 76$). Following forskolin pretreatment, stimulation with $10 \mu\text{M}$ CCh resulted in a rapid reduction in I_{SC} (Figure 2), the maximal decrease at 30 s being greatest after $1 \mu\text{M}$ forskolin ($-21.8 \pm 4.3 \mu\text{A cm}^{-2}$, $n = 5$); subsequently, I_{SC} recovered to a peak at 2–3 min after agonist addition. Only in cells treated with a low forskolin concentration (100 nM or 300 nM) did this peak response represent an overall increase above the initial elevated I_{SC} levels (Figure 2). 10 s upward ($+4.4 \pm 1.6 \mu\text{A cm}^{-2}$, $n = 5$) or

downward spikes ($-5.8 \pm 1.7 \mu\text{A cm}^{-2}$, $n = 5$; revealed on an extended time scale in the inset in Figure 3) were also often observed, akin to the CCh responses in otherwise unstimulated cells.

HCO₃⁻ free Unstimulated epithelia in HCO₃⁻ free solution exhibited basal resistances ($30.4 \pm 0.8 \Omega \text{ cm}^2$, $n = 102$), I_{SC} ($14.3 \pm 1.6 \mu\text{A cm}^{-2}$, $n = 53$) and responses to $10 \mu\text{M}$ CCh that were similar to those in KH buffer (Figure 3A; Table 1). The 10 s downward spike after CCh was observed less often (four out of nine responses), while the 30 s upward spike was more

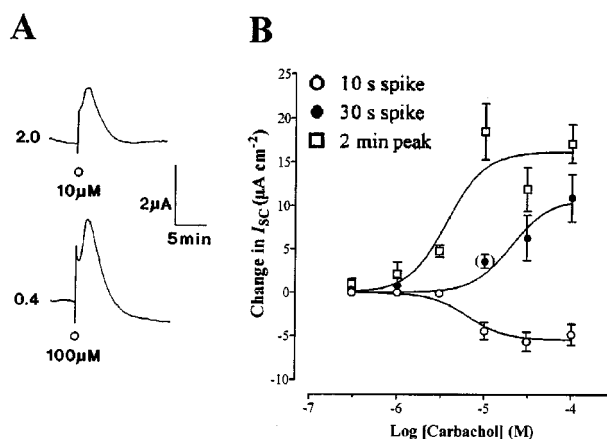


Figure 1 Responses to CCh in Colony 1 epithelial layers. (A) shows representative traces in which Colony 1 cells in KH buffer were stimulated with $10 \mu\text{M}$ (upper trace) or $100 \mu\text{M}$ (lower trace) CCh, with initial baseline currents (in μA) given to the left of each example. In (B) single additions of increasing CCh concentration ($0.3\text{--}100 \mu\text{M}$; $n = 4\text{--}14$) are represented as a series of concentration response relationships showing the 10 s downward spike, the 30 s spike and the 2 min transient increase in I_{SC} . A separate 30 s component could not be distinguished after $3 \mu\text{M}$ CCh and was observed in only a minority of cases (4 out of 14, bracketed value) after $10 \mu\text{M}$ agonist. EC₅₀ values are quoted in the text.

Table 1 Summary of the effects of different solutions, charybdotoxin and apical Ba²⁺ on CCh responses

Pretreatment	Solution	Responses to $10 \mu\text{M}$ CCh ($\mu\text{A cm}^{-2}$)		
		10 s spike	30 s spike	2 min peak
None	KH	-4.4 ± 1.1 (14)	$+3.6 \pm 0.8$ [4]	$+18.5 \pm 3.2$ (14)
ChT (50 nM, bl)	KH	-13.0 ± 2.0 (3)	—	$+11.3 \pm 5.4$ (3)
ChT (50 nM, ap/bl)	KH	-9.7 ± 1.8 (3)	—	$+7.2 \pm 3.9$ (3)
None	HCO ₃ ⁻ free	-3.0 ± 1.6 [4]	$+3.2 \pm 1.1$ (9)	$+9.5 \pm 1.8$ (9)
ChT (50 nM, bl)	HCO ₃ ⁻ free	-9.1 ± 1.9 (4)	—	$+10.4 \pm 2.7$ (4)
Ba ²⁺ (5 mM, ap)	HCO ₃ ⁻ free	0.0 ± 0.0 (4)	$+4.8 \pm 0.4$ (4)	$+3.3 \pm 0.8$ (4)
ChT (bl)/Ba ²⁺ (ap)	HCO ₃ ⁻ free	-3.4 ± 0.5 (4)	—	$+4.4 \pm 1.5$ (4)
None	Cl ⁻ free	0.0 ± 0.0 (5)	—	$+1.5 \pm 0.2$ (5)

Colony 1 cells were bathed in KH solution or buffers lacking either HCO₃⁻ or Cl⁻ anions, and pretreated with charybdotoxin (ChT) and Ba²⁺ added apically (ap) or basolaterally (bl) as indicated. Peak changes in I_{SC} are given for each measurable phase of the subsequent $10 \mu\text{M}$ CCh response; in a number of cases the 30 s spike was absent. Values in parenthesis indicate either the total number of observations (rounded brackets) or the number of times a particular component could be distinguished [square brackets]. Significant differences are presented elsewhere in the text or in Figures 7 and 8.

prominent and easily distinguished from the slower transient elevation in I_{SC} . Forskolin-induced increases in I_{SC} ($10 \mu\text{M}$; $+49.2 \pm 8.3 \mu\text{A cm}^{-2}$, $n=8$) were smaller than but not signifi-

cantly different from those observed in KH buffer. After 30 min prestimulation with $10 \mu\text{M}$ forskolin, $10 \mu\text{M}$ CCh elicited a rapid 30 s spike ($+8.2 \pm 1.0 \mu\text{A cm}^{-2}$), followed by a transient increase (peak at 2 min: $+1.9 \pm 1.8 \mu\text{A cm}^{-2}$; $P<0.05$ compared to unstimulated control responses) and a sustained decrease in I_{SC} ($-4.2 \pm 1.3 \mu\text{A cm}^{-2}$; $n=8$; Figure 3B).

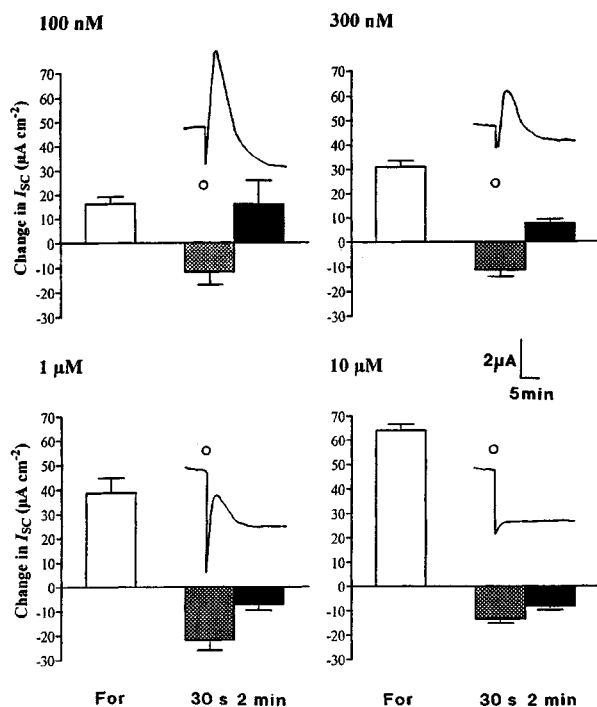


Figure 2 The effect of forskolin stimulation on CCh-induced changes in I_{SC} . Histograms show the increases in I_{SC} after four different concentrations of forskolin (For) in epithelia bathed in KH solution (100 nM – $10 \mu\text{M}$; $n=4$ – 76 ; open bars) and the responses to $10 \mu\text{M}$ CCh added 30 min subsequently, summarized as the 30 s peak decrease in I_{SC} (hatched bars) and the maximum at 2 min after agonist addition relative to initial levels (solid bars). Example traces are given as an inset to each histogram, with CCh added at the time indicated.

Cl^- free In Cl^- free solution, all components of the response to CCh ($10 \mu\text{M}$) in unstimulated epithelial layers were significantly reduced ($P<0.05$, Table 1 and Figure 3A). Responses to $10 \mu\text{M}$ forskolin were also inhibited ($+10.0 \pm 1.6 \mu\text{A cm}^{-2}$, $n=5$; $P<0.01$ compared to responses in KH solution), and the residual increase in I_{SC} was insensitive to the loop diuretic piretanide ($200 \mu\text{M}$). After $10 \mu\text{M}$ forskolin, CCh decreased I_{SC} ($-1.1 \pm 0.4 \mu\text{A cm}^{-2}$ at 2 min, $n=5$, Figure 3B).

The effects of K^+ channel inhibitors on CCh and forskolin responses

293B The chromanol derivative 293B has been used in previous studies as a specific inhibitor of basolateral cyclic AMP-gated K^+ channels in a variety of epithelial cell types (Busch & Suessbrich, 1997). Consistent with this action, basolateral pretreatment with $10 \mu\text{M}$ 293B (in HCO_3^- free solution) did not alter the Ca^{2+} -mediated I_{SC} changes following $10 \mu\text{M}$ CCh addition (Figure 4), but the sustained increases in I_{SC} after $10 \mu\text{M}$ forskolin were significantly attenuated (Figure 5A; $P<0.01$ compared to controls). In the presence of 293B, the responses to $10 \mu\text{M}$ CCh added 30 min after forskolin were strikingly potentiated (Figure 5B); the 30 s spike component was increased 10 fold compared to control CCh responses after forskolin ($+81.3 \pm 14.3 \mu\text{A cm}^{-2}$, $n=5$; $P<0.001$) and in the two cases where it could be distinguished, the peak at 2 min was also greater ($+50.8 \mu\text{A cm}^{-2}$).

Basolateral Ba^{2+} The effects of basolateral Ba^{2+} ions (in HCO_3^- free buffer) were essentially identical to those observed

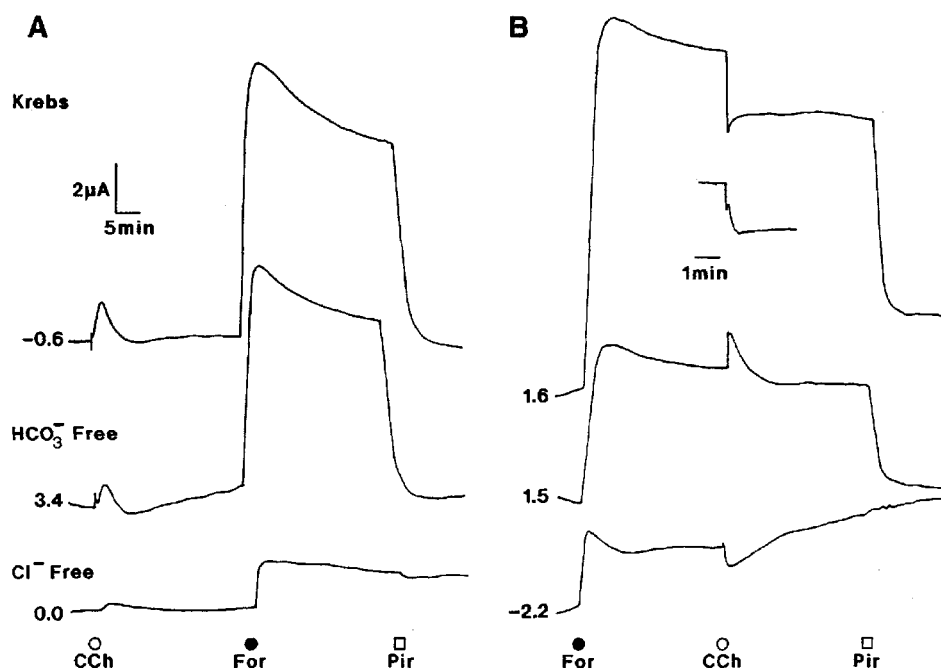


Figure 3 CCh and forskolin responses in Krebs, HCO_3^- and Cl^- -free buffers. (A) The effects of CCh ($10 \mu\text{M}$), forskolin ($10 \mu\text{M}$; For) and piretanide ($200 \mu\text{M}$; Pir) at 30 min intervals are shown in representative traces from cells bathed in KH, HCO_3^- free or Cl^- substituted solutions. In (B), the order of agonist addition is reversed, and an inset to the uppermost trace (KH buffer) displays the changes in I_{SC} after CCh on an extended time-scale to highlight the additional spike component present in the response. Basal I_{SC} is given in μA to the left of each trace.

after treatment with 293B. CCh responses in otherwise unstimulated cells were unaffected (Figure 4); at higher Ba^{2+} concentrations (5 and 10 mM), the 30 s spike component was

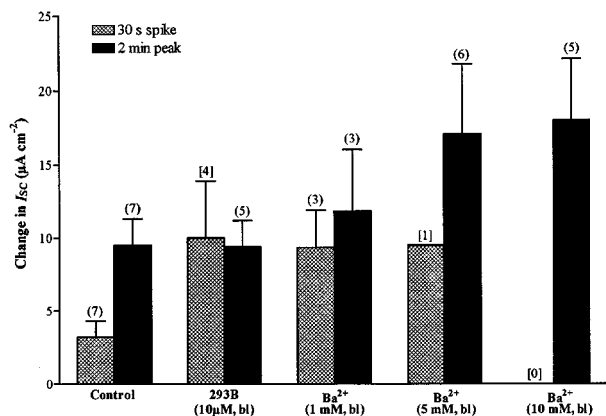


Figure 4 The effect of 293B and Ba^{2+} on CCh stimulation. Epithelial layers in HCO_3^- free buffer were pretreated for 10 min with basolateral 293B (10 μM) or Ba^{2+} (as BaCl_2 , 1–10 mM) followed by 10 μM CCh. The 30 s spike, where clearly defined, and the 2 min peak increase in I_{SC} are illustrated; the 10 s spike was rarely observed (not shown). *N* values (rounded brackets) or the number of times a component was observed (square brackets) are given above each bar.

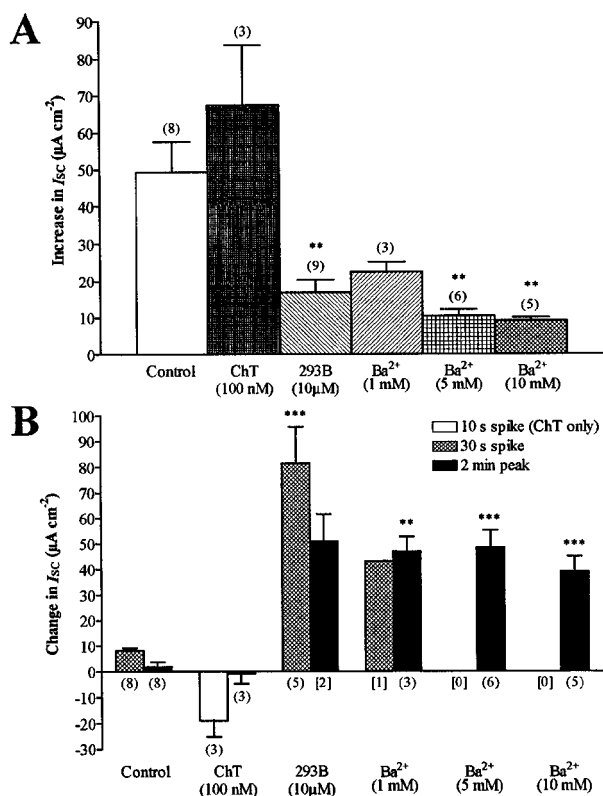


Figure 5 Inhibition of forskolin-induced I_{SC} increases by K^+ channel blockade and the subsequent effects on CCh stimulation. (A) illustrates the effect on 10 μM forskolin responses of 10 min pretreatment with basolateral charybdotoxin (ChT, 100 nM), 293B (10 μM) or Ba^{2+} (1–10 mM). (B) shows the responses to 10 μM CCh added 30 min after forskolin in the same experiments, with each bar representing the 10 s spike (charybdotoxin treated cells only), 30 s spike and 2 min peak. The total number of observations and the frequency of individual components are indicated in round and square brackets respectively; ** $P < 0.01$ and *** $P < 0.001$ compared to controls.

only observed in a minority of cases, but a 'shoulder' in the majority of the responses suggested it was still present. Forskolin-induced I_{SC} increases were in contrast substantially inhibited (significantly so after 5 mM and 10 mM Ba^{2+} ; Figures 5A and 6), while subsequent CCh responses were potentiated (e.g. 2 min peak after 5 mM Ba^{2+} : $+48.3 \pm 6.7 \mu\text{A cm}^{-2}$, $n = 6$; $P < 0.001$ compared to controls; Figures 5B, 6); under these conditions the 30 s spike was rarely distinguished.

Charybdotoxin and apical Ba^{2+} Charybdotoxin (50 nM, basolateral), a specific inhibitor of Ca^{2+} -gated K^+ channels in T84 cells (Devor & Frizzell, 1993), brought about a profound qualitative change in CCh responses in KH buffer (Figure 7). The initial 10 s spike was significantly larger (Table 1, $P < 0.01$ compared to controls) and prolonged; the 30 s spike was apparently absent, while the increase in I_{SC} at 2–3 min was unchanged (Table 1, $P = 0.35$ compared to controls). Pretreatment with apical as well as basolateral charybdotoxin (both 50 nM) had little or no additional effect on 10 μM CCh responses (Figure 7, Table 1), although the changes in I_{SC} observed for both the 10 s spike and the 2 min peak were marginally smaller (though not significantly) than those observed in cells treated with basolateral charybdotoxin alone.

In HCO_3^- free buffer, the response to CCh in cells pretreated with charybdotoxin (50 nM, basolateral) also consisted of an augmented 10 s spike, no 30 s upward spike and a transient increase in I_{SC} (Figure 8, Table 1). Apical Ba^{2+} (5 mM) in combination with basolateral charybdotoxin

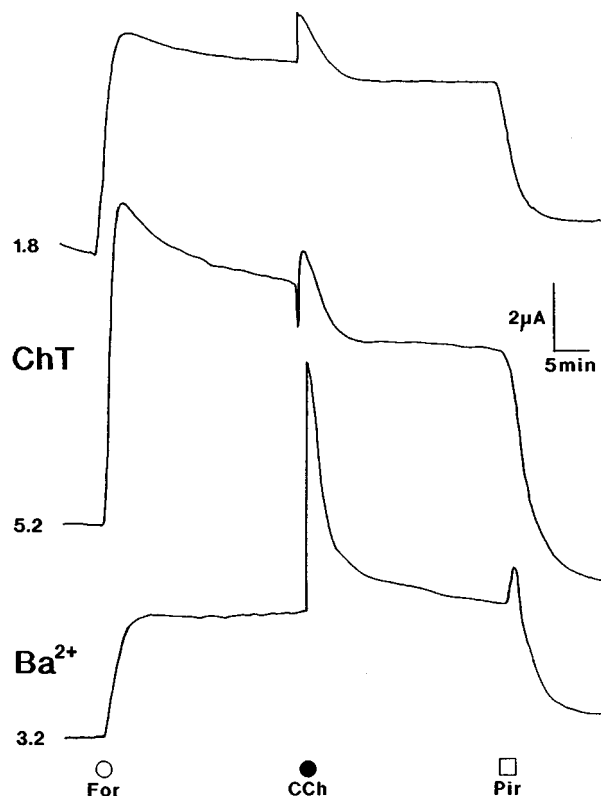


Figure 6 Forskolin and CCh responses in the presence of charybdotoxin or Ba^{2+} . Example experiments from Colony 1 epithelia in HCO_3^- free buffer show the effect of 10 min pretreatment with either 50 nM charybdotoxin (ChT) or 1 mM Ba^{2+} compared to untreated cells (upper trace). Agents were added at the following concentrations: forskolin (10 μM ; For), CCh (10 μM) and piritanide (200 μM ; Pir). Initial I_{SC} levels are indicated on the left (in μA) of each representative trace.

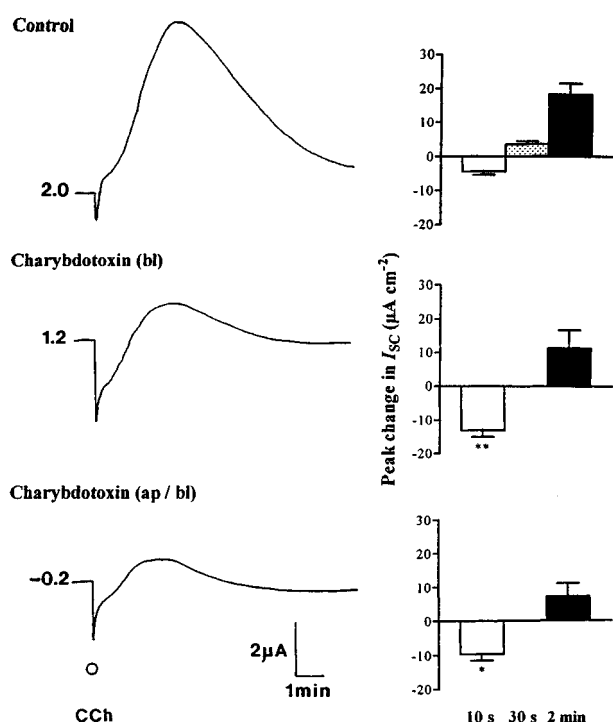


Figure 7 Alteration of CCh responses by charybdotoxin. Representative traces illustrate the CCh-induced changes in I_{sc} from the basal levels indicated, in control cells in KH solution or in those incubated with basolateral (bl) or both apical (ap) and basolateral charybdotoxin (50 nM) for 10 min. To the right of each trace, the histograms show the size of the 10 s spike (open bars), 30 s spike (shaded bars) or the 2 min increase in I_{sc} (solid bars). Significant differences between control values ($n=14$) and those in charybdotoxin-pretreated cells (both $n=3$) are indicated by * $P<0.05$ and ** $P<0.01$.

substantially reduced both these phases of the CCh response (e.g. 10 s spike; $P<0.05$ compared to cells inhibited with charybdotoxin only; Figure 8). When added alone, apical Ba^{2+} did not alter the size of the 30 s spike after 10 μM CCh, but did reduce the 2 min peak compared to control responses in untreated cells ($P<0.05$; Table 1). 10 μM forskolin responses were unaffected by a higher concentration of charybdotoxin (100 nM; Figure 5A); however, subsequent changes in I_{sc} after CCh were altered in a manner similar to that observed in unstimulated cells (Figures 5B and 6), consisting of a rapid decrease ($-19.0 \pm 6.4 \mu A cm^{-2}$), followed by a recovery to approximately initial I_{sc} levels ($-1.0 \pm 3.8 \mu A cm^{-2}$) and a sustained decrease in I_{sc} ($-9.2 \pm 1.9 \mu A cm^{-2}$, $n=3$).

Effect of Ba^{2+} and 293B on piretanide responses

Addition of the loop diuretic piretanide after forskolin and CCh stimulation reduced I_{sc} to approximately basal levels. In both control and charybdotoxin treated epithelial layers, these decreases were monophasic (e.g. $-35.9 \pm 8.5 \mu A cm^{-2}$ in control cells, $n=8$; Figure 6). However, piretanide responses were consistently biphasic in nature following Ba^{2+} or 293B pretreatment. In 293B inhibited cells, piretanide addition gave a transient increase in I_{sc} of $+18.4 \pm 10.6 \mu A cm^{-2}$ ($n=5$) followed by a sustained decrease ($-13.8 \pm 2.5 \mu A cm^{-2}$); equivalent measurements in epithelia exposed to 5 mM Ba^{2+} were $+4.2 \pm 1.7 \mu A cm^{-2}$ and $-6.4 \pm 0.9 \mu A cm^{-2}$ ($n=6$) respectively (Figure 6, lowest trace).

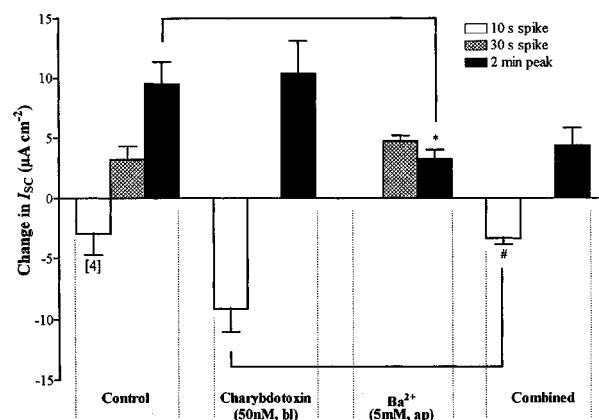


Figure 8 Inhibition of CCh-stimulated changes in I_{sc} by apical Ba^{2+} and charybdotoxin. The histogram shows the separate phases of 10 μM CCh responses in untreated cells in HCO_3^- free solution (control, $n=9$) or those exposed to 50 nM basolateral (bl) charybdotoxin ($n=4$), 5 mM apical (ap) Ba^{2+} ($n=4$) or both treatments ($n=4$) for 10 min. Note that the 10 s spike component of the control group was present in only 4 out of 9 observations, and that the 30 s spike was absent in charybdotoxin-treated cells. Significant differences are indicated as follows: * $P<0.05$ compared to control cells; # $P<0.05$ compared to pretreatment with charybdotoxin only.

Role of carbonic anhydrase

When Colony 1 cells were bathed in HCO_3^- free rather than KH solution, differences in the time-profile of the CCh responses were observed, particularly after first stimulating the cells with forskolin. To examine the extent to which these differences might originate from an inhibition of HCO_3^- transport, epithelia bathed in KH buffer were treated with the carbonic anhydrase inhibitor acetazolamide (450 μM , added both apically and basolaterally). In unstimulated cells, acetazolamide produced a small decrease in I_{sc} ($-0.7 \pm 0.1 \mu A cm^{-2}$, $n=10$) which was also transient. Following forskolin (10 μM ; $+64.0 \pm 2.7 \mu A cm^{-2}$, $n=76$), a sustained reduction in I_{sc} of $-4.2 \pm 0.7 \mu A cm^{-2}$ ($n=4$) was observed which accounted for approximately 5% of the forskolin response, the remainder of which was inhibited by piretanide ($-66.3 \pm 8.8 \mu A cm^{-2}$, $n=4$). Figure 9 shows the effect of acetazolamide pretreatment on 10 μM CCh responses in cells previously treated with forskolin (300 nM), compared with control traces from cells in KH or HCO_3^- free solution. In epithelia treated with acetazolamide, CCh addition resulted in a biphasic response consisting of a 30 s spike ($+3.8 \pm 1.5 \mu A cm^{-2}$) and a transient increase in I_{sc} ($+6.8 \pm 0.7 \mu A cm^{-2}$, $n=3$); this time-profile was essentially the same as that obtained in HCO_3^- free solution (30 s spike: $+6.6 \pm 1.1 \mu A cm^{-2}$; 2 min peak: $+9.0 \pm 1.5 \mu A cm^{-2}$, $n=4$).

Action of muscarinic antagonists

To determine whether the separate components of the CCh response following forskolin were mediated by different receptors, the effects of a number of muscarinic antagonists were investigated (Figure 10). Pretreatment for 10 min with either the non-selective antagonist atropine (100 nM) or 4-DAMP (100 nM; M_3 and M_1 selective), dramatically reduced the changes in I_{sc} associated with CCh, while 1 μM gallamine (M_2 selective) and 1 μM pF-HHSiD (M_3 selective) were without effect. Following addition of pF-HHSiD (10 μM) or

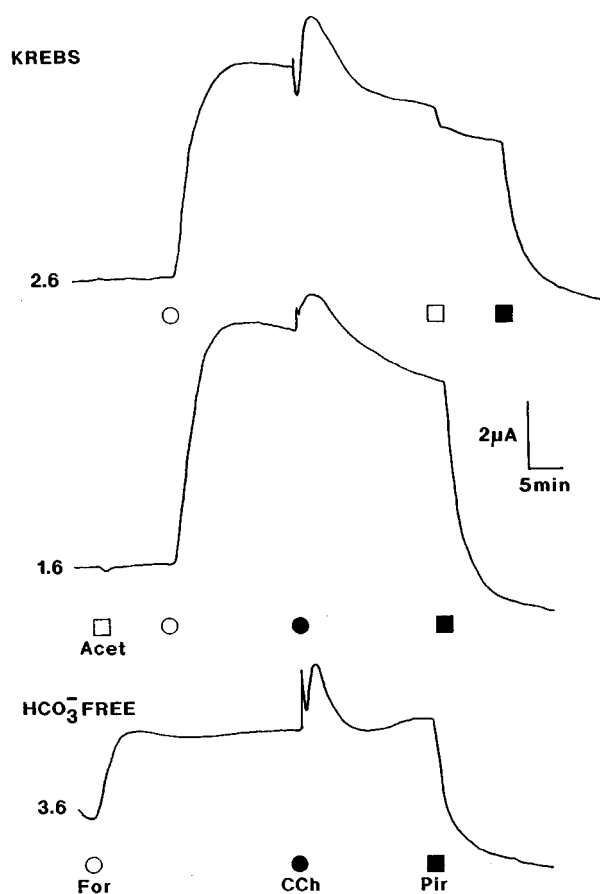


Figure 9 The effect of acetazolamide pretreatment. Example traces show the effect of acetazolamide addition (450 μM , added apically and basolaterally; Acet) on forskolin (300 nM; For) and CCh responses (10 μM) in KH compared with those from otherwise untreated cells bathed in either KH, or HCO_3^- free buffer (lowest trace only). Piretanide (200 μM , Pir) was added at the end of each experiment. Basal I_{sc} (in μA) in each recording is indicated on the left.

pirenzepine (1 μM , M_1 selective), the time-profile of the CCh response was significantly altered, the major component being a small transient increase in I_{sc} at 2 min. Identical responses were obtained when forskolin treated cells were stimulated with a 10 fold lower concentration of CCh (1 μM , Figure 10).

Discussion

CCh responses $\pm$ forskolin

Responses to CCh in Colony 1 epithelial layers consisted of transient increases in I_{sc} , which were attributable to the net electrogenic secretion of Cl^- anions and were in general typical of other colonic carcinoma cell lines, such as T84 (Dharmasathaphorn & Pandol, 1986) and the parent cell line HCA-7 (Cuthbert *et al.*, 1987; MacVinish *et al.*, 1993). In otherwise unstimulated cells, this transient elevation in I_{sc} was preceded by two components. In common with HCA-7 responses and those of other subpopulations (MacVinish *et al.*, 1993), there was an upward spike with a maximum at 30 s after agonist addition; however a very rapid initial reduction in I_{sc} , peaking at 10 s, was also observed and is so far unique to Colony 1 cells. Secondly, interactions between CCh and forskolin in these epithelia do not appear to have synergistic actions on transepithelial ion transport (Holliday *et al.*, 1997), in marked contrast to most other epithelial cell lines studied. In KH solution, forskolin-stimulated epithelial layers showed a marked initial decrease in I_{sc} after CCh followed by a recovery, the extent of which depended on the prior forskolin concentration.

K⁺ channel inhibition of forskolin responses

Basolateral 293B (10 μM) did not significantly affect CCh responses but substantially inhibited the increases in I_{sc} after forskolin, a selective blockade which is in agreement with previous studies. 293B inhibits forskolin stimulated secretion in rat colonic crypts (Warth *et al.*, 1996), and recently it has

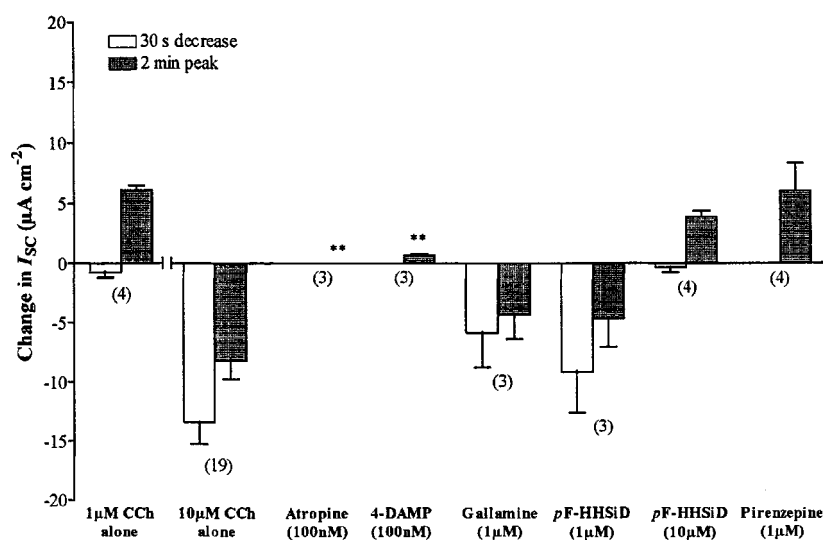


Figure 10 Inhibition of CCh responses by muscarinic antagonists. CCh (10 μM) was added to epithelia bathed in KH solution and prestimulated for 30 min with forskolin (10 μM), with the exception of a separate data group where 1 μM CCh controls are included solely for comparison. Antagonists were applied at the concentrations indicated 10 min prior to the addition of 10 μM CCh, and the subsequent peak changes in I_{sc} in each case are represented by open (initial 30 s decrease) and shaded bars (2 min peak). Responses were compared either with control increases in I_{sc} after 1 μM CCh (atropine, 4-DAMP, pF-HHSiD (10 μM) and pirenzepine), or with control decreases in I_{sc} after 10 μM CCh (gallamine, 1 μM pF-HHSiD). Significant differences are highlighted by asterisks (** $P < 0.01$); numbers in parenthesis denote n values.

been shown to specifically target cyclic AMP-gated I_{K_s} channels formed by the KvLQT1 and I_{sk} subunits (Busch & Suessbrich, 1997; Loussouarn *et al.*, 1997). The presence of Ba^{2+} (1–10 mM) in the basolateral reservoir also selectively attenuated cyclic AMP-induced Cl^- secretion, as previously observed in T84 monolayers (Dharmasathaphorn & Pandolf, 1986) and HT-29 cl. 16E cells (Merlin *et al.*, 1995), and consistent with the Ba^{2+} sensitivity of the I_{K_s} current (Busch & Suessbrich, 1997). Although Ba^{2+} has also been reported to inhibit certain Ca^{2+} -gated K^+ channels (Bleich *et al.*, 1996), the complementary actions of both 293B and Ba^{2+} provide strong evidence that Colony 1 cells express at least two different basolateral K^+ conductances (Figure 11), of which one is unaffected by either agent and sufficient to enable Ca^{2+} -mediated Cl^- secretion.

Effects of charybdotoxin on CCh responses

Charybdotoxin, a potent inhibitor of epithelial Ca^{2+} -activated K^+ channels identified in basolateral membranes of T84 cells (Ba^{2+} -insensitive; Devor & Frizzell, 1993) and colonic crypts (Ba^{2+} -sensitive; Bleich *et al.*, 1996), did not affect the cyclic AMP-mediated increases in I_{SC} after addition of forskolin. However striking differences in the time-profile of the CCh responses were observed, in charybdotoxin pretreated cells bathed in either KH or HCO_3^- free buffer. These consisted of a larger and more prolonged initial reduction in I_{SC} compared to control responses, and the apparent absence of the 30 s spike component. The transient increase in I_{SC} , with a maximum at 2–3 min after agonist addition, was slightly smaller than control responses but not significantly so. The changes in I_{SC} therefore could reflect an initial net electrogenic cationic secretion followed by net anionic secretion. It is clear that the initial decreases in I_{SC} are not dependent on the

presence of HCO_3^- anions. Although the responses are less readily observed in control cells bathed in HCO_3^- free buffer, they are augmented in an identical manner when cells are exposed to basolateral charybdotoxin. In addition, all the components associated with the CCh response in otherwise unstimulated Colony 1 cells are critically dependent on the presence of extracellular Cl^- ions, complete inhibition is achieved with Cl^- replacement by gluconate. This experimental evidence leads to a model for secretion in Colony 1 cells in which an increase in the intracellular Ca^{2+} ion concentration leads to the activation of both basolateral and apical K^+ conductances (Figure 11). The basolateral Ca^{2+} -gated K^+ channels, responsible for the 30 s spike component of the CCh response, are inhibited by charybdotoxin. However the opening of apical K^+ channels results in electrogenic K^+ secretion, corresponding to the initial decrease in I_{SC} . This process relies on the entry of K^+ through the basolateral cotransporter and is therefore dependent on extracellular Cl^- . Crucially, the hyperpolarization afforded by the activation of an apical K^+ conductance is sufficient to drive electrogenic Cl^- secretion, and because of the stoichiometry of the $Na^+/K^+/2Cl^-$ cotransporter, a net anionic secretion is predicted overall (observed in charybdotoxin-treated cells as the slower transient rise in I_{SC}). The presence of apical K^+ channels may also partially account for the residual increase in I_{SC} observed after forskolin in 293B or Ba^{2+} treated cells, since subsequent piretanide responses were consistently biphasic, a transient elevation in I_{SC} preceding an overall decrease. These effects of piretanide (not seen in control or charybdotoxin-inhibited epithelia) may represent a reduction in K^+ transport before the reduction in Cl^- secretion.

Effects of apical Ba^{2+}

The exchange of drugs that may occur between apical and basolateral bathing solutions presents potential problems for further characterization of the apical K^+ conductance in Colony 1 epithelial layers. However addition of both apical and basolateral charybdotoxin did not significantly change the time-profile or size of CCh responses from those observed after basolateral application alone, thus suggesting that the apical K^+ channels are charybdotoxin-insensitive (Figure 11). In contrast to the insensitivity of Colony 1 CCh responses to basolateral Ba^{2+} , apical addition of $BaCl_2$ (5 mM, HCO_3^- free) did not alter the size of the 30 s spike but it did significantly reduce the subsequent increase in I_{SC} . Although this reduced response might partially result from an elevated apical Cl^- concentration, apical Ba^{2+} also significantly inhibited the initial decrease in I_{SC} induced by CCh when this was revealed by charybdotoxin pretreatment. It might be expected that inhibition of an apical K^+ conductance would lead to an increase in the size of the I_{SC} responses through a reduction in K^+ secretion, but this change is dependent on the time-course over which such secretion occurs. In addition, apical K^+ channels might make a significant contribution to the overall driving force for Cl^- transport, depending on their density. These findings suggest that apical K^+ channels in Colony 1 cells resemble those recently characterized in the rat distal colon (Butterfield *et al.*, 1997), which were inhibited by Ba^{2+} ions and were sensitive to the Ca^{2+} ion concentration in the 'cytosolic' patch solution. The selective actions of charybdotoxin and Ba^{2+} distinguish apical and basolateral K^+ channels from each other and suggest that this distribution may be physiologically relevant. The existence of distinct conductances precludes the possibility that basolateral channels are aberrantly targeted to the apical

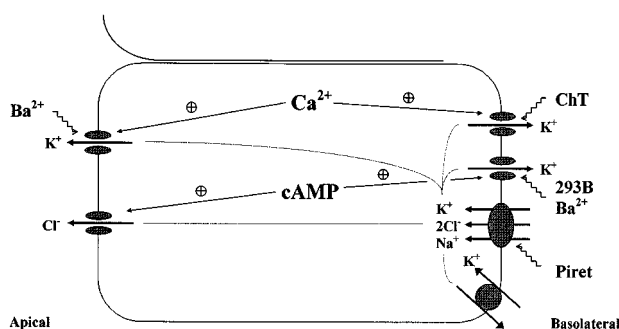


Figure 11 Proposed model for epithelial K^+ and Cl^- secretion in Colony 1 epithelia. The diagram indicates the apical and basolateral membrane conductances that are at present sufficient to explain the observed changes in I_{SC} after either carbachol or forskolin in HCO_3^- free buffer, and the effects on these responses of the range of K^+ channel blockers tested. Elevation of cyclic AMP levels activates basolateral Ba^{2+} - and 293B-sensitive K^+ channels, creating a favourable electrochemical gradient for Cl^- exit through an apical cyclic AMP-gated Cl^- conductance. Na^+ ions, which enter via the $Na^+/K^+/2Cl^-$ cotransporter inhibited by piretanide (Piret), recycle across the basolateral membrane through the Na^+/K^+ ATPase. Ca^{2+} -mediated agonists increase electrogenic Cl^- secretion by opening a separate basolateral K^+ conductance inhibited by charybdotoxin (ChT), but also activate a population of apical K^+ channels that may at least be partially sensitive to Ba^{2+} , allowing electrogenic K^+ secretion. The existence of these apical channels underlies the distinctive biphasic responses to carbachol that are observed after basolateral blockade by charybdotoxin. In Krebs solution, increases in both the cyclic AMP and Ca^{2+} ion concentration may also modulate bicarbonate transport (not shown), as described in the text.

membrane as a consequence of the Colony 1 carcinoma phenotype.

Apical Cl^- permeability as a determinant of Ca^{2+} - and cyclic AMP-mediated secretion

The lack of potentiation observed between CCh and forskolin does not result from the absence of separate basolateral K^+ conductances activated by Ca^{2+} and cyclic AMP and since a net anionic secretion is predicted even in the presence of apical K^+ transport processes, activation of apical K^+ channels can also not be a factor. Indirect elevation of cyclic AMP levels by CCh through eicosanoid formation (as occurs in the HCA-7 Colony 3 and Colony 29 subpopulations; MacVinish *et al.*, 1993) does not occur in Colony 1 cells, since control CCh responses are not inhibited by either 293B or Ba^{2+} . Crosstalk at the intracellular level between the cyclic AMP and Ca^{2+} messenger systems might influence the resulting secretory response, and indeed attenuation of Ca^{2+} influx by elevated cyclic AMP levels has been observed in HT-29 cells (Fischer *et al.*, 1996). However this interaction between cyclic AMP and Ca^{2+} ions is apparently outweighed in this cell line by their cooperative activation of membrane conductances. We therefore favour a third mechanism which is consistent with our current data and requires that under forskolin-stimulated conditions the rate of Cl^- secretion is determined by the permeability of the apical membrane to Cl^- , rather than K^+ transport processes. Under these conditions additional activation of Ca^{2+} -gated K^+ channels by CCh would have little or no effect on anion secretion and therefore on the changes in I_{SC} attributable to Cl^- movement. This assumption implies that Ca^{2+} -activated Cl^- channels are not selectively targeted to the apical membrane in Colony 1 cells, so that the predominant effect of increased intracellular Ca^{2+} is on K^+ transport and not apical Cl^- permeability. Consistent with this view is the observation, in HCO_3^- free buffer, that the slower transient rise in I_{SC} induced by CCh is significantly reduced in size if Colony 1 cells are first prestimulated with 10 μM forskolin. Studies on HT-29 cells also suggest that polarization of HT-29 epithelia does not restrict the distribution of Ca^{2+} -gated Cl^- channels (Morris *et al.*, 1994), and modulation of Cl^- secretion by CCh in both T84 cells (Dharmasathaphorn & Pandol, 1986) and the rat distal colon (Strabel & Diener, 1995) arises principally through its activation of K^+ conductances. Potentiation of CCh-induced Cl^- secretion by forskolin only occurs in the presence of either Ba^{2+} or 293B, both of which reduce basolateral K^+ transport under cyclic AMP-stimulated conditions. Entry of Cl^- across the basolateral membrane therefore becomes the rate limiting step, and may be accelerated through the effects of CCh on Ca^{2+} -activated K^+ channels.

Electrogenic responses in HCO_3^- free buffer

Investigations with Ba^{2+} required the use of HCO_3^- free buffer as the bathing solution for much of the work on K^+ channel inhibitors in Colony 1 cells. However it is clear that CCh responses are qualitatively different in the absence of HCO_3^- anions after prestimulation with forskolin. Acetazolamide, an inhibitor of carbonic anhydrase, had no sustained effect on the basal I_{SC} of cells bathed in KH solution but did reduce forskolin responses by approximately 5%. Strikingly, the reductions in forskolin-stimulated I_{SC} induced by CCh were also eliminated, such that they became essentially identical to those in HCO_3^- free solution. It is possible that changes in intracellular pH (which may result from both these treatments) could partially

account for the differences observed, for example through influences on K^+ conductances (Klaerke, 1997). However the profiles of CCh responses following basolateral charybdotoxin pretreatment suggest that apical Ca^{2+} -gated K^+ channels may still be activated in HCO_3^- free buffer. Alternatively both CCh and forskolin may modulate HCO_3^- transport across Colony 1 epithelial layers, through a mechanism at least partially dependent on carbonic anhydrase. Involvement of the intracellular isoform of this enzyme in HCO_3^- secretion has been previously demonstrated in the rat colon (Feldman, 1994), in guinea-pig duodenum (Muallem *et al.*, 1994), in rat epididymal epithelia (Chan *et al.*, 1996) and in airway epithelial cells (Smith & Welsh, 1992). Surprisingly, the decreases in I_{SC} after CCh in forskolin-stimulated cells which are sensitive to acetazolamide may represent a reduction in HCO_3^- secretion (or an increase in its absorption). HCO_3^- secretion occurs in colonic epithelia in concert with Na^+ and Cl^- absorption, *via* a carbonic anhydrase dependent mechanism (for a review, see Binder & Sandle, 1994) and in this context, it is interesting to note that flux studies in porcine distal colon have shown that CCh, but not the cyclic AMP-mediated agonist VIP, inhibits Na^+ and Cl^- absorption (Traynor *et al.*, 1991). Such a mechanism, if present in Colony 1 cells would explain the reduction in HCO_3^- secretion after CCh and also its dependence on the presence of Cl^- ions, because of the requirement for apical Cl^- HCO_3^- exchange (Feldman & Stephenson, 1990). One limitation is that this transport process is predicted to be electroneutral overall, and therefore it is clear that an elucidation of the exact pathways for HCO_3^- transport in Colony 1 epithelia will require further study.

Muscarinic antagonists

4-DAMP (100 nM), an antagonist with high affinity for human M_1 and M_3 receptors (Eglen *et al.*, 1996), and the non-selective muscarinic antagonist atropine both abolished 10 μM CCh responses in Colony 1 cells prestimulated with forskolin. A higher concentration of gallamine (1 μM , M_2 selective) was without effect, while pirenzepine (1 μM , M_1 selective) altered the profile of the CCh response. The latter action reflects the change in time-course that occurs with increasing agonist concentration, and not a selective inhibition of a component of the CCh-induced changes in I_{SC} . The increased potency of 4-DAMP relative to pirenzepine is indicative of the presence of a single M_3 -like receptor in Colony 1 cells. Interestingly pF-HHSiD, a reported M_3 muscarinic antagonist, did not affect CCh responses except at high concentration (10 μM), but this apparent low affinity does not necessarily indicate an atypical muscarinic receptor subtype. Other functional studies with colonic smooth muscle have shown that pF-HHSiD has a low affinity for human M_3 receptors; in circular smooth muscle this antagonist was in fact less potent than pirenzepine (Kerr *et al.*, 1995). So far therefore there is no evidence to suggest that the receptors in Colony 1 cells differ from the M_3 subtype characterized in rat colonic epithelia (O'Malley *et al.*, 1995) and the human adenocarcinoma cell lines T84 (Dickinson *et al.*, 1992) and HT-29 (Kopp *et al.*, 1989). Activation of this single receptor population is therefore solely responsible for the complex electrogenic CCh responses recorded.

Colony 1 epithelial layers therefore possess distinct charybdotoxin-sensitive and 293B-inhibitable classes of basolateral K^+ channels which participate in secretory responses; however in contrast to other cell lines, potentiation between Ca^{2+} - and cyclic AMP-elevating agonists is limited by apical permeability to Cl^- . Apical membranes may also contain a Ba^{2+} -sensitive K^+ conductance, the activation of which

underlies many of the distinctive features of the M₃-like receptor response to CCh. Thus Colony 1 cells may prove a useful model in the study of the cellular mechanisms of potassium adaptation in epithelia (Butterfield *et al.*, 1997).

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Interaction of amylin with calcitonin gene-related peptide receptors in the microvasculature of the hamster cheek pouch *in vivo*

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1 This study used intravital microscopy to investigate the receptors stimulated by amylin which shares around 50% sequence homology with the vasodilator calcitonin gene-related peptide (CGRP) in the hamster cheek pouch microvasculature *in vivo*.

2 Receptor agonists dilated arterioles (diameters 20–40 μ m). The $-\log$ of the concentrations ($\pm$ s.e. mean; $n=8$) causing 50% increase in arteriole diameter were: human β CGRP (10.8 ± 0.3), human α CGRP (10.8 ± 0.4), rat α CGRP (10.4 ± 0.3). Rat amylin and the CGRP₂ receptor selective agonist [Cys(ACM^{2,7})-human α CGRP] were 100 fold less potent (estimates were 8.5 ± 0.4 and 8.2 ± 0.3 respectively).

3 The CGRP₁ receptor antagonist, CGRP_{8–37} (300 nmol kg^{−1}; i.v.) reversibly inhibited the increase in diameter evoked by human α CGRP (0.3 nM) from $178 \pm 22\%$ to $59 \pm 12\%$ ($n=8$; $P<0.05$) and by rat amylin (100 nM) from $138 \pm 23\%$ to $68 \pm 24\%$ ($n=6$; $P<0.05$). CGRP_{8–37} did not inhibit vasodilation evoked by substance P (10 nM; $n=4$; $P>0.05$).

4 The amylin receptor antagonist, amylin_{8–37} (300 nmol kg^{−1}; i.v.) did not significantly inhibit the increase in diameter evoked by human α CGRP (0.3 nM) which was $112 \pm 26\%$ in the absence, and $90 \pm 29\%$ in the presence of antagonist ($n=4$; $P<0.05$); nor that evoked by rat amylin (100 nM) which was $146 \pm 23\%$ in the absence and $144 \pm 32\%$ in the presence of antagonist ($n=4$; $P>0.05$).

5 The agonist profile for vasodilatation and the inhibition of this dilatation by CGRP_{8–37}, although not the amylin_{8–37} indicates that amylin causes vasodilatation through interaction with CGRP₁ receptors in the hamster cheek pouch.

Keywords: CGRP; amylin; CGRP_{8–37}; microvasculature; amylin_{8–37}; sensory nerves; blood flow; amylin antagonist; CGRP antagonist; vasodilation

Abbreviations: CGRP, calcitonin gene-related peptide; CGRP_{8–37}, human α CGRP_{8–37}, RAMPs, receptor activity modifying proteins

Introduction

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide expressed primarily in neural tissue (Poyner, 1992). Peripherally, CGRP-containing fibres are predominantly perivascular primary afferent unmyelinated C-fibres which often co-express the tachykinins substance P and neurokinin A (Holzer, 1988). Amylin (insulinoma amyloid peptide; islet amyloid polypeptide) originally discovered co-localized with insulin in the β cells of the pancreas in patients with non-insulin dependent diabetes mellitus (Castillo *et al.*, 1995) has been reported to also be expressed in sensory neurons (Mulder *et al.*, 1995).

CGRP and amylin share around 50% sequence homology and have some biological activities in common, including vasodilatation, inhibition of neuronal activity in the periphery and positive inotropic effects on the heart (Poyner, 1992; Hall & Brain, 1996). There is currently uncertainty regarding the pharmacological classification of the receptor types and subtypes stimulated by CGRP, amylin and related peptides; and as yet, convincing evidence for the cloning of *bona fide* CGRP and amylin receptors is still awaited (Hall & Smith, 1998). For CGRP, limited pharmacological studies have led to the proposal of two subtypes of receptor, CGRP₁ receptors for which the antagonist fragment human α CGRP_{8–37} (CGRP_{8–37}) has a relatively high affinity (pK_B around 7), and CGRP₂

receptors for which this fragment has a lower affinity ($pK_B < 6$) but for which the agonist [Cys(ACM^{2,7})-human α CGRP] is more selective (Dennis *et al.*, 1989; 1990). For amylin, although there have been proposals for the existence of separate amylin receptors, there is evidence that some of the actions of amylin are mediated through stimulation of CGRP receptors (Poyner, 1995). This evidence comes mainly from studies where responses to amylin have been reported to be inhibited by the CGRP₁ receptor antagonist CGRP_{8–37} (Chin *et al.*, 1994; Giuliani *et al.*, 1992; Tomlinson & Poyner, 1996) and in radioligand binding studies where specific labelled CGRP receptor binding is displaced by amylin and fragments (Veale *et al.*, 1994; Aiyar *et al.*, 1995). This receptor cross-talk is not limited to amylin and CGRP, but also to the structurally-related peptide adrenomedullin (Muff *et al.*, 1995), and is considered to be a consequence of the sequence homology between the peptides which resides in a six-residue ring structure formed by an intramolecular disulphide linkage, an area considered important for receptor interaction, and also in the C-terminal sequence (Hall & Brain, 1996; Poyner, 1992; 1995).

Our preliminary experiments showed that in the hamster cheek pouch microvasculature, CGRP and amylin cause vasodilatation (Hall and Brain, 1993). The aim of the present study was to examine the effects of CGRP and amylin in the microvasculature by intravital microscopy of the hamster cheek pouch *in vivo*, and to determine the nature of the

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receptors activated by these peptides in the microcirculation. We have approached this by studying the effects of receptor agonists and peptide antagonists for CGRP and amylin receptors including CGRP₈₋₃₇ (Dennis *et al.*, 1990) and amylin₈₋₃₇ (Deems *et al.*, 1991).

Preliminary accounts of this work has been presented to the European Neuropeptide Club Meeting, Cambridge, U.K. (Hall & Brain, 1993) and to CGRP '98. New Horizons in CGRP and Related Peptides Research, Shaftesbury, U.K.

Methods

Male golden (Syrian) hamsters (80–120 g) were anaesthetized with sodium pentobarbitone (Sagatal 60 mg kg⁻¹, i.p.) and anaesthesia was maintained with 15 mg kg⁻¹ pentobarbitone as required. A single layer of vascular membrane was prepared as described by Duling (1973) with some modifications (Hall & Brain, 1994). Briefly, the hamster was placed on a specially designed stage with a central depressed well, the right cheek was carefully everted and placed in the well and pinned on a silicon-rubber ring encircling the window. A single vascular layer was dissected out keeping an intact blood supply, and all connective tissue was removed. The left jugular vein was cannulated (Portex 18 gauge) for i.v. administration of antagonists. The tissue was superfused with Krebs-bicarbonate solution (composition mM: NaCl 120; KCl 4.7; MgCl₂ 0.12, CaCl₂ 0.18, KH₂PO₄ 0.4, NaHCO₃ 23, glucose 10) at a rate of 4 ml min⁻¹, and the Krebs solution, warmed to 35°C, was gassed with 5% CO₂ in air maintained at pH 7.4. An area of microvasculature was selected which allowed an arteriole and adjacent venule (each between 20 and 40 µm in diameter) to be viewed concomitantly; in some experiments two arterioles were monitored in the same cheek pouch. The microvessels were viewed with a Leitz Dialux or a Zeiss Axioscop microscope having a 20 or 25× salt-water dipping objective with ×10 eye pieces. Diameters of microvessels were measured with an imaging system (Kompira Limited, Strathclyde, U.K.), or manually with a ruler.

In all experiments, arterioles were pre-constricted (by *ca.* 50%) with human endothelin-1 (usually 30–300 pM) superfused 20 min before, and throughout, the experiment. Agonists were dissolved in Krebs solution and applied topically by superfusion. Receptor antagonists (300 nmol kg⁻¹, i.v.), dissolved in 0.5 ml saline: bovine serum albumin (BSA; 0.1%), was applied i.v. over 1 min, followed by i.v. 0.5 ml saline:BSA over 2 min, 5 min before administration of agonist. Following application of test agent, vessel diameter was measured at 15 s, 30 s, 45 s, 1 min, and every minute up to 10 min, then at 5 min intervals thereafter, until the dilator response had fully reversed and pre-dilator vessel diameter was recovered. Responses to sodium nitroprusside (100 nM) were obtained at the end of every experiment to confirm tissue viability.

Agonist studies

Up to five agonists (human αCGRP, human βCGRP, rat αCGRP, [Cys(ACM^{2,7})-human αCGRP, rat amylin) were applied to each cheek pouch using a cumulative dosing protocol with a random order of peptides between hamsters, and at least 20 min between subsequent agonists. With a cumulative dosing protocol, maximal dilator responses to all peptides developed within 3 min of drug application, so each concentration of peptide was applied for 3 min. In a separate series of experiments investigating the reproducibility of

agonist responses, full cumulative concentration-response curves were obtained every 20 min for human CGRPα for 2.5 h (the duration of the longest experiment).

Antagonist studies

Concentrations of human αCGRP and rat amylin amide were chosen that produced a submaximal vasodilatation of around 100% increase in arteriolar diameter. Responses were obtained to human αCGRP (0.3 nM), rat amylin (100 nM), repeated 20 min later after infusion of human CGRP₈₋₃₇ or human amylin₈₋₃₇ (300 nmol kg⁻¹; i.v.) 5 min before agonist. CGRP₈₋₃₇ was tested in the same way against submaximal responses to substance P (10 nM); saline; 0.1% BSA was tested in the same way against submaximal responses to human αCGRP (0.1 nM), and in some experiments, rat amylin₈₋₃₇ was tested in the same way against responses to amylin (100 nM). Recovery of responses was monitored by obtaining responses 20 and 40 min after antagonist administration. One agonist and one antagonist combination only was tested in each cheek pouch preparation.

Source of drugs

Drugs were obtained as follows: pentobarbitone sodium (Sagatal; Rhône Mérieux Ltd, Essex, U.K.) substance P, endothelin-1, human αCGRP, human βCGRP, rat αCGRP, human αCGRP₈₋₃₇, rat amylin, rat amylin₈₋₃₇, human amylin₈₋₃₇, [Cys(ACM^{2,7})-human αCGRP (Bachem, Essex, U.K. or Peninsula Labs, Merseyside, U.K.). All peptides were dissolved in distilled water to 1 mM stock solutions and stored at -20°C, peptide dilutions and other drugs were made up in Krebs' solution. Antagonists were made up in saline:0.1% BSA. All salts were of analytical grade and were obtained from B.D.H., U.K.

Expression of results and statistical analysis

Arteriolar vasodilatation was calculated as per cent of maximal increase in arteriole diameter (compared to pre-constricted diameter). Data from individual experiments were combined, and results are expressed as mean ± s.e.mean. The value *n* refers to number of arterioles in experiments carried out in at least four animals for each study. Estimates of the concentration causing 50% increase in vessel diameter were calculated as log₁₀ means with s.e.means, and were obtained from individual log concentration-response curves. For comparison of the time course for dilator responses, the control dilator responses following single (non-cumulative) concentrations of rat amylin (100 nM) and human αCGRP (0.3 nM) were analysed. Thus, the time to reach 50% and maximal dilation, and 50% and reversal of dilation were averaged for each agonist. Tests for significant differences were made using Students paired *t*-tests.

Results

Agonist studies

The various forms of CGRP, [Cys(ACM^{2,7})-human αCGRP and rat amylin evoked arteriolar vasodilatation (Figure 1), but were without significant effect on venule diameter (data not shown). The -log of the concentrations (± s.e.mean; *n* = 8) causing 50% increase in arteriole diameter were: human βCGRP (10.8 ± 0.3), human αCGRP (10.8 ± 0.4), rat αCGRP

(10.4 ± 0.3). Rat amylin and [Cys(ACM^{2,7})-human α CGRP also caused vasodilation but were 100 fold less potent ($-\log$ concentrations causing 50% increase in arteriole diameter were 8.5 ± 0.4 and 8.2 ± 0.3 respectively; $n=8$), and the lower maximal responses indicates that the two agonists are indeed partial agonists at CGRP receptors in this model. The time-course for dilatation was similar for all agonists studied, responses reached a maximum within 3–4 min and reversed after 10–20 min. Data are shown in Table 1 for human α CGRP and rat amylin. There was no significant change in the log-concentration-response curve to human α CGRP with time ($n=4$; data not shown).

Antagonist studies

The CGRP₁ receptor antagonist, CGRP₈₋₃₇ (300 nmol kg^{-1} , i.v.), in its own right produced a small constriction in arteriolar tone, which was maximal (at $11 \pm 2\%$) 1.75 min after i.v. injection, but which had essentially reversed after 5 min. CGRP₈₋₃₇ (300 nmol kg^{-1} , i.v.) reversibly inhibited vasodilator responses evoked by human α CGRP (0.3 nM) from $178 \pm 22\%$ to $59 \pm 12\%$ increase in arteriole diameter ($n=8$; $P<0.05$; Figure 2a). Vasodilator responses to rat amylin (100 nM) were also reversibly inhibited by CGRP₈₋₃₇ (300 nmol kg^{-1} , i.v.) from 138 ± 23 – $68 \pm 24\%$ increase in arteriole diameter ($n=6$; $P<0.05$; Figure 2b). Submaximal vasodilator responses to substance P (10 nM) were unaffected by CGRP₈₋₃₇ (300 nmol kg^{-1} , i.v. dilation was $63 \pm 14\%$ in the absence and $67 \pm 11\%$ in the presence of antagonist; $n=6$; data not shown). In contrast, the amylin receptor antagonist

human amylin₈₋₃₇ (300 nmol kg^{-1} , i.v.) had no effect on arteriole diameter in its own right, and did not inhibit vasodilator responses to human α CGRP (0.3 nM ; dilation was $112 \pm 26\%$ in the absence and $90 \pm 29\%$ in the presence of antagonist; $n=6$; Figure 2c) or to amylin (100 nM) (dilation was $146 \pm 23\%$ in the absence and $144 \pm 32\%$ in the presence of antagonist $n=7$; $P>0.05$; Figure 2d), and rat amylin₈₋₃₇ (300 nmol kg^{-1} , i.v.) did not inhibit vasodilator responses to amylin (100 nM) (dilation was $131 \pm 27\%$ in the absence and $121 \pm 29\%$ in the presence of antagonist $n=8$; $P>0.05$; data not shown). The antagonist vehicle (BSA : saline) had no effect on responses to human α CGRP (0.1 nM ; dilation was $98 \pm 23\%$ in absence and $91 \pm 21\%$ in presence of vehicle $n=4$; $P>0.05$; data not shown).

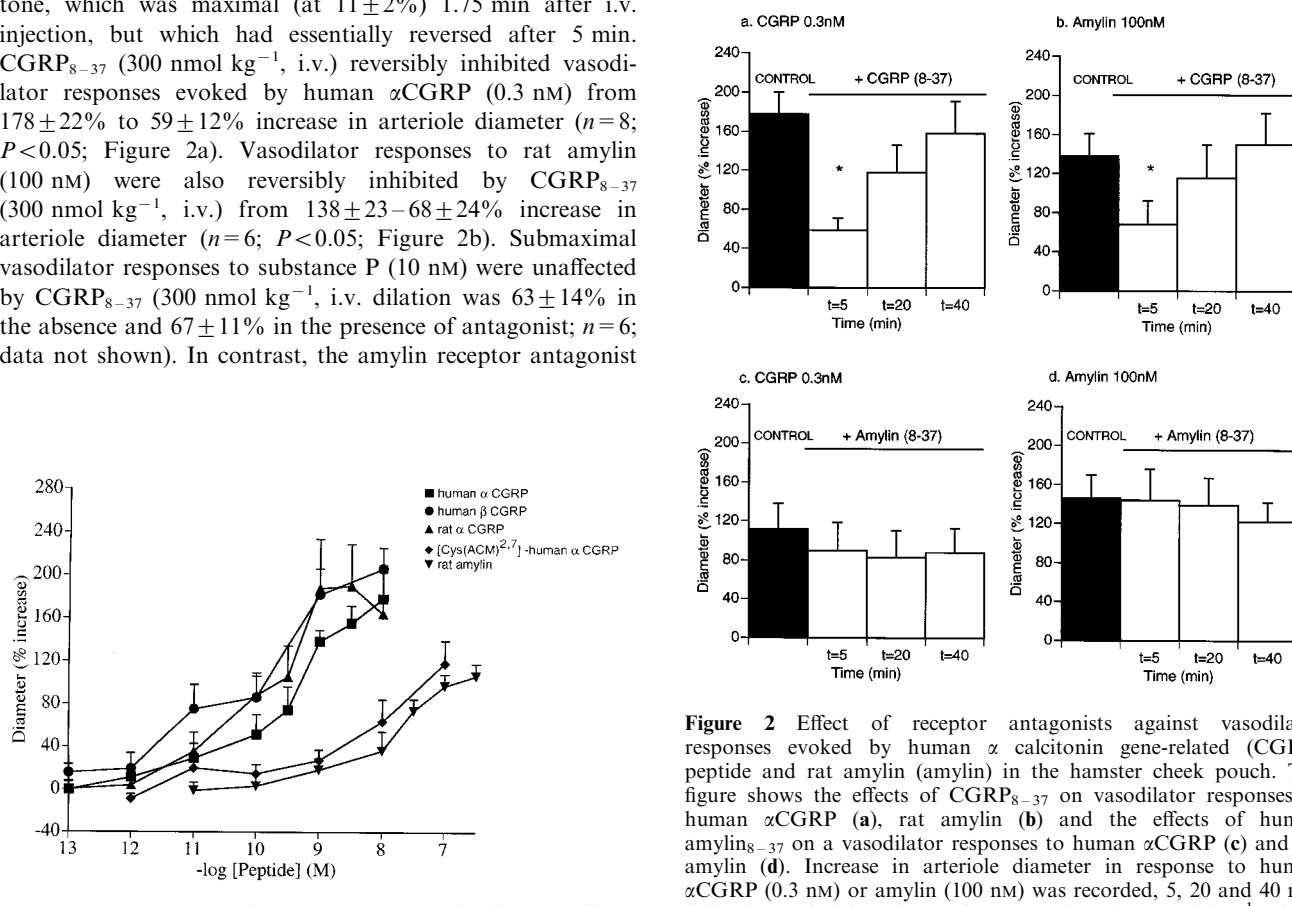


Figure 1 Log concentration-response curves showing vasodilator responses for calcitonin gene-related peptide and analogues and rat amylin on arterioles in the hamster cheek pouch microvasculature. Agonists were applied by superfusion at each concentration for 3 min and responses shown are peak responses measured at that time for each concentration. Responses are shown as mean $\pm$ s.e. mean of eight arterioles for experiments carried out in seven animals.

Figure 2 Effect of receptor antagonists against vasodilator responses evoked by human α calcitonin gene-related (CGRP) peptide and rat amylin (amylin) in the hamster cheek pouch. The figure shows the effects of CGRP₈₋₃₇ on vasodilator responses to human α CGRP (a), rat amylin (b) and the effects of human amylin₈₋₃₇ on a vasodilator responses to human α CGRP (c) and rat amylin (d). Increase in arteriole diameter in response to human α CGRP (0.3 nM) or amylin (100 nM) was recorded, 5, 20 and 40 min following administration of antagonist (300 nmol kg^{-1} , i.v.). Responses are shown as the mean $\pm$ s.e. mean of 6–11 vessels monitored in 4–7 hamsters. * $P<0.05$ vs control. Figure 2a is reprinted with permission from Pharmacology of Calcitonin Gene-Related Peptide, in *Neurogenic Inflammation*, Chapter 9, 101–114, Geppetti, P & Holzer, P (eds), Copyright CRC Press, Boca, Raton Florida 1996.

Table 1 Comparison of time course of vasodilator responses to CGRP and amylin in hamster cheek pouch

Agonist	Time (min) 50% dilation	Time (min) 100% dilation	Time (min) 50% reversal	Time (min) 100% reversal
CGRP	1.6 ± 0.25	3.3 ± 0.47	10.8 ± 1.4	14.6 ± 2.1
Amylin	2.0 ± 0.23	3.9 ± 0.39	7.4 ± 0.5	13.1 ± 1.7

The table shows the mean time (min $\pm$ s.e. mean) for arteriolar dilator responses to human α calcitonin gene-related peptide (CGRP; 0.3 nM) and rat amylin (amylin; 100 nM) to reach 50% and 100% maximum dilation and subsequently times to reverse by 50% and 100%. Responses were measured during, and after, 3 min superfusion of CGRP or amylin to the hamster cheek pouch, and values shown are for 7–9 arterioles.

Discussion

This study demonstrates that various forms of CGRP and rat amylin are potent arteriolar vasodilators in the microvasculature of the hamster cheek pouch. Human α CGRP and rat amylin mediate their vasodilator effects through stimulation of receptors sensitive to inhibition by the CGRP receptor antagonist CGRP₈₋₃₇ but not by the amylin receptor antagonist amylin₈₋₃₇. The agonist profile of CGRP and analogues and the inhibition of vasodilator responses to both CGRP and amylin by CGRP₈₋₃₇ suggest that the receptors involved are of the CGRP₁ type.

The time-course of the arteriolar dilatation was similar for all agonists tested with dilatation reaching a maximum at 3–4 min and reversing after 10–20 min. The rate of onset and offset of responses of the two peptides is relatively slow when compared to certain other vasodilators such as substance P (Hall & Brain, 1994), though similar to the structurally similar peptide adrenomedullin₁₃₋₅₂ (Hall *et al.*, 1995). CGRP is well known to produce long-lasting microvascular vasodilatation, characterized by a pronounced and well maintained erythema in human skin and a maintained increase in blood flow in rabbit skin (Brain *et al.*, 1985), and prolonged decreases in perfusion pressure in isolated preparations such as the rat mesentery (Claing *et al.*, 1992) and rat kidney (Chin *et al.*, 1994). The similarity in time-course of vasodilator responses to CGRP and analogues and amylin, as well as adrenomedullin, suggests a similar mechanism of vasodilatation. The lower maximal dilator responses we found with rat amylin and with [Cys(ACM^{2,7})-human α CGRP indicates that these two agonists are partial agonists at the CGRP₁ receptor in this model.

In support of the proposal that CGRP and amylin evoke vasodilatation *via* a common mechanism, the CGRP receptor antagonist CGRP₈₋₃₇ significantly inhibited the vascular effects of both rat amylin and human α CGRP. These results with the CGRP receptor antagonist CGRP₈₋₃₇ are consistent with an interaction of both CGRP and amylin with CGRP₁ receptors since this antagonist is selective for the CGRP₁ over the CGRP₂ receptor subtype (Dennis *et al.*, 1989). The dose of CGRP₈₋₃₇ we used to show inhibition of vasodilatation, (300 nmol kg⁻¹, i.v.) in the hamster cheek pouch, is comparable with that used previously to exhibit a selective inhibition of CGRP and adrenomedullin₁₃₋₅₂-induced vasodilator responses in the hamster cheek pouch (Hall *et al.*, 1995) and rat skin (Escott & Brain, 1993; Hall *et al.*, 1995); and in the present experiments, CGRP₈₋₃₇ did not inhibit submaximal vasodilator responses to substance P, indicating a selective block of CGRP receptors. The observation that the CGRP₂ receptor agonist [Cys(ACM^{2,7})-human α CGRP was 100 fold less active than the various forms of CGRP further supports the proposal that CGRP₁, rather than CGRP₂ are involved. Further support for an interaction of amylin with CGRP₁ receptors was the observation that amylin was 100 fold less potent than CGRP (Baskaya *et al.*, 1995) and that at a similar dose (300 nmol kg⁻¹, i.v.), both the human and rat forms of

the amylin receptor antagonist amylin₈₋₃₇, did not significantly inhibit vasodilator responses to amylin. We used the same dose of both antagonists since, in systems where there is evidence for antagonism by amylin₈₋₃₇, amylin₈₋₃₇ and CGRP₈₋₃₇ are equiactive. Thus, in the rat skeletal muscle amylin and CGRP are approximately equiactive in stimulating a decrease in glycogen metabolism and here amylin₈₋₃₇ (human or rat acetylated form) and CGRP₈₋₃₇ are equiactive at inhibiting the response (Deems *et al.*, 1991). Further, in porcine nucleus accumbens where radiolabelled amylin and CGRP have a similar binding affinity (low nM) and amylin₈₋₃₇ and CGRP₈₋₃₇ have a similar potency at inhibiting ¹²⁵I-CGRP binding (Aiyar *et al.*, 1995). In a recent study, Wisskirchen and colleagues (1998) investigated the response of CGRP and amylin in the rat microvasculature (the rat pulmonary artery) *in vitro*. In this tissue, rat amylin was 50 fold less potent than CGRP while [Cys(ACM^{2,7})-human α CGRP (100 nM–0.1 mM) was at least 300 fold less potent. These authors drew a similar conclusion to us that a CGRP₁ receptor is present on the microvasculature. They did not investigate the activity of amylin₈₋₃₇, or the effects of CGRP₈₋₃₇ on amylin responses, but did demonstrate that CGRP₈₋₃₇ inhibited responses to CGRP. They also provided evidence that peptidase inhibitors did not affect responses to the peptides. Sheykhzade and co-workers (Sheykhzade & Berg Nyborg, 1998) reached a similar conclusion after study of the rat intramural coronary artery, but again these authors did not investigate the activity of amylin₈₋₃₇.

Taken overall, these data indicate an interaction of amylin and CGRP with CGRP₁ receptors in the microvasculature of the hamster cheek pouch. These present data are consistent with the proposal that the metabolic effects of amylin appear to be mediated through interaction with amylin receptors (Deems *et al.*, 1991; Wang *et al.*, 1993), but the cardiovascular effects of amylin are mediated *via* an interaction with CGRP₁ receptors (Beaumont *et al.*, 1995; Vine *et al.*, 1996). This could be of importance in the microvasculature in inflammatory disease. Amylin has been shown to be upregulated in the dorsal root ganglion following adjuvant-induced inflammation in the rat paw (Mulder *et al.*, 1997). In the absence of non-peptide receptor antagonists, determination of the residues within the sequence of CGRP, amylin and adrenomedullin that are important for agonist activity at CGRP₁ receptors has relevance to the design of receptor antagonists for analytical and therapeutic purposes. The design of selective discriminative ligands for receptors for CGRP, amylin and adrenomedullin is especially important in the light of the recent cloning of accessory proteins known as RAMPs (Receptor Activity Modifying Proteins) which have the ability to change the recognition properties of receptors that recognize CGRP and related peptides (McLatchie *et al.*, 1998).

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Selective effects of a 4-oxystilbene derivative on wild and mutant neuronal chick $\alpha 7$ nicotinic receptor

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1 We assessed the pharmacological activity of triethyl-(β -4-stilbenoxy-ethyl) ammonium (MG624), a drug that is active on neuronal nicotinic receptors (nicotinic AChR). Experiments on the major nicotinic AChR subtypes present in chick brain, showed that it inhibits the binding of [¹²⁵I]- α Bungarotoxin (α Bgtx) to the $\alpha 7$ subtype, and that of [³H]-epibatidine (Epi) to the $\alpha 4\beta 2$ subtype, with K_i values of respectively 106 nM and 84 μ M.

2 MG624 also inhibited ACh elicited currents (I_{ACh}) in the oocyte-expressed $\alpha 7$ and $\alpha 4\beta 2$ chick subtypes with half-inhibitory concentrations (IC_{50}) of respectively 109 nM and 3.2 μ M.

3 When tested on muscle-type AChR, it inhibited [¹²⁵I]- α Bgtx binding with a K_i of 32 μ M and ACh elicited currents (I_{ACh}) in the oocyte-expressed $\alpha 1\beta 1\gamma\delta$ chick subtype with an IC_{50} of 2.9 μ M.

4 The interaction of MG624 with the $\alpha 7$ subtype was investigated using an $\alpha 7$ homomeric mutant receptor with a threonine-for-leucine 247 substitution (L247T $\alpha 7$). MG624 did not induce any current in oocytes expressing the wild type $\alpha 7$ receptor, but did induce large currents in the oocyte-expressed L247T $\alpha 7$ receptor. The MG624 elicited current (I_{MG624}) has an EC_{50} of 0.2 nM and a Hill coefficient nH of 1.9, and is blocked by the nicotinic receptor antagonist methyllycaconitine (MLA).

5 These binding and electrophysiological studies show that MG624 is a potent antagonist of neuronal chick $\alpha 7$ nicotinic AChR, and becomes a competitive agonist following the mutation of the highly conserved leucine residue 247 located in the M2 channel domain.

Keywords: MG624; neuronal nicotinic receptors; muscle nicotinic receptors; subtypes; α Bungarotoxin; epibatidine; methyllycaconitine; oocytes; chick

Abbreviations: ACh, Acetylcholine; Abs, Antibodies; α Bgtx, α Bungarotoxin; Epi, epibatidine; Carb, carbamylcholine; KD, kilodalton; K_d , dissociation constant; MLA, methyllycaconitine; mAbs, monoclonal antibodies; d-Tub, d-Tubocurarine

Introduction

Molecular biology studies have revealed the presence of several nicotinic AChR subtypes in vertebrate brain and ganglia, each of which has a distinct pattern of expression (Sargent, 1993; Role & Berg, 1996).

These subtypes are believed to be pentameric ion channels consisting of distinct homologous subunits that are coded by 11 genes ($\alpha 2$ – $\alpha 9$ and $\beta 2$ – $\beta 4$). Expression studies have demonstrated that these subtypes are both pharmacologically and functionally distinguishable, and can be formed homomERICALLY from the $\alpha 7$, $\alpha 8$ and $\alpha 9$ subunits, or as heteromeric complexes containing one or more α subunits (different from those forming the homomeric receptors) and one or more β subunits. The diversity and complexity of the nAChR present in vertebrate brain and ganglia has also been confirmed using biochemical and immunological techniques. An α Bgtx receptor containing both the $\alpha 7$ and $\alpha 8$ subunits is present (Gotti *et al.*, 1994) together with nAChR subtypes containing three or four different nAChR subunits, such as $\alpha 3\alpha 4\beta 2$, $\alpha 4\alpha 5\beta 2$ and $\alpha 3\alpha 5\beta 2\beta 4$, (Conroy *et al.*, 1992; Vernallis *et al.*, 1993; Conroy & Berg, 1995). An $\alpha 4\beta 2\beta 3\beta 4$ subtype has been found in rat cerebellum (Forsayeth & Kobrin, 1997).

Pharmacological and functional studies have shown that the major nicotinic AChR subtypes present in vertebrate brain are the $\alpha 4\beta 2$ and $\alpha 7$ subtypes (Albuquerque *et al.*, 1997; Gotti *et al.*, 1997a; McGehee & Role, 1995). The $\alpha 4\beta 2$ subtype

represents more than 80% of the high affinity agonist binding sites, and is known to be located at least presynaptically where it can modulate neurotransmitter release (Wonnacott, 1997). The $\alpha 7$ subtype, which is as abundant as the $\alpha 4\beta 2$ subtype, binds α Bgtx with high affinity and contains the $\alpha 7$ subunit. When expressed in oocytes, the $\alpha 7$ subunit forms functional homomeric receptor-channels with a low affinity for ACh, desensitizes very rapidly, and has a non linear current-voltage relationship (Couturier *et al.*, 1990; McGehee & Role, 1995). This homomeric $\alpha 7$ subtype is not only highly permeable to Ca^{2+} , but Ca^{2+} binding to sites located on the extracellular part of the $\alpha 7$ receptor can potentiate its responses to the natural ACh neurotransmitter (Galzi *et al.*, 1996).

A mutated form of the $\alpha 7$ subunit has been engineered (L247T $\alpha 7$), in which the substitution of the conserved leucine 247 for threonine in the M2 channel domain greatly changes the functional and pharmacological properties of $\alpha 7$ receptors leading to a higher affinity for ACh, a reduced rate of desensitization, and no current rectification. Furthermore a number of competitive antagonists of the $\alpha 7$ wild type become agonists of the L247T $\alpha 7$ receptor. These alterations in the functional and pharmacological properties of the mutated receptor make it a good model for studying the structure and function of the $\alpha 7$ subtype and its drug interactions (Revah *et al.*, 1991; Bertrand *et al.*, 1993; 1997).

As the pharmacological properties and physiological functions of nicotinic AChR subtypes are still largely unknown, considerable efforts have been made to investigate their

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structure in order to provide information that can be used to design more selective compounds. Much of the recent increase in nicotinic ligand research is due to the growing evidence that nicotinic AChRs are involved in brain function and play a central role in the physiopathology of a number of disorders (Lèna & Changeux, 1997; Dani & Heinemann, 1996; Lindstrom, 1997; Kuryatov *et al.*, 1997; Gotti *et al.*, 1997a).

It has long been known that 4-oxystilbene derivatives have ganglioplegic activity and are able to antagonise nicotine-induced tremors in rabbits (Mantegazza & Tommasini, 1955). We therefore decided to test the nicotinic AChR selectivity of these compounds by means of binding and electrophysiological studies on the two subtypes most expressed in vertebrate brain: the $\alpha 7$ and $\alpha 4\beta 2$ subtypes. We chose triethyl-(β -4-stilbenoxy-ethyl) ammonium (MG624) (Figure 1) because it retains the pharmacological activity of the 4-oxystilbene compounds (Mantegazza & Tommasini, 1955) and it is the most potent 4-oxystilbene derivative in inhibiting the binding of [^{125} I]- α Bgtx to a heterogeneous receptor population of $\alpha 7$ -containing receptors and the least potent in inhibiting the binding of [^{3} H]-Epi to the $\beta 4$ and $\beta 2$ -containing receptors (Gotti *et al.*, 1998).

The aim of this study was to gain more information on the nicotinic specificity of this compound, by investigating its binding to the individual neuronal subtypes, and comparing the results with those obtained in the same subtypes expressed in oocytes. In order to clarify its mechanism of action, we also investigated its effects on the mutated L247T $\alpha 7$ receptor.

Methods

Antibody production and characterization

The peptides and antibodies (Abs) were produced as previously described (Gotti *et al.*, 1994). Anti- $\alpha 7$ and anti- $\beta 2$ chick-specific antibodies (Abs) were produced in rabbits by means of immunization with peptides chosen from the most divergent region of the $\alpha 7$ and $\beta 2$ subunits (the cytoplasmic loop between M3 and M4), and also against a peptide located at the COOH terminal of the $\beta 2$ subunit.

The anti-muscle type Ab was mAb73, a mAb that is specific for the $\beta 1$ subunit (Tzartos *et al.*, 1986), which was prepared as previously described (Gotti *et al.*, 1997b).

The specificity of anti- $\alpha 7$ Abs has been previously reported by Gotti *et al.* (1994; 1997c). The specificity of the anti- $\beta 2$ Abs, those directed against the cytoplasmic peptide, as well as those directed against the COOH peptide, was measured in immunoprecipitation experiments using receptors labelled with the nicotinic agonist [^{3} H]-Epi (Gerzanich *et al.*, 1995). Both of our Abs were capable of immunoprecipitating $95 \pm 2\%$ of the [^{3} H]-Epi high affinity labelled receptors, an amount that was almost identical to that immunoprecipitated by mAb 270, which specifically recognizes the $\beta 2$ subunit (Whiting *et al.*,

1987) and, in our hands, immunoprecipitated 90% of the [^{3} H]-Epi high affinity sites.

mAb 270, which was raised against chicken brain nicotinic AChR and directed against the $\beta 2$ subunit (Whiting *et al.*, 1987), and mAb35, which was raised against muscle-type AChR, recognizes the $\alpha 1$ subunit and cross-reacts with the $\alpha 5$ subunit (Conroy *et al.*, 1992), was purified from a hybridoma cell line obtained from the American Type Culture Collection. mAb 299, which was raised against rat brain nicotinic AChR and directed against the $\alpha 4$ subunit (Whiting & Lindstrom, 1988), and mAb 313, which was raised against the fusion protein containing the putative cytoplasmic of the $\alpha 3$ subunit (Whiting *et al.*, 1991b), were both purchased from RBI.

Anti-muscle type receptor subunits mAbs, 155 (anti $\alpha 1$), 148 (anti $\beta 1$), 162 (anti γ) and 137 (anti δ) (Tzartos *et al.*, 1986) were a generous gift of Dr Tzartos.

Receptor immobilization by subunit-specific antibodies

The affinity-purified Abs were bound to the wells at a concentration of 10 μ g/ml by means of overnight incubation at 4°C in phosphate buffer pH 7.5. On the following day, the wells were washed in order to remove the excess of unbound Abs, and then incubated overnight at 4°C with 200 μ l of 2% Triton X-100 extract obtained from COL membranes and prepared as described below (in the case of the $\alpha 7$ and $\beta 2$ -containing receptors), or with purified AChR foetal calf muscle in the case of mAb 73. After overnight incubation with the extract, the wells were washed and the presence of immobilized receptor was revealed by means of [^{125} I]- α Bgtx (in the case of the $\alpha 7$ subtype or muscle AChR) or [^{3} H]-Epi binding (in the case of the $\alpha 4\beta 2$ subtype).

AChR subtype preparation

The extracts containing the neuronal $\alpha 7$ and $\alpha 4\beta 2$ subtypes were obtained from chick optic lobes (COLs) as previously described (Gotti *et al.*, 1994). For every experiment, 18 g of COL, were homogenized in an excess of Na phosphate pH 7.4 50 mM, NaCl 1 M, EDTA 2 mM, EGTA 2 mM and PMFS 2 mM for 2 min in an ultra Turrax homogenizer; the homogenate was then diluted and centrifuged for 1.5 h at 60,000 $\times$ g.

This homogenization, dilution and centrifugation procedure was performed three times, after which the pellets were collected, rapidly rinsed with Na phosphate 50 mM, NaCl 50 mM, EDTA 2 mM, EGTA 2 mM, PMFS 2 mM, and then resuspended in the same buffer containing a mixture of 5 μ g ml $^{-1}$ of each of the following protease inhibitors: leupeptin, bestatin, pepstatin A and aprotinin (Sigma). Triton X-100, at a final concentration of 2%, was added to the washed membrane, and the membrane extracted for 2 h at 4°C. The extract (100 ml) was then centrifuged for 1.5 h at 60,000 $\times$ g and recovered.

$\alpha 7$ subtype Previous experiments (Gotti *et al.*, 1994) have shown that 60–70% of the α Bgtx receptors present in COL extract contain the $\alpha 7$ subunit ($\alpha 7$ subtype), and 20–25% contain both the $\alpha 7$ and $\alpha 8$ subunits ($\alpha 7$ – $\alpha 8$ subtype). In order to obtain an extract containing only the $\alpha 7$ subtype, we immunodepleted the original extract by means of incubation with anti- $\alpha 8$ antibodies bound to Sepharose 4B.

Immunoprecipitation experiments with anti- $\alpha 7$ and anti- $\alpha 8$ subunit-specific Abs were performed in order to verify the depletion of the subtypes containing the $\alpha 8$ subunit, and the depleted extract was plated on wells coated with anti- $\alpha 7$ Abs.

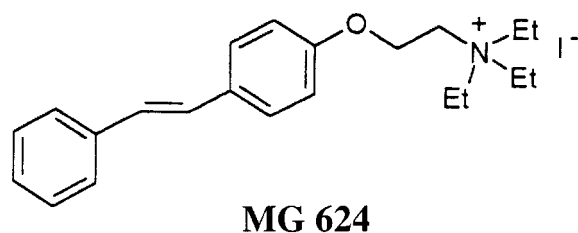


Figure 1 Chemical structure of the MG624 compound.

For the immunopurification of the $\alpha 7$ subtype, the $\alpha 8$ -depleted extract was then incubated overnight with anti- $\alpha 7$ Abs bound to Sepharose and after the resin had been extensively washed, the bound receptors were eluted with 0.2 M glycine pH 2.2, dialyzed, concentrated and lyophilized.

$\alpha 4\beta 2$ subtype Using [^3H]-Epi labelled receptors and Abs directed against the different nicotinic AChR subunits, we found that, although COL extract contains a number of nicotinic AChR subtypes ($\alpha 3\beta 2$, $\alpha 3\beta 4$, and $\alpha 4\alpha 5\beta 2$, $\alpha 4\beta 2$), the $\alpha 4\beta 2$ subtype represents 65–70% of the high affinity agonist binding sites.

In order to remove the $\alpha 3$ and $\beta 4$ -containing receptors, the extract (20 ml for each round) was first incubated with 5 ml of Sepharose-4B and bound anti- $\alpha 3$ Abs, and then with 5 ml of Sepharose-4B and bound anti- $\beta 4$ Abs (1 mg ml $^{-1}$ of purified Abs). The flowthrough was then incubated twice with Sepharose 4B with bound anti- $\alpha 5$ Abs in order to remove the $\alpha 4\alpha 5\beta 2$ subtype. Immunoprecipitation experiments with anti-subunit specific Abs and mAbs were performed in order to verify the depletion of the subtypes containing the $\alpha 3$, $\beta 4$ and $\alpha 5$ subunits, and the extract was then plated on wells coated with anti $\beta 2$ Abs.

For $\alpha 4\beta 2$ subtype immunopurification, the $\alpha 3$, $\beta 4$, and $\alpha 5$ depleted extract was incubated overnight with anti- $\beta 2$ Abs bound to Sepharose, and the receptors recovered as described for the $\alpha 7$ subtype.

Muscle subtype The $\alpha 1\beta 1\gamma\delta$ subtype was purified from foetal calf muscles, as previously described with minor modifications; the purified receptor was then plated on wells coated with mAb 73, a mAb directed against the $\beta 1$ subunit present in the muscle-type receptor (Gotti *et al.*, 1997b).

For $\alpha 1\beta 1\gamma\delta$ subtype immunopurification, the receptors purified on the α Bgtx column were incubated overnight with anti- $\beta 1$ mAbs bound to Sepharose, and the receptors processed as described for the $\alpha 7$ subtype.

Binding of nicotinic ligands to immobilized subtypes

The subtypes immobilized by the corresponding subunit-specific Abs were incubated overnight at 20°C with 250 μl of [^3H]-Epi or with 250 μl [^{125}I]- α Bgtx. All of the incubations were performed using a buffer containing (mM) Tris-HCl pH 7.5; NaCl 150; KCl 5; MgCl $_2$ 1; CaCl $_2$ 2.5; 2 mg ml $^{-1}$ BSA and 0.05% Tween 20 (buffer C). The [^3H]-Epi saturation binding experiments to the $\alpha 4\beta 2$ subtype were carried out in triplicate at concentrations ranging from 0.005–5 nM; for each [^3H]-Epi concentration, unspecific binding in the presence of 100 nM of cold Epi was evaluated. At the concentration of 2.5 nM [^3H]-Epi, a parallel inhibition experiment using 5–6 different concentration of cold Epi (from 1 nM–1000 nM) was performed.

The [^{125}I]- α Bgtx saturation binding experiments were performed in triplicate using concentrations of 0.01–10 nM with non-specific binding being determined in parallel by means of incubation in the presence of 1 μM α Bgtx. At the concentration of 3 nM [^{125}I]- α Bgtx, a parallel inhibition experiment using 5–6 different concentrations of cold α Bgtx (from 1 nM–1000 nM) was performed.

Testing direct saturation binding (the first 7–8 concentrations of respectively 0.005–5 nM for [^3H]-Epi and 0.01–10 nM for [^{125}I]- α Bgtx) and displacement (the last 5–6 concentrations of 1–1000 nM for both ligands) (Rovati *et al.*, 1991) in the same experiment make it possible to use the LIGAND programme to fit both the saturation and competition data

in order to search for the possible presence of sites with different affinity for Epi. This is because high Epi concentrations can be reached without using excessive amounts of labelled ligand (the competition part of the curve) and at the same time, adequate radioactivity can be retained in the lower concentration range (the saturation part of the curve).

In order to assess the pharmacological profiles of the immuno-immobilized subtypes we performed competition experiments using MG624, d-Tubocurarine (d-Tub) and MLA.

The drugs were dissolved in buffer C just before use and serial dilutions were preincubated for 30 min at RT. Subsequently a final concentration of 0.1 nM [^3H]-Epi was added to the $\alpha 4\beta 2$ subtype, and a final concentration of 0.5 nM or 0.3 nM of [^{125}I]- α Bgtx was added to the $\alpha 7$ or $\alpha 1\beta 1\gamma\delta$ subtypes. The $\alpha 4\beta 2$ and $\alpha 7$ subtypes were incubated overnight at 20°C, the $\alpha 1\beta 1\gamma\delta$ subtype was incubated for 48 h at 20°C.

After incubation, the wells were washed seven times with ice-cold PBS containing 0.05% Tween 20, and the bound radioactivity was recovered by means of incubation with 200 μl of 2N NaOH for 2 h. The bound radioactivity was then determined by means of liquid scintillation counting in a β counter in the case of [^3H]-Epi, or direct counting in a γ counter in the case of [^{125}I]- α Bgtx.

Data analysis The experimental data obtained from the saturation binding experiments were analysed by means of a non-linear least square procedure using the LIGAND program as described by Munson & Rodbard (1980). The parameters of the [^3H]-Epi binding to the $\alpha 4\beta 2$ subtype and [^{125}I]- α Bgtx binding to the $\alpha 7$ and $\alpha 1\beta 1\gamma\delta$ receptors obtained from at least three separate experiments were analysed using the LIGAND program. An 'extra sum of squares' F-test was performed by the program LIGAND to evaluate the different binding models statistically.

The K_i values of all of the tested drugs were determined by means of LIGAND, using the data obtained from three independent experiments. The experimental data from all of the subtypes were fitted to models with one and two binding site classes in order to test whether the fits were significantly better for the one- or two-binding site model (Munson & Rodbard, 1980).

cDNAs and oocyte injection

The cDNAs encoding the chick neuronal nicotinic AChR subunits and the chick muscle-type subunits were kindly provided by Dr Marc Ballivet. Full length cDNAs encoding wild type $\alpha 7$, L247T $\alpha 7$, the neuronal $\alpha 4\beta 2$ subtype or the muscle-type $\alpha 1\beta 1\gamma\delta$ receptor were expressed as previously described (Palma *et al.*, 1996a). The preparation of oocytes and nuclear injection procedures are detailed elsewhere (Bertrand *et al.*, 1991). Stage VI oocytes were injected intranuclearly (10 nl) with cDNA clones (0.2 mg ml $^{-1}$) using a pressure micro injector (Eppendorf, Germany) and a Singer micro manipulator (U.K.).

Electrophysiological recordings

Membrane currents were recorded 2–4 days after injection using a voltage-clamp technique with two microelectrodes filled with 3 M KCl. The oocytes were placed in a recording chamber (volume, 0.1 ml) and continuously perfused with Ringer's oocyte medium (mM): NaCl 82.5; KCl 2.5; CaCl $_2$ 2.5; MgCl $_2$ 1; HEPES 5/adjusted to pH 7.4 with NaOH) at

controlled room temperature (20–22°C). The recordings were usually made in the presence of atropine (0.5 μM) in order to prevent the stimulation of the muscarinic AChRs sometimes present in native oocytes (Miledi *et al.*, 1989). Since atropine can affect nicotinic responses, the results obtained in five oocytes (two donors) pre-treated or not with MG624 (100 nM) for 30 s before applying ACh (100 μM) in the presence of atropine (0.5 μM) were compared with those elicited in oocytes not treated with atropine. Atropine did not alter I_{ACh} nor reduce the I_{ACh} induced by MG624 in any of the examined oocytes. To construct dose/response relationships, the oocyte membrane potential was held at -60 mV, and the drugs applied at 3 min intervals. After each determination, control currents were elicited, twice in order to check for possible current rundown. The drugs were dissolved in Ringer's medium and applied by superfusing the oocytes at a rate of 12 ml min $^{-1}$. In the experiments designed to block nicotinic AChRs, the nicotinic blocker MLA (Palma *et al.*, 1996a) was coapplied with the neurotransmitter after MLA pre-treatment for 40 s. The solutions were exchanged by using electromagnetic valves (Type III; General Valve, U.S.A.). MG624 was dissolved just before the experiments.

Experimental analysis

The current records were digitized at 50–150 Hz using an analogue-to-digital converter (Digidata 1200 Interface, Axon Instruments, U.S.A.), and computer stored for subsequent analysis using pClamp 6.0.2 routines (Axon Instruments, U.S.A.). To estimate the half-inhibitory concentration (IC_{50}) of MG624, as well as the half dissociation constant (EC_{50}) of ACh, the data were fitted, by means of least-square routines (included in Sigma Plot, Jandel, Germany), to the following Hill equations:

$$I/I_{\text{max}} = \text{IC}_{50}n_{\text{H}}/([\text{MG624}]n_{\text{H}} + \text{IC}_{50}n_{\text{H}}) \quad (1)$$

$$I/I_{\text{max}} = [\text{ACh}]n_{\text{H}}/([\text{ACh}]n_{\text{H}} + \text{EC}_{50}n_{\text{H}}) \quad (2)$$

where [MG624] and [ACh] are the drug doses, n_{H} the Hill coefficient, and I_{max} the maximum response. For more details, see Miledi *et al.*, 1989 and Palma *et al.*, 1996a.

Materials

The lyophilized αBgtx , anti-protease inhibitors, cholinergic ligands, Triton X-100, anti-rabbit and anti-rat antisera were purchased from Sigma, U.S.A.; non-radioactive Epi, MLA, mAb 313, mAb 299 from RBI; CnBr-activated Sepharose 4BCL from Pharmacia, Sweden; [^{125}I]- αBgtx , [^{125}I]-Prot.A and [^3H]-Epi from Amersham, U.K.; and the reagents for gel electrophoresis from Bio-Rad Laboratories, U.S.A.

Results

Subunit composition of immunoimmobilized subtypes

In order to check the subunit composition of the receptors immuno-immobilized by our anti- $\alpha 7$, anti- $\beta 2$ and anti- $\beta 1$ Abs, we used the same Abs to immunopurify the receptors and determine their subunit composition by Western blotting. Figure 2A shows the results of the $\alpha 7$ subtype probed with anti- $\alpha 7$ (lane 4), anti- $\alpha 8$ Abs (lane 5), anti- $\alpha 3$ (mAb 313, lane 1), anti- $\alpha 4$ (mAb 299, lane 2), anti- $\alpha 5$ (mAb 35, lane 3) and anti- $\beta 2$ (lane 6) and anti- $\beta 4$ Abs (lane 7). Only the anti- $\alpha 7$ Abs were specifically able to recognize a peptide of the expected M_r of 57 KD.

Figure 2B shows the results of the $\alpha 4\beta 2$ immunopurified receptors probed with the same Abs and mAbs. Only the $\alpha 4$ -specific mAb 299 (lane 9) and our anti- $\beta 2$ Abs (lane 12) were able to recognize single polypeptides of respectively 66 and 54 KD. As expected, the anti- $\alpha 7$ Abs (lane 11) did not recognize any peptide (in agreement with previously reported data from Vernallis *et al.*, 1993; Gotti *et al.*, 1994), thus showing that the $\alpha 7$ -containing receptors are not promiscuous with the heteromeric receptors in terms of subunit(s).

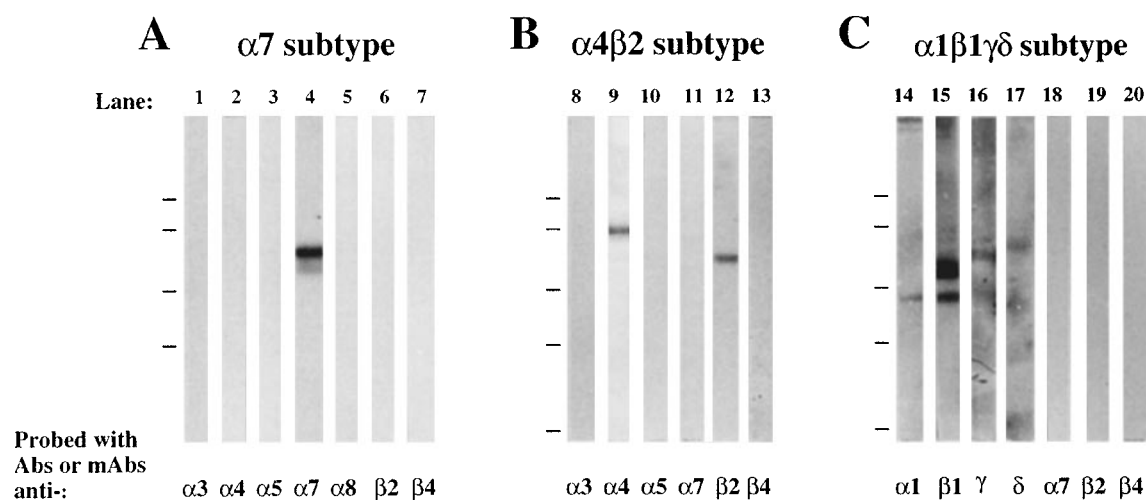


Figure 2 Western blot analysis of the subunit composition of the immuno-immobilized nicotinic AChR subtypes: $\alpha 7$ (A), $\alpha 4\beta 2$ (B) and $\alpha 1\beta 1\gamma\delta$ (C). The receptors, which were immunopurified as described in Methods, were separated on 9% acrylamide SDS gels, electrotransferred to nitrocellulose and probed with the indicated subunit-specific Abs or mAbs used at concentrations of 5–10 $\mu\text{g}/\text{ml}$. The anti- $\alpha 7$, $\alpha 8$, $\beta 2$ and $\beta 4$ Abs were those produced by us and described in Methods, whereas anti- $\alpha 3$ (mAb 313), anti- $\alpha 4$ (mAb 299) and anti- $\alpha 5$ (mAb 35) were commercially available mAbs. When mAbs were used, the blots were incubated for a further 2 h with anti-rat IgG diluted 1:1000. The bound Abs were revealed by means of [^{125}I]-Protein A. The molecular weight markers (top to bottom) are 97 KD, 66 KD, 45 KD, 31 KD and 21 KD.

We have also probed the $\alpha 7$ and $\alpha 4\beta 2$ subtypes for the presence of the $\alpha 2$ subunit (data not shown) using mAb 323 (a generous gift of Dr Lindstrom and used for the localization of the $\alpha 2$ subunit by Ullian & Sargent (1995) but we did not have any labelling. This mAb certainly recognizes the $\alpha 2$ subunit in immunolocalization but there are no data as to whether it works on Western blotting, and so we do not know if the lack of signal was due to the absence of the subunit or a lack of recognition.

We did not check for the presence of the $\alpha 6$ subunit in these subtypes because no specific Abs are available, but on the basis of the work of Fucile *et al.* (1998), showing undetectable levels of mRNA for the $\alpha 6$ subunit in the chick optic tectum, we believe that, if the $\alpha 6$ -containing subtype is present in COL it is certainly a minor subtype.

We also probed the purified $\alpha 1\beta 1\gamma \delta$ muscle-type subtype with mAbs specific for the $\alpha 1$ (mAb 155), $\beta 1$ (mAb 148), γ (mAb 162) and δ (mAb 137) subunits (Tzartos *et al.*, 1986); the results are shown in Figure 2C. Apart from mAb 148 (lane 15), which recognized the $\alpha 1$ subunit of M_r 42 KD in addition to the $\beta 1$ subunit of M_r 49 KD, the other mAbs only recognized the peptides of M_r 42, 55 and 58 KD corresponding to the $\alpha 1$ (lane 14), γ (lane 16) and δ subunits (lane 17).

Since it has been reported that the $\alpha 7$ subunit is present in embryonic muscle (Corriveau *et al.*, 1995), we probed the $\alpha 1\beta 1\gamma \delta$ receptors with our anti- $\alpha 7$ Abs produced against the chick $\alpha 7$ subunit, as well as with our anti- $\beta 2$ and anti- $\beta 4$ Abs, but we could not determine the presence of any of the three subunits (lanes 18, 19, 20) in this subtype.

Ligand binding to $\alpha 7$ and $\alpha 4\beta 2$ chick neuronal subtypes

The binding of [^{125} I]- α Bgtx to the immuno-immobilized $\alpha 7$ subtype has a K_d of 0.58 nM [Coefficient of variation (CV) = 17%], and MG624 competitively displaced this [^{125} I]- α Bgtx binding in a concentration-dependent manner with a K_i of 106 nM (CV 20%) (see Table 1). In the same experiments, we also tested two nicotinic antagonists as controls, (the $\alpha 7$ selective MLA and the non-selective d-Tub) for their ability to inhibit [^{125} I]- α Bgtx binding to the $\alpha 7$ subtype, and found K_i

values of 1.15 nM (CV 21%) for MLA and 1.57 μ M (CV 26%) for d-Tub (see Table 1). Figure 3A shows a representative binding experiment and the inhibition of the binding induced by MG624, MLA and d-Tub.

Several nicotinic AChR subtypes containing the $\beta 2$ subunit ($\alpha 3\beta 2$, $\alpha 3\beta 2\beta 4$, $\alpha 4\alpha 5\beta 2$, $\alpha 4\beta 2$) are present in COL membrane extract. The K_i value on the $\beta 2$ -containing subtypes that we have previously reported (K_i 69.5 μ M; Gotti *et al.*, 1998) is an average of the K_i values of the different subtypes. In order to determine the K_i value of only the $\alpha 4\beta 2$ subtype, we performed selective immunodepletion experiments to obtain an extract that only contained $\alpha 4\beta 2$ receptors. The $\alpha 4\beta 2$ subtype bound [^{3} H]-Epi with a K_d of 73 pM (CV 18%); the K_i values of MG624, MLA and d-Tub were respectively 84 μ M > 50 μ M and 19.6 μ M (see Table 1).

Figure 3B shows the results of a representative experiment of [^{3} H]-Epi binding to the $\alpha 4\beta 2$ subtype, and the inhibition of this binding by MG624, MLA and d-Tub.

Ligand binding to the muscle $\alpha 1\beta 1\gamma \delta$ subtype

Binding experiments showed that MG624 has a selective action toward the neuronal $\alpha 7$ subtype which, like the $\alpha 8$ and $\alpha 9$ subtypes, binds α Bgtx. Given that the superfamily of nicotinic AChRs includes the muscle $\alpha 1\beta 1\gamma \delta$ subtype (which, in addition to the $\alpha 7$, $\alpha 8$ and $\alpha 9$ subtypes, also binds α Bgtx), we tested the effects of MG624 on it by means of binding and inhibition experiments. The K_d of [^{125} I]- α Bgtx binding to the $\alpha 1\beta 1\gamma \delta$ subtype obtained from foetal calf muscle was 0.33 nM (CV 29%); the K_i of MG624 in competing with the [^{125} I]- α Bgtx binding was 32 μ M (CV 28%). As a control, we also tested the MLA and d-Tub antagonists, and found that their K_i were > 50 μ M and 98 nM (see Table 1).

Figure 3C shows a representative experiment of [^{125} I]- α Bgtx binding to the immuno-immobilized $\alpha 1\beta 1\gamma \delta$ subtype and the inhibition of the binding by MG624, MLA and d-Tub.

All of the competition data relating to the binding of the three drugs to the $\alpha 7$, $\alpha 4\beta 2$ and $\alpha 1\beta 1\gamma \delta$ subtypes were fitted for one or two classes of binding site: and there was always a statistically better fit for the presence of only a single class of binding sites.

Effects of MG624 on wild type neuronal $\alpha 7$ nicotinic AChRs

Oocytes injected with wild type $\alpha 7$ subunit cDNA responded to ACh with an inward current (I_{ACh}) whose peak amplitude depended on the concentration of the transmitter. With 100 μ M ACh (EC_{50} ACh), applied to oocytes held at -60 mV, the mean current amplitude averaged 1.1 μ A (23 oocytes; three donors, 23/3; range: 99 nA to 3.9 μ A); and decayed with a time to 10% decay ($T_{0.1}$) of 0.13 ± 0.01 s (mean $\pm$ s.d.). Neither the non-injected oocytes nor the oocytes expressing $\alpha 7$ nicotinic AChRs, responded to MG624 (10 nM–1 μ M) with a detectable response above a noise level of 4 ± 1 nA. When MG624 was co-applied with ACh, the I_{ACh} amplitude was no different from the control. On the contrary, when the oocytes were pretreated with MG624 for 20–30 s before co-applying ACh, the I_{ACh} amplitude was considerably reduced. As no further reduction was observed when MG624 pretreatment was extended as much as 30–60 min, all of the other experiments were carried out using an exposure time of 30 s before the co-application of ACh. With this procedure MG624 (100 nM) reduced I_{ACh} peak amplitude by 59.7% at 100 μ M ACh without affecting current decay ($T_{0.1} = 0.13 \pm 0.03$ s). The inhibition of I_{ACh} by MG624 was dose-dependent; the IC_{50} was 109 nM

Table 1 Relative affinities (K_i) of MG624, MLA and d-Tub for immunoimmobilized subtypes, and MG624 relative inhibitory potencies (IC_{50}) on ACh-evoked responses in oocyte-expressed chick subtypes

Subtype	MG624 K_i (nM)	MLA K_i (nM)	d-Tub K_i (nM)	MG624 IC_{50} (nM)
$\alpha 7$	106 (20)	1.15 (21)	1570 (26)	109
$\alpha 4\beta 2$	84 000 (20)	> 50 000 (21)	19 600 (38)	3200
$\alpha 1\beta 1\gamma \delta$	32 200 (26)	> 50 000 (18)	98 (25)	2900

The K_d s of [^{125} I]- α Bgtx for the $\alpha 7$ and $\alpha 1\beta 1\gamma \delta$ subtypes were respectively 0.58 nM (CV = 17%) and 0.33 nM (CV = 29%), and the K_d of [^{3} H]-Epi for the $\alpha 4\beta 2$ was 73 pM (CV = 18%). The K_i values were derived from [^{125} I]- α Bgtx saturation and competition binding curves to the $\alpha 7$ and $\alpha 1\beta 1\gamma \delta$ subtypes, and [^{3} H]-Epi saturation and competition binding curves to the $\alpha 4\beta 2$ subtype. The curves obtained from three separate experiments were fitted using a non-linear least squares analysis program and the F test (Munson & Rodboard (1980)). The numbers in brackets represent the per cent CV. The MG624 IC_{50} values were obtained from inhibition experiments performed in oocyte-expressed subtypes using increasing concentrations of MG624 for 30 s before the application of ACh at their EC_{50} values (100 μ M for the $\alpha 7$ subtype, 2 μ M for the $\alpha 4\beta 2$ and 10 μ M for the $\alpha 1\beta 1\gamma \delta$ subtype).

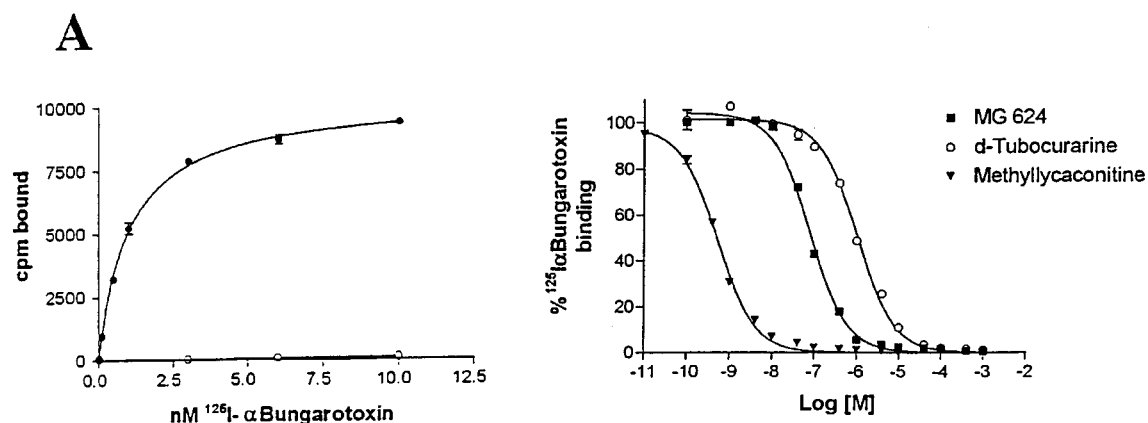
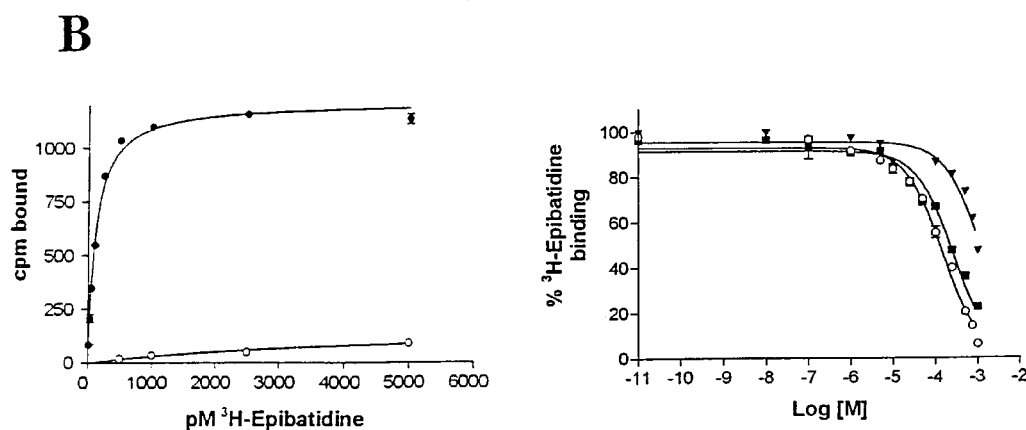
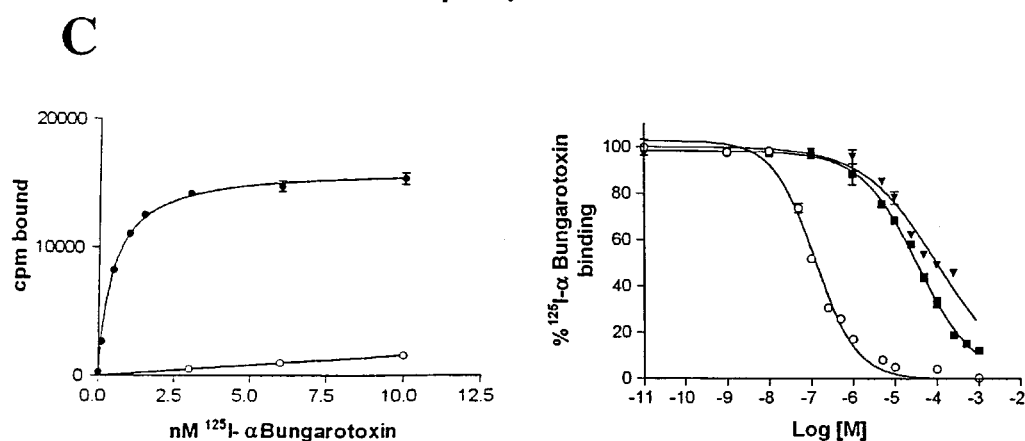
$\alpha 7$ subtype $\alpha 4\beta 2$ subtype $\alpha 1\beta 1\gamma\delta$ subtype

Figure 3 Binding of nicotinic ligands to immuno-immobilized subtypes. (A) Saturation curves of total and specific [^{125} I]- α Bgtx binding to the $\alpha 7$ subtype (left), and the inhibition curves of specific [^{125} I]- α Bgtx binding to the immuno-immobilized subtype by nicotinic antagonists (right). For total binding (●), the immuno-immobilized $\alpha 7$ subtype was incubated overnight at 20°C with the indicated concentrations of [^{125} I]- α Bgtx and, for aspecific binding (○), also in the presence of 1 μ M cold α Bgtx. The total, aspecific binding and inhibition curves shown are those obtained from a representative experiment. (B) Saturation curves of total and aspecific [^{3} H]-Epi binding to $\alpha 4\beta 2$ (left) and the inhibition curves of specific [^{3} H]-Epi binding to the $\alpha 4\beta 2$ immuno-immobilized subtype by nicotinic antagonist (right). For total binding (●), the immuno-immobilized subtype was incubated overnight at 20°C with the indicated concentrations on [^{3} H]-Epi and, for aspecific binding (○), also in the presence of 100 nM cold α Bgtx. The total, aspecific binding and inhibition curves shown are those obtained from a representative experiment. (C) Saturation curves of total (●) and aspecific (○) [^{125} I]- α Bgtx binding to the $\alpha 1\beta 1\gamma\delta$ subtype (left), and the inhibition curves of specific [^{125} I]- α Bgtx binding to the immuno-immobilized subtype by nicotinic antagonists (right). The binding experiments were performed as described in A, except for the fact that the incubation time was 48 h at 20°C.

(Figure 4A, lower part) and remained similar when the Ca^{2+} was substituted by an equimolar concentration of Ba^{2+} (three oocytes, data not shown).

In order to exclude a possible channel block by MG624, we tested MG624 inhibition at different potentials because one of the characteristics of a channel blocker is that the block is voltage dependent. Our findings showed that the block of MG624 was not voltage dependent (Figure 5A) and the degree of block at different voltages was constant (as shown in Figure 5B). In addition, as previously reported (Palma *et al.*, 1996a, b), the I_{ACh} -voltage relationship showed a high degree of rectification at positive potentials, and this pattern was not modified by MG624 (100 nM) even though the current amplitudes were markedly reduced (Figure 5A). All of these findings together indicate that the effect of MG624 on $\alpha 7$ nicotinic AChR function is consistent with voltage-independent inhibition, and thus not compatible with a channel block.

Inhibitory effects of MG624 on the $\alpha 4\beta 2$ and on the muscle $\alpha 1\beta 1\gamma\delta$ subtypes

In order to compare the potency of MG624 in inhibiting the function of wild type $\alpha 7$ vs $\alpha 4\beta 2$ or $\alpha 1\beta 1\gamma\delta$ nicotinic AChR subtypes, experiments were performed in oocytes injected with

either $\alpha 4\beta 2$ or $\alpha 1\beta 1\gamma\delta$ subunit cDNAs. The upper part of Figure 4B shows a typical recording obtained from oocytes injected with $\alpha 4\beta 2$ subunits exposed to 2 μM ACh (left), 2 μM ACh + 1 μM MG624 (middle) and the recovery after 3 min washes (right). Upper part of Figure 4C shows a typical recording obtained from oocytes injected with $\alpha 1\beta 1\gamma\delta$ subunits exposed to 10 μM ACh (left), 10 μM ACh + 1 μM MG624 (middle) and the recovery after 3 min washes (right). The lower part of the same figures shows the dose-dependent inhibition of I_{ACh} by MG624 in the two subtypes. The inhibition was obtained by incubation with increasing concentrations of MG624 for 30 s before the application of ACh at about its EC_{50} value. The IC_{50} and n_{H} values were 3.2 μM and 1.2 ($n = 7$) for the $\alpha 4\beta 2$ subtype (ACh, 2 μM ; Fucile *et al.*, 1997, and 2.9 μM and 0.9 (ACh, 10 μM ; Mileo *et al.*, 1995) for the $\alpha 1\beta 1\gamma\delta$ subtype, thus indicating that the half inhibitory concentrations of MG624 for $\alpha 4\beta 2$ or $\alpha 1\beta 1\gamma\delta$ nicotinic AChRs were much higher than those of $\alpha 7$ nicotinic AChRs.

Effects of MG624 on L247T $\alpha 7$ nicotinic AChR

In order to obtain more information concerning the nature of the inhibitory activity of MG624, we tested it on the mutated L247T in which it is known that some nicotinic antagonists

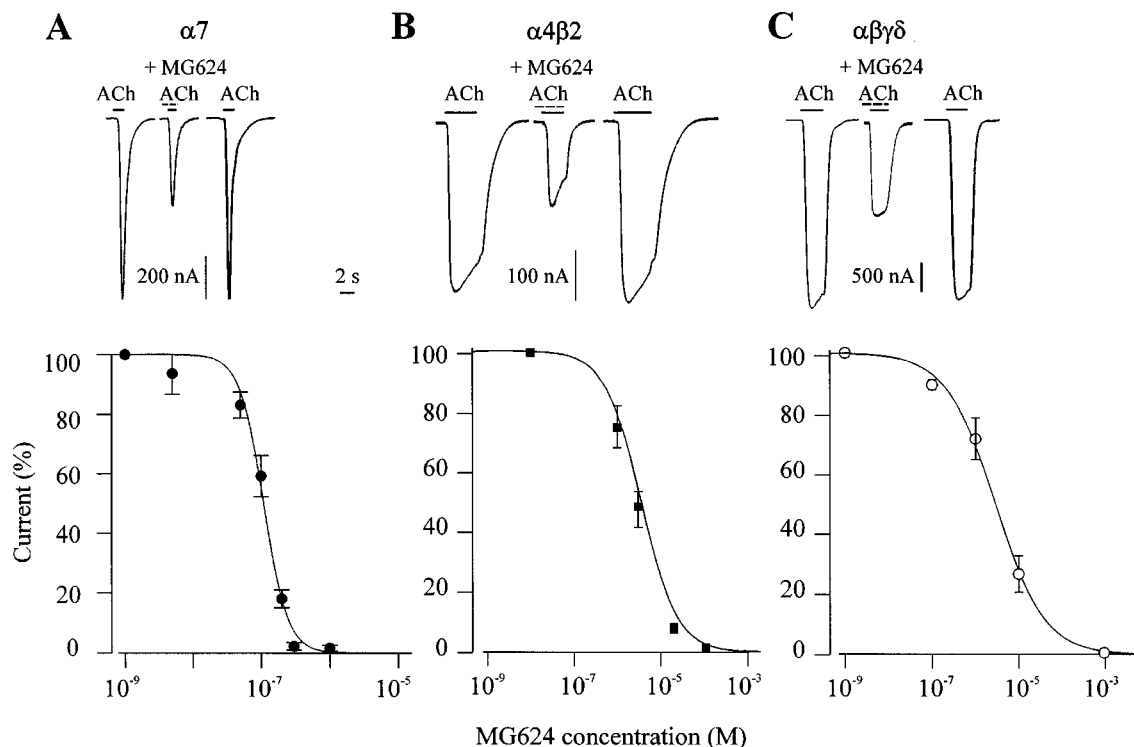


Figure 4 MG624 dose- I_{ACh} response curves for neuronal and muscle nicotinic AChRs expressed in *Xenopus* oocytes. (A) $\alpha 7$ subunit cDNA-injected *Xenopus* oocyte; Top: current traces in an oocyte voltage-clamped at -60mV (ACh 100 μM , horizontal bar) before (left), during (middle), and after (right) treatment with MG624 (100 nM). Dashed or continuous line, MG624 and ACh applications, respectively. Note similar I_{ACh} decay in control vs treated oocyte. Bottom: peak current (mean $\pm$ s.e.mean) evoked by ACh coapplied with MG624 at the concentrations indicated in the abscissa, were normalized (in percentages) to the response to 100 μM ACh. Ordinate: normalized percentage I_{ACh} . The oocytes ($n = 12$, three donors) were pretreated for 30 s with MG624 at the same concentrations as those used for the MG624 coapplications with ACh. $\text{IC}_{50} = 109$ nM and $n_{\text{H}} = 2.5$ (estimated using equation 1, see Methods. (B) $\alpha 4\beta 2$ subunit cDNAs-injected oocytes. Top: ACh 2 μM , and MG624 1 μM . Bottom: peak current (mean $\pm$ s.e.mean) ($n = 7$, two donors) evoked by ACh coapplied with MG624 at the concentrations indicated in the abscissa, were normalized (in percentages) to the response to 2 μM ACh. The best fit gave an IC_{50} value for MG624 of 3.2 μM and an $n_{\text{H}} = 1.2$. Abscissa, ordinate, drug pretreatment, holding potentials and bars as in A. (C) $\alpha 1\beta 1\gamma\delta$ subunits cDNA-injected oocytes. Top: ACh, 10 μM and MG624 1 μM . Bottom: peak current (mean $\pm$ s.e.mean) ($n = 6$, two donors) evoked by ACh coapplied with MG624 at the concentrations indicated in the abscissa, were normalized (in percentages) to the response to 10 μM ACh. The best fit gave an IC_{50} value for MG624 of 2.9 μM and $n_{\text{H}} =$ value of 0.9. Abscissa, ordinate, drug pretreatment, holding potentials and bars as in A and B.

behave as agonists. In contrast to its failure to generate currents in oocytes expressing wild type $\alpha 7$ nicotinic AChR, MG624 applied alone to oocytes expressing L247T $\alpha 7$ nicotinic AChRs gave rise to an inward current (I_{MG624}) whose amplitude depended on the concentration of the drug. The MG624 dose- I_{MG624} relationship (Figure 6A) gave EC_{50} and n_{H} values for MG624 of 0.2 nM and 1.9, thus indicating that the level of cooperativity was similar, but the apparent affinity of MG624 for the L247T $\alpha 7$ mutant was 1000 times greater than that of ACh (MG624 EC_{50} = 0.2 nM and ACh EC_{50} = 0.2 μM ; Palma *et al.*, 1996a,b). The mean amplitude of I_{MG624} was 2.5 μA (range: 434 nA to 6 μA ; holding potential, -60 mV; 15/3) when MG624 was bath-applied at a concentration of 0.2 nM (EC_{50} MG624), and that of I_{ACh} was 2.1 μA (range: 80 nA to 5.8 μA , same oocytes) with an ACh concentration of 0.2 μM (EC_{50} ACh) (Figure 6B left and middle).

It is well known that the potent competitive nAChR antagonist MLA reduces the holding current required to clamp the cell expressing L247T $\alpha 7$ nicotinic AChRs vs wild type $\alpha 7$ (Betrand *et al.*, 1997). As expected the L247T $\alpha 7$ mutant-injected oocytes acutely exposed to MLA (500 pM, 6 oocytes/two donors) gave rise to an MLA-induced outward current and became unresponsive to MG624 (Figure 6C).

In order to investigate whether ACh alters the apparent affinity and cooperativity of the L247T $\alpha 7$ mutant for MG624,

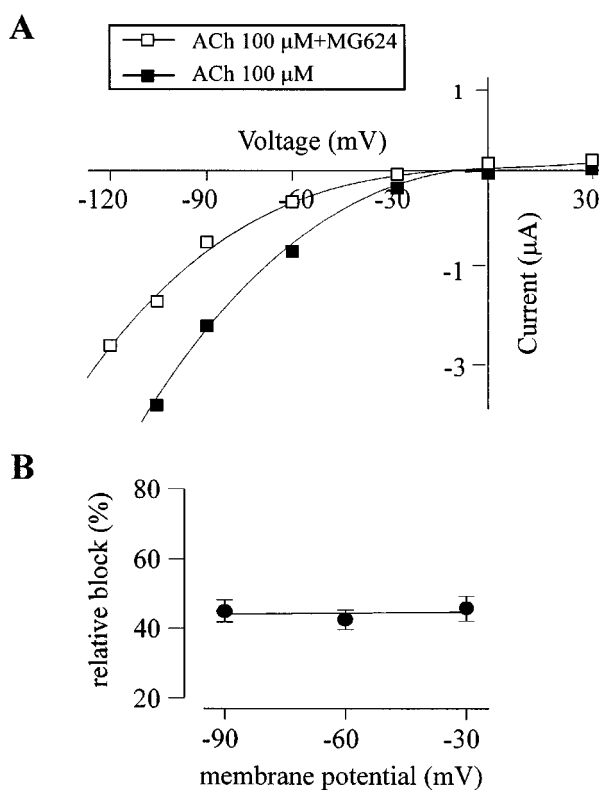


Figure 5 Voltage independent inhibition of I_{ACh} by MG624 in oocytes expressing homomeric wild type $\alpha 7$ nAChRs. (A) Current-voltage relationships in an oocyte held at -60 mV. Peak currents evoked at various test potentials by ACh (100 μM , ■) or by ACh plus MG624 (100 nM, □) (representative of ten experiments, two donors). The solid lines represent two-order polynomial data fits. Note rectification at positive potentials and similar curve profiles. (B) Inhibition of I_{ACh} (ACh 100 μM) by MG624 (100 nM) at various holding potentials. The relative block was calculated as $[1 - (I_{\text{ACh}} + \text{MG624})/I_{\text{ACh}}] \times 100$. Each point is the mean $\pm$ s.e.mean of ten oocytes. ACh was applied at 3 min intervals, and the oocytes were pretreated with MG624 as in Figure 4.

the MG624 dose- I_{MG624} relationship was examined in the presence of ACh. The dose-response relationship fitted to a Hill equation shifted to the right in the presence of 0.2 μM ACh (Figure 6A), giving EC_{50} and n_{H} values of 0.67 nM and 1.5 respectively (10 oocytes/two donors) a finding that is consistent with a competitive action of MG624 on the L247T $\alpha 7$ mutant receptor.

Analysis at different transmembrane voltages showed that, as in the case of I_{ACh} , the amplitude of I_{MG624} increased almost linearly with potential (Figure 7). This linearity was maintained even at high MG624 concentrations, a behaviour that is incompatible with an open channel blockade. Furthermore, the reversal potential (V_r) of I_{MG624} was similar to that previously reported for I_{ACh} (Palma *et al.*, 1996b), thus indicating that the ionic basis of the responses to both substances was the same.

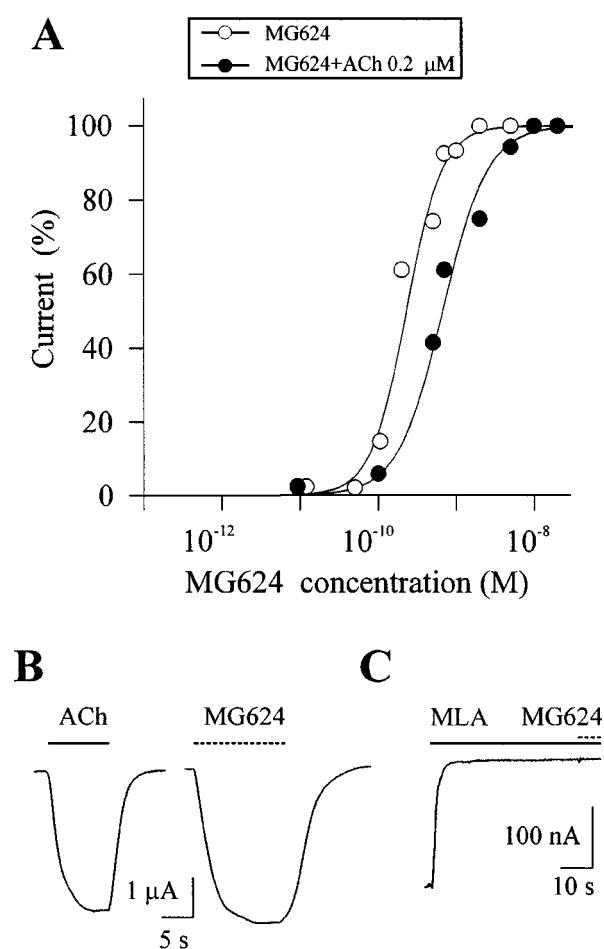


Figure 6 Current responses in oocytes expressing L247T $\alpha 7$ nicotinic AChRs. (A) Dose-response relationships for MG624 (○) and MG624 plus 0.2 μM ACh (●). The peak currents were normalized to the current evoked by MG624 at 10 nM. Each point represents the mean value ($n=10$, two donors); for the sake of simplicity, s.e.mean values $<0.3\%$ are not reported. The oocytes were held at -60 mV. (○): EC_{50} = 0.23 nM and n_{H} = 1.9 (estimated using equation 2, see Methods). (●): EC_{50} = 0.67 nM and n_{H} = 1.5. The average I_{max} was -3708 ± 551 nA for the MG624 currents, and -3273 ± 746 nA for ACh + MG624. (B) Current responses in the same oocyte held at -60 mV to 0.2 μM ACh (left), 0.2 nM MG624 (right); (C) I_{MG624} (MG624, 10 nM) blocked by MLA (500 pM). Note the outward current evoked by MLA and the absence of response to MG624. (Representative of six experiments, two donors). Dashed lines: MG624, continuous line: MLA application; holding potential as in A.

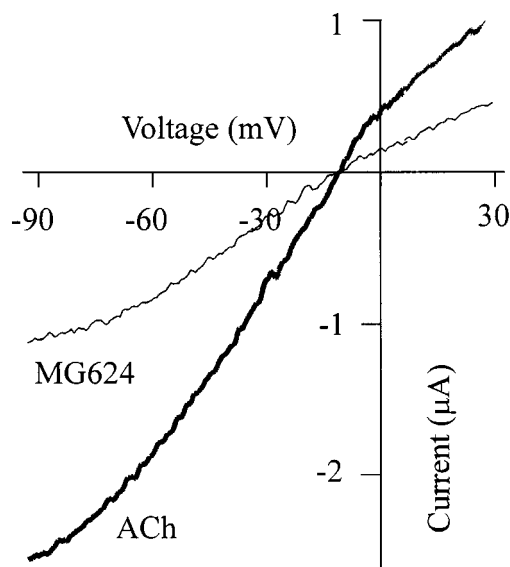


Figure 7 I_{MG624} and I_{ACh} membrane voltage relationships in an oocyte injected with L247T $\alpha 7$ subunit cDNA. The curves were obtained using voltage-ramps ranging from -90 mV to $+30$ mV. The MG624 and ACh concentrations were 0.2 nM and 0.2 μ M, respectively. Note the same curve profiles for both drugs and the same equilibrium potential (-13 mV).

Discussion

In a previous study, we demonstrated that two oxystilbene derivatives (MG624 and F3: Gotti *et al.*, 1998) are selective ligands for neuronal α Bgtx receptors and provided a preliminary characterization of the antagonistic effects of this class of compounds. In the present study, the interaction of MG624 with the $\alpha 7$ subtype has been better characterized by means of binding and electrophysiological studies, and its antagonistic selectivity towards the $\alpha 7$ homomeric chick subtype further demonstrated by comparing its effects with those obtained using the neuronal $\alpha 4\beta 2$ and $\alpha 1\beta 1\gamma\delta$ muscle chick AChR subtypes. Furthermore, in order to obtain more information concerning the compound's mechanism of action, we tested its effect on the mutated L247T $\alpha 7$ subtype.

The binding studies showed that MG624 has a K_i of 106 nM, which is lower than that reported in our previous study (27 nM). This difference is probably due to the fact that the binding experiments in this study were performed using a purified $\alpha 7$ subtype (see Figure 2), whereas the previous data were obtained in a mixed population of $\alpha 7$ and $\alpha 7$ - $\alpha 8$ subtypes. In order to have a more complete pharmacological profile of the $\alpha 7$, $\alpha 4\beta 2$ and $\alpha 1\beta 1\gamma\delta$ subtypes used in the binding studies, we also tested two other nicotinic antagonists: the $\alpha 7$ -selecting antagonist MLA and the non selective d-Tub. We found that the K_i of MLA for the $\alpha 7$ subtype was in close agreement with published data (Ward *et al.*, 1990), whereas the K_i values for the $\alpha 4\beta 2$ subtype and the $\alpha 1\beta 1\gamma\delta$ muscle were higher than previously reported. The K_i values determined for d-Tub were very similar to those reported by other authors using the same nicotinic subtypes (Anand *et al.*, 1993; Whiting *et al.*, 1991a; Colquhoun & Rang 1976).

The specificity and selectivity of MG624 revealed by the binding studies was confirmed using the oocyte-expressed chick $\alpha 7$ subtype, MG624 had a dose-dependent inhibitory action on $\alpha 7$ nicotinic AChR, and this block did not increase at

hyperpolarizing potentials; the constant degree of the block at different voltages strongly suggests that the effect of this drug is not due to an open channel blocking mechanism. Moreover, when inhibited by MG624, the $\alpha 7$ nicotinic AChR showed positive cooperativity and the difference between the binding affinity and the IC_{50} to block $\alpha 7$ homomers was minimal. These results suggest that MG624 is a competitive inhibitor of the $\alpha 7$ receptor subtype.

Further information on the MG624 $\alpha 7$ subunit binding site comes from the experiments made using the mutated L247T $\alpha 7$ subtype (Revah *et al.*, 1991; Bertrand *et al.*, 1992). In particular, when tested on this mutated receptor, $\alpha 7$ antagonists can be classified into at least two main groups: the first includes the classical nicotinic antagonists (such as hexamethonium, d-Tub and dihydro- β -erythroidine), which elicit current responses; the second includes the selective $\alpha 7$ antagonists, (α Bgtx and MLA) which do not induce any currents but are capable of blocking the currents induced by hexamethonium, d-Tub and dihydro- β -erythroidine. Our results show that, when applied alone to L247T $\alpha 7$ mutant receptors at concentrations of even 500 times less than the IC_{50} of the $\alpha 7$ wild type, MG624 is still capable of giving rise to large currents that are blocked by low concentrations of MLA.

The current elicited by MG624 is linearly related to voltage and, as has been previously suggested by Bertrand *et al.*, 1992, incompatible with an open channel block mechanism. This effect distinguishes MG624 from both d-Tub and hexamethonium, both of which elicit currents that are reduced at hyperpolarized potentials. Furthermore, unlike that of 5-hydroxytryptamine (Palma *et al.*, 1996b), the action of MG624 on the L247T $\alpha 7$ receptor appears to be competitive in nature, since the receptor affinity for MG624 is influenced by ACh. The current elicited by MG624 in L247T $\alpha 7$ mutant receptors is blocked by the competitive antagonist MLA; moreover, these receptors show similar positive cooperativity despite the fact that they have a higher affinity for MG624 than for ACh. These findings indicate that MG624 activates L247T $\alpha 7$ receptors by acting on the same ACh site in the extracellular domain.

In conclusion, the MG624-induced competitive inhibition of the [^{125} I]- α Bgtx binding on $\alpha 7$ containing receptor (Gotti *et al.*, 1998), the linearity of the I-V relationship on the $\alpha 7$ receptors also in the presence of MG624, and the effects of ACh on the concentration-response relationship for MG624 in the mutated L247T $\alpha 7$, strongly suggest that MG624 is a selective competitive antagonist of the $\alpha 7$ receptor. The binding site of this drug only partially overlaps that of MLA (which blocks the effect of MG624 on the mutated receptor) because, unlike MLA, MG624 is capable of activating the mutated $\alpha 7$ receptor.

The binding studies showed that MG624 also binds to the chick neuronal $\alpha 4\beta 2$ and muscle $\alpha 1\beta 1\gamma\delta$ subtypes, and this was confirmed by its antagonist effect on oocyte-expressed subtypes. However, MG624 was respectively 30 and 27 times less potent on the $\alpha 4\beta 2$ and $\alpha 1\beta 1\gamma\delta$ subtypes than on the $\alpha 7$ subtype.

Whereas the K_i and IC_{50} for the $\alpha 7$ subtype were almost identical, we found a difference between the K_i values and the IC_{50} for the $\alpha 4\beta 2$ and $\alpha 1\beta 1\gamma\delta$ subtypes (see Table 1) and can only speculate that this may be due to the presence of some essential and still uncloned subunit(s) in native receptors that can influence the affinity of drug binding (although we do not have any proof of such a presence) and/or the variability in the results obtained when different expression systems are used for electrophysiological experiments (Covernton *et al.*, 1994; Lewis *et al.*, 1997; Fucile *et al.*, 1997).

There is increasing evidence that the brain contains a large number of pharmacologically distinct nicotinic AChR subtypes that may be involved in mediating specific functions and behaviours. Experimental findings show that the $\alpha 7$ receptor is certainly involved in the modulation of synaptic transmission, CNS development and sensory gating (reviewed in Albuquerque *et al.*, 1997; Role & Berg 1996; Gotti *et al.*, 1997a), but further *in vivo* studies are necessary in order to clarify the complete physiological role of this subtype.

In vivo studies of $\alpha 7$ -mediated functions are hampered by the limited availability of $\alpha 7$ -specific ligands. Although a number of nicotinic agonists have recently been discovered (GTS-21, ABT418, Epibatidine) (Decker *et al.*, 1995; Gotti *et al.*, 1997a), all of them are more potent towards the $\alpha 4\beta 2$ subtype than towards the $\alpha 7$ subtype. The characterization of anabaseine as a potent agonist of α Bgtx-sensitive receptors has recently been reported (Kem *et al.*, 1997), but it has low K_i and EC_{50} values not only for $\alpha 7$ -containing receptors, but also for the $\alpha 4\beta 2$ and muscle type receptors.

The currently available $\alpha 7$ -selective antagonists are: α Bgtx, MLA and α conotoxin IMI; α Bgtx and MLA bind to and block the $\alpha 7$ subtype with very high affinity, specificity and potency, and both are more potent than MG624 in blocking $\alpha 7$ -

containing receptors, with an IC_{50} of respectively 0.52 nM and 25 pM (Johnson *et al.*, 1995; Palma *et al.*, 1996a). α Conotoxin IMI also specifically blocks the $\alpha 7$ homomeric receptor with an IC_{50} of 220 nM (Johnson *et al.*, 1995) a value that is higher than that of α Bgtx and MLA but very close to that of MG624. Although they are very specific, due to their toxic effects, these toxins are not very easy to use in *in vivo* studies aimed at delineating the roles of different nicotinic AChR subtypes in normal physiology.

In conclusion, MG624 is a powerful and selective competitive antagonist for the $\alpha 7$ subtype, and could serve as a lead structure for the development of new drugs with even greater $\alpha 7$ selectivity.

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Lack of interaction between nitric oxide and the redox modulatory site of the NMDA receptor

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1 The inhibitory effects of nitric oxide (NO) on N-methyl-D-aspartate (NMDA) receptor function have been proposed to be mediated *via* the interaction of this gas with a redox-sensitive thiol moiety on the receptor. Here, we evaluated this suggested mechanism by examining the actions of various NO donors on native neuronal receptors as well as in wild-type and cysteine-mutated recombinant NMDA receptors expressed in Chinese hamster ovary (CHO) cells.

2 The NO donor N-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)ethanamine (NOC-12; 100 μ M) produced a rapid and readily reversible inhibition of whole-cell currents induced by NMDA (30 μ M) in cultured cortical neurons. The inhibition was apparent at all holding potentials, though a more pronounced block was observed at negative voltages. The effects of NOC-12 disappeared when the donor was allowed to expire. A similar receptor block was observed with another NO-releasing agent, S-nitroso-N-acetylpenicillamine (SNAP; 1 mM).

3 The blocking effects of NO released by SNAP, 3-morpholinosydnonimine (SIN-1; 1 mM), and 3-[2-hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propanamine (NOC-5; 100 μ M) on currents mediated by recombinant NR1/NR2B receptors were virtually indistinguishable from those observed on native receptors. Furthermore, mutating cysteines 744 and 798 of NR1, which constitute the principal redox modulatory site of the NR1/NR2B receptor configuration, did not affect the inhibition produced by NO.

4 The NR2A subunit may contribute its own redox-sensitive site. However, the effects of NO on NR1/NR2A receptors were very similar to those seen for all other receptor configurations evaluated. Hence, we conclude that NO does not exert its inhibition of NMDA-induced responses *via* a modification of any of the previously described redox-sensitive sites on the receptor.

Keywords: NMDA receptor; nitric oxide; redox modulatory site; cortical neurons; recombinant expression; patch-clamping

Abbreviations: CHO, Chinese hamster ovary; cys, cysteine; NMDA, N-methyl-D-aspartate; NO, nitric oxide; NOC-5, 3-[2-hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propanamine; NOC-12, N-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)ethanamine; NR, NMDA receptor; SIN-1, 3-morpholinosydnonimine; SNAP, S-nitroso-N-acetylpenicillamine

Introduction

Disulfide reducing agents can potentiate physiological responses mediated by the N-methyl-D-aspartate (NMDA) receptor while thiol oxidants can reverse the effects of reductants and, in some instances, depress native responses (Tang & Aizenman, 1993a). Several redox-active substances of endogenous origin can modulate NMDA receptor function (Aizenman, 1994), and further, it has been observed that the redox state of the receptor can change without the addition of exogenous reductants or oxidants *in vitro* (Aizenman *et al.*, 1989; Gozlan & Ben-Ari, 1995; Sinor *et al.*, 1997). Hence, NMDA receptor function in the brain may be modulated by redox substances, thereby affecting the physiological and pathophysiological consequences of receptor activation. Indeed, substances known to oxidize the redox modulatory site *in vitro* have neuroprotective and anticonvulsive activity *in vivo* (Jensen *et al.*, 1994; Quesada *et al.*, 1997). It has been suggested that more than one redox-sensitive site may be present in a functional NMDA receptor (Sullivan *et al.*, 1994; Köhr *et al.*, 1994; Brimecombe *et al.*, 1997; Arden *et al.*, 1998; but see Paoletti *et al.*, 1997). However, only one site has been unequivocally demonstrated to be formed by a pair of cysteines, cys-744 and cys-798, which are localized in an

extracellular region between two transmembrane domains in the NR1 subunit (Sullivan *et al.*, 1994).

Nitric oxide (NO), or a closely related derivative (Butler *et al.*, 1995), has been shown to inhibit NMDA receptor-mediated physiological responses in a number of preparations (Hoyt *et al.*, 1992; Manzoni *et al.*, 1992; Lei *et al.*, 1992; Fagni *et al.*, 1995). Several mechanisms have been proposed to account for this inhibition, including voltage-dependent channel block (Fagni *et al.*, 1995) and interference with down-stream calcium homeostatic processes (Hoyt *et al.*, 1992). However, a widely-held view is that the gas can directly interact with the receptor's redox modulatory site (Lei *et al.*, 1992), and specifically, with the one formed by cys-744 and cys-798 of NR1 (Stamler *et al.*, 1997). This hypothesis is based partly on sequence homology to proposed targets for NO in other proteins (Stamler *et al.*, 1997). The putative ensuing cysteine nitrosylation has been suggested to be responsible for the inhibition of the receptor-mediated responses (Lipton *et al.*, 1993; Stamler *et al.*, 1997). However, our group (Hoyt *et al.*, 1992), as well as others (Fagni *et al.*, 1995), previously suggested that the redox modulatory site does not, in fact, mediate any of the actions of NO at the NMDA receptor. In the present study, we re-examine the effects of NO in native, as well as in wild-type and cysteine mutated recombinant NMDA receptors in order to test directly the degree to which NO can

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modulate receptor function in the presence or absence of a functional redox site.

Methods

Tissue culture

All tissue culture reagents were purchased from Sigma (St. Louis, MO, U.S.A.), with the exception of iron-supplemented bovine calf serum (Hyclone Laboratories, Logan, UT, U.S.A.), and minimal essential medium, (Gibco BRL, Gaithersburg, MD, U.S.A.). Chinese hamster ovary cells (CHO-K1; ATTC CCL61) were grown in Ham's F-12 nutrient medium with 10% foetal bovine serum and glutamine (1 mM) at 37°C in 5% CO₂. These cells were passaged every 2 days at a 1 : 10 dilution for a maximum of 30 times. Cerebral cortices were dissociated from E-16 Sprague Dawley rats according to methods described previously (Hartnett *et al.*, 1997). After dissociation, cells were plated at a density of $3-5 \times 10^5$ cells ml⁻¹ in growth medium (v/v 80% Dulbecco's modified Eagle's minimum, 10% Ham's F12 nutrient mixture, 10% heat-inactivated iron-supplemented bovine calf serum, HEPES 25 mM, penicillin 24 U ml⁻¹, streptomycin 24 mg ml⁻¹ and L-glutamine 2 mM) into 35-mm tissue culture dishes containing 12-mm poly-L-lysine-coated glass coverslips. The cells were fed three times a week with growth medium. Non-neuronal cell proliferation was inhibited with cytosine arabinoside (2 mM) at 15 days *in vitro* after which the growth medium was changed to 2% serum and no F-12. Cells were maintained at 37°C in 5% CO₂. Neurons were utilized for electrophysiological measurements between 25–29 days *in vitro*.

Transfection protocol

Generation of mammalian expression vectors for the NMDA receptor (NR) subunits (Boeckman & Aizenman, 1994, 1996) and green fluorescent protein (Brimecombe *et al.*, 1997) have been previously described. The double mutant NR1 (C744A, C798A) plasmid was a kind gift from Dr S. Traynelis (Emory University, Atlanta, GA, U.S.A.). CHO cells were seeded at 3×10^5 cells well⁻¹ in 6-well plates (35-mm wells) containing CHO media approximately 24 h prior to transfection. Serum-free CHO media (1 ml) containing 1.3 mg of total DNA and 6 ml of LipofectAMINE (Gibco-BRL) was added to each well. A ratio of 1 : 4.3 was used for GFP to total NR plasmid DNA, and a ratio of 1 : 3 was used for NR1 to NR2 subunits (Cik *et al.*, 1993). After a 4 h incubation at 37°C in 5% CO₂ with the transfection solution, cells were re-fed with serum-containing CHO medium plus 300 µM ketamine (Boeckman & Aizenman, 1996). Recordings were performed approximately 2 days after transfection.

Whole-cell voltage-clamp recordings

Electrophysiological measurements were obtained from cortical neurons and transiently transfected CHO cells using the whole-cell configuration of the patch-clamp technique. Methods for data acquisition and analysis have been previously described (Tang & Aizenman, 1993a). The extracellular recording solution consisted of (in mM) NaCl 150, KCl 2.8, CaCl₂ 1.0, HEPES 10 and 10 µM glycine (pH 7.2). For cortical neuron recordings, 0.25 µM tetrodotoxin (CalBiochem, La Jolla, CA, U.S.A.) was also added to this solution. Patch electrodes (2 MΩ) were filled with (in mM) CsF 140, EGTA 10, CaCl₂ 1, and HEPES 10 (pH 7.2). Drugs were

applied onto cells by a multi-barrel fast perfusion system (Warner Instrument Corp., Hamden, CT, U.S.A.). Freshly-prepared stock solutions of 3-[2-Hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propanamine (NOC-5; CalBiochem) and N-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)ethanamine (NOC-12; CalBiochem) were made in NaOH (10 mM), diluted in external recording solution and used immediately. 3-Morpholinosydnonimine (SIN-1; BIOMOL, Plymouth Meeting, PA, U.S.A.) and S-nitroso-N-acetylpenicillamine (SNAP; BIOMOL) were dissolved directly in external recording solution and used immediately. When necessary, NO was allowed to expire by allowing the NO donor-containing solution to be in direct contact with air and exposed to light overnight.

Results

Whole-cell currents were obtained from cortical neurons during the application of NMDA (30 µM) alone or in the presence of freshly prepared NOC-12 (100 µM) at several holding potentials. The half-life of NO release by this compound is 327 min at 22°C in physiological solutions, according to the supplier (CalBiochem). We observed that inclusion of the NO donor produced a readily reversible inhibition of the NMDA-induced responses at all holding voltages tested (Figure 1A); Current-voltage relationships were generated by measuring the steady state current amplitude in the absence or presence of NOC-12 (Figure 1B); a more pronounced block was apparent at negative membrane potentials when compared to positive voltages. The block at -60 mV for this drug was near 25% ($n=5$; Table 1). In a separate group of neurons ($n=6$), we tested for the ability of expired NOC-12 to block the NMDA receptor-mediated responses. As is evident from Figure 1C, the spent donor did not influence the currents to any measurable extent. These results corroborate the previously noted actions of other NO donors such as SIN-1, SNAP and isosorbide dinitrate which demonstrate that the gas can reversibly block the NMDA receptor in both a voltage-dependent and voltage-independent manner, and that the use of NO-expired donors, or NO scavengers such as haemoglobin, can prevent the actions of the gas on the responses mediated by this receptor (Fagni *et al.*, 1995). Similar NMDA receptor inhibition was observed in the present investigation with the use of another NO donor, SNAP (1 mM; $n=5$), on a separate group of cortical neurons (Table 1).

Table 1 Comparison of the NO-mediated block of whole-cell NMDA currents (-60 mV)*

Receptor subtype	SIN-1 (1 mM)	SNAP (1 mM)	NOC-12 (0.1 mM)	NOC-5 (0.1 mM)
Neuronal		78.6 ± 5.1 (5)	77.0 ± 5.3 (5)	
NR1/NR2B	77.7 ± 7.7 (6)	67.0 ± 4.7 (4)		59.0 ± 9.5 (3)
NR1(C744A, C798A)/NR2B	66.7 ± 8.9 (5)	66.6 ± 5.5 (4)		
NR1/NR2A	70.5 ± 8.6 (8)	70.8 ± 10.2 (4)		

*Values shown represent the mean ± s.d. per cent of control current remaining in the presence of the NO donor indicated. The number of cells tested for each conditions is in parentheses.

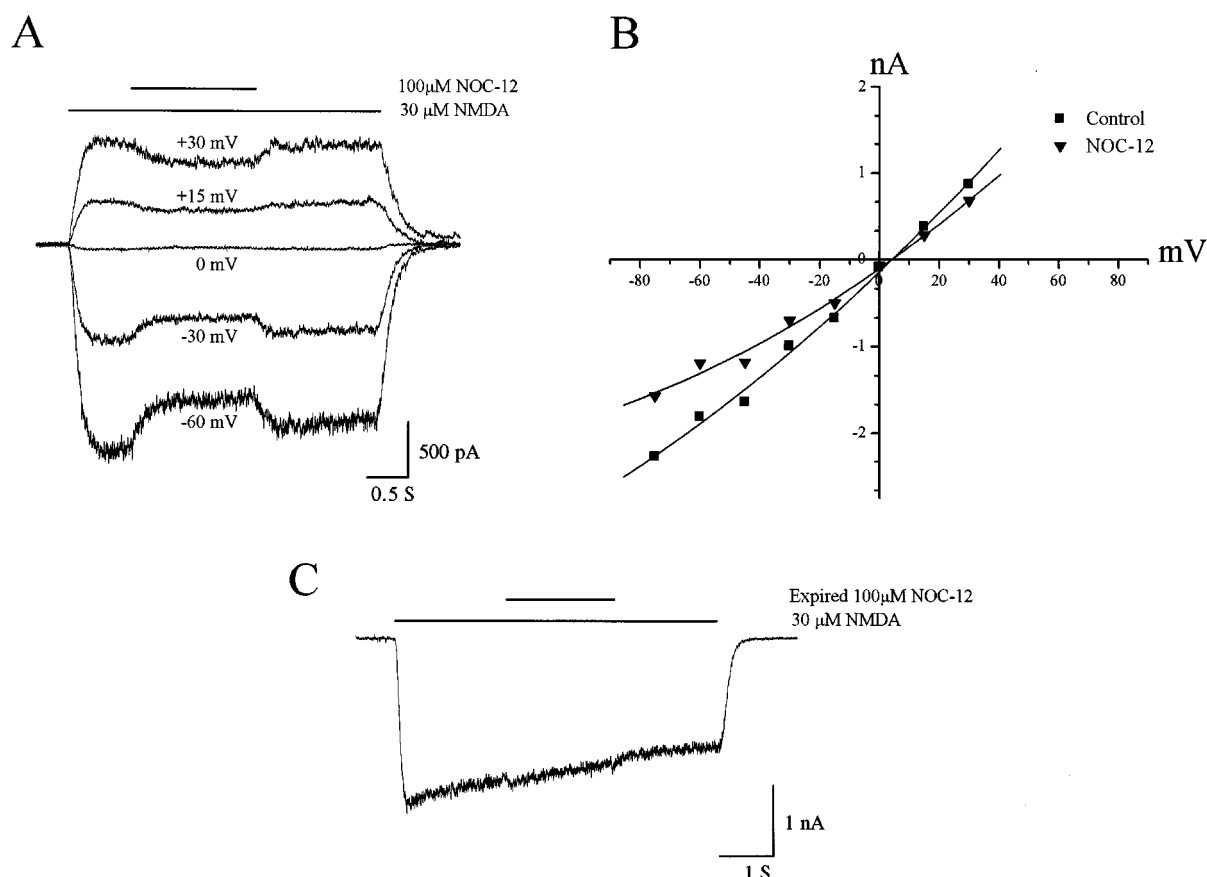


Figure 1 Inhibition of NMDA-activated currents by NO in neurons. (A) Representative whole-cell current traces obtained at -60 , -30 , 0 , $+15$ and $+30$ mV in rat cortical neurons in culture during activation by NMDA ($30 \mu\text{M}$) in the absence and presence of freshly-prepared NOC-12 ($100 \mu\text{M}$). (B) Current-voltage relationship of steady-state NMDA responses in the absence and presence of NOC-12 for the same cell shown in A. (C) Whole-cell current trace during activation by NMDA at -60 mV in the absence and presence of NOC-12 ($100 \mu\text{M}$) which had been allowed to expire overnight.

We next turned our attention to the effects of NO on NMDA-induced responses mediated by recombinant receptors. We chose first to utilize the NR1/NR2B receptor configuration as cys-744 and cys-798 of NR1 constitute the principal redox-sensitive site of this subunit combination (Sullivan *et al.*, 1994). As seen with native receptors in neurons, NO, released by the donors NOC-5 ($100 \mu\text{M}$; $n=3$), SIN-1 (1 mM ; $n=6$), and SNAP (1 mM ; $n=4$), produced similar block of the whole cell currents mediated by this receptor configuration (Table 1). The half-life of NO release by NOC-5 is only 93 min (CalBiochem) and thus may transiently produce a higher concentration of the gas than a comparable amount of another donor. This likely accounts for the slightly greater degree block observed with this donor (Table 1). Figure 2A shows the typical current block observed with the NO donor SIN-1. The current-voltage relationship obtained from NR1/NR2B receptor-mediated responses (Figure 2B) demonstrate that the voltage-dependence of the inhibition is similar to what was observed with native neuronal receptors. Although SIN-1 also produces superoxide anions, a previous investigation reported that the effects of this donor on NMDA receptor-mediated currents are not affected by the addition of superoxide dismutase and catalase (Fagni *et al.*, 1995). A separate set of CHO cells were then transfected with the double mutant NR1(C744A, C798A) together with NR2B. In these, the pattern NMDA response inhibition produced by the NO donors SIN-1 (Figure 2B,C; $n=5$) and SNAP ($n=4$) was

virtually indistinguishable to that seen in the wild-type receptors and native channels (Table 1). Thus, the presence or absence of the redox site on NR1 had no influence on the actions of NO on NR2B-containing receptors.

The redox sensitivity of NR1/NR2A receptors is not critically dependent on cys-744 and cys-798 of NR1 (Sullivan *et al.*, 1994; Arden *et al.*, 1998). Although individual thiol-reactive residues in NR2A have not yet been localized (Köhr *et al.*, 1994), results of a recent study from our laboratory suggest that this subunit does indeed have unique redox attributes (Arden *et al.*, 1998). However, the effects of both SIN-1 ($n=8$) and SNAP ($n=4$) on this subunit configuration were almost identical to those seen in neuronal receptors and in wild-type and cysteine mutated NR1/NR2B receptors (Table 1). This suggests that the NO inhibition of NMDA receptor currents is not subunit dependent.

Discussion

Cysteine residues 744 and 798 on NR1 appear to constitute the principal redox modulatory site of receptors composed of this subunit when it is co-expressed with either NR2B, NR2C or NR2D (Sullivan *et al.*, 1994). Receptors which are assembled with NR1 and NR2A contain a second redox sensitive site which has yet to be localized at the molecular level (Köhr *et al.*, 1994; Arden *et al.*, 1998). Our present results suggest that NO

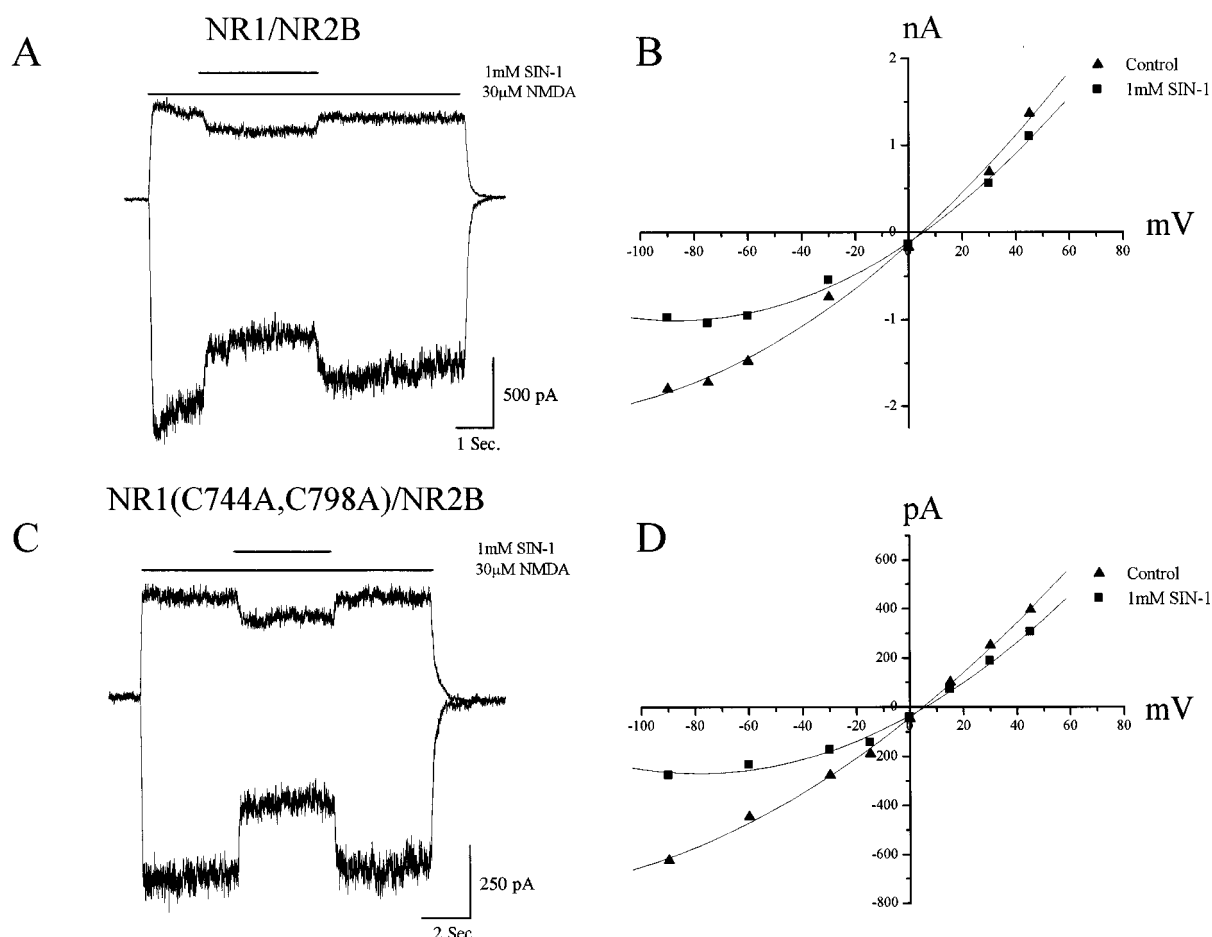


Figure 2 Inhibition of NMDA-activated currents by NO in transfected CHO cells does not require the presence of a functional redox site. (A) Representative whole-cell currents obtained at -60 and $+30$ mV in a CHO cell expressing NR1/NR2B receptors during application of NMDA ($30 \mu\text{M}$) in the absence and presence of SIN-1 (1 mM). (B) Current-voltage relationship of steady-state NMDA responses in the absence and presence of NOC-12 for the same cell shown in A. (C) Representative whole-cell currents obtained in a CHO cell expressing NR1 (C744A, C798A)/NR2B receptors, which lack a functional redox site, during application of NMDA with or without SIN-1. (D) Current-voltage relationship of steady-state NMDA responses in the absence and presence of NOC-12 for the same cell shown in C.

does not interact with the redox modulatory site formed by cys-744 and cys-798 of NR1. In fact, given the almost identical effects of NO on native receptors and on recombinant receptors composed of either NR1/NR2A or NR1/NR2B, it is unlikely that the gas interacts with any of the previously investigated sites on the NMDA receptor which are sensitive to redox reagents. This is in direct contrast to the widely accepted mechanism of NO inhibition of NMDA receptors that has been suggested by other investigators (Lei *et al.*, 1992; Lipton *et al.*, 1993; Stamler *et al.*, 1997).

Our conclusion, however, is not altogether surprising as the actions of NO on the NMDA receptor do not seem to fit the pattern of effects seen for other agents known to interact with the redox site. For instance, the inhibitory effects of thiol oxidants on the NMDA receptor do not spontaneously reverse (Aizenman *et al.*, 1989; Tang & Aizenman, 1993a). That is, a subsequent treatment with a disulfide reducing agent is necessary to reverse the effects of oxidants. In contrast, as seen in this and prior studies (Hoyt *et al.*, 1992; Manzoni *et al.*, 1992; Lei *et al.*, 1992; Fagni *et al.*, 1995), the effects of NO appear to be readily and spontaneously reversible. Inhibition by NO is also present even after prior oxidation of the receptor with a thiol agent (Hoyt *et al.*, 1992). Additionally, previous

studies have shown that after chemical alkylation and subsequent functional elimination of the redox site in neurons (Tang & Aizenman, 1993a), the inhibitory effects of NO remain virtually intact (Hoyt *et al.*, 1992; Fagni *et al.*, 1995). Finally, redox agents do not alter the conductance of native or recombinant single NMDA receptor channels (Tang & Aizenman, 1993a; Brimecombe *et al.*, 1997), while one of the principal reported actions of NO on unitary currents is to decrease their amplitude (Fagni *et al.*, 1995).

Lei *et al.* (1992) first suggested that NO could interact with the redox modulatory site of the NMDA receptor. This group reported that NO donors could depress NMDA receptor-mediated responses in neurons in a partially reversible manner. This reversibility, inconsistent with an effect at the redox site, was attributed to a rapid decomposition of putative S-nitrosothiol groups formed by the interaction of NO with the cysteine moieties that formed this site. However, these investigators also noted that NO could reversibly depress responses even after exposure of the receptor to thiol oxidants, which would eliminate any available free thiols for NO to nitrosylate. Lei and co-workers argued that NO may have reacted with thiols spared by the oxidants. However, this argument is not consistent with the fact that (i) oxidants

depress NMDA-induced responses by interacting with the redox site, and (ii) this inhibitory actions of thiol oxidants have been shown to be completely abolished following alkylation of the receptor (Tang & Aizenman, 1993a,b; Aizenman *et al.*, 1994). As mentioned earlier, in our previous studies (Hoyt *et al.*, 1992) as well as in those of others (Fagni *et al.*, 1995), it was observed that alkylation did not alter, to any extent, the inhibitory effects of NO. Indeed, a close look at the data presented by Lei *et al.* (1992) reveal that the effects of NO in depressing whole-cell currents before (38%) and after (26%) treatment with an alkylating agent are not dramatically different. Hence, we must conclude that the inhibitory effects

of NO on NMDA receptor function appear not to be determined by the subunit composition of the receptor, nor by the presence or absence of the redox site on NR1. The present findings refute the hypothesis that cys-744 and cys-798 on the NR1 subunit are the target of NO (Stamler *et al.*, 1997), at least with respect to the functional inhibition of the channel.

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Modulation of acute and chronic inflammatory processes by cacospongionolide B, a novel inhibitor of human synovial phospholipase A₂

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1 Cacospongionolide B is a novel marine metabolite isolated from the sponge *Fasciospongia cavernosa*. In *in vitro* studies, this compound inhibited phospholipase A₂ (PLA₂), showing selectivity for secretory PLA₂ (sPLA₂) versus cytosolic PLA₂ (cPLA₂), and its potency on the human synovial enzyme (group II) was similar to that of manoalide.

2 This activity was confirmed *in vivo* in the 8 h zymosan-injected rat air pouch, on the secretory enzyme accumulating in the pouch exudate. Cacospongionolide B, that is bioavailable when is given orally, reduced the elevated levels of sPLA₂ present in paw homogenates of rats with adjuvant arthritis.

3 This marine metabolite showed topical anti-inflammatory activity on the mouse ear oedema induced by 12-*O*-tetradecanoylphorbol acetate (TPA) and decreased carrageenin paw oedema in mice after oral administration of 5, 10 or 20 mg kg⁻¹.

4 In the mouse air pouch injected with zymosan, cacospongionolide B administered into the pouch, induced a dose-dependent reduction in the levels of eicosanoids and tumour necrosis factor α (TNF α) in the exudates 4 h after the stimulus. It also had a weak effect on cell migration.

5 The inflammatory response of adjuvant arthritis was reduced by cacospongionolide B, which did not significantly affect eicosanoid levels in serum, paw or stomach homogenates and did not induce toxic effects.

6 Cacospongionolide B is a new inhibitor of sPLA₂ *in vitro* and *in vivo*, with anti-inflammatory properties in acute and chronic inflammation. This marine metabolite was active after oral administration and able to modify TNF α levels, and may offer an interesting approach in the search for new anti-inflammatory agents.

Keywords: Inflammation; phospholipase A₂; rat and mouse air pouch; adjuvant arthritis; manoalide; cacospongionolide B

Abbreviations: COX, cyclo-oxygenase; cPLA₂, cytosolic PLA₂; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; iNOS, inducible nitric oxide synthase; IP₃, inositol triphosphate; IL-1 β , interleukin-1 β ; LDH, lactate dehydrogenase; LTB₄, leukotriene B₄; LPS, lipopolysaccharide; 5-LO, 5-lipoxygenase; MAC-1, CD11b/CD18; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NSAID, non-steroidal anti-inflammatory drug; PTK, palmityl trifluoromethyl ketone; PBS, phosphate buffered saline; PLA₂, phospholipase A₂; PLC, phospholipase C; PG, prostaglandin; PGD₂, prostaglandin D₂; PGE₂, prostaglandin E₂; sPLA₂, secretory phospholipase A₂; TPA, 12-*O*-tetradecanoylphorbol acetate; TXA₂, thromboxane A₂; TXB₂, thromboxane B₂; TNF α , tumour necrosis factor α

Introduction

The activation of different phospholipases is a critical step in the biosynthesis of lipid mediators. Phospholipase A₂ (PLA₂) is a class of enzymes that hydrolyze the acyl group from the *sn*-2 position of glycerophospholipids, yielding free fatty acids and lysophospholipids. These products or their metabolites are bioactive lipids modulating different cellular processes. Mammalian cells contain diverse PLA₂ which may play a distinct role in cell activation and signal transduction. Moreover, in pathologic states, increased PLA₂ activity causes alteration of membrane structure and function as well as an excessive production of lipid mediators and toxic species that contributes to tissue injury. Secretory PLA₂ (sPLA₂, groups I, II, III and V), cytosolic PLA₂ (cPLA₂, group IV) and calcium-independent PLA₂ have been studied (for review see Serhan *et al.* (1996); Dennis (1997)). Calcium-independent PLA₂ is present in the myocardium and other tissues. This enzyme may regulate the incorporation of arachidonic acid into

membrane phospholipids in P388D₁ macrophages (Balsinde *et al.*, 1995) and could participate in arachidonic acid release and cell spreading in murine peritoneal macrophages (Teslenko *et al.*, 1997).

It has been reported that cPLA₂ play an important role in arachidonic acid release in a number of cell systems, e.g. human platelets stimulated with thrombin (Bartoli *et al.*, 1994) or calcium ionophore (Riendeau *et al.*, 1994), permeabilized human neutrophils (Bauldry & Wooten, 1996) or mouse peritoneal macrophages challenged with zymosan or 12-*O*-tetradecanoylphorbol acetate (TPA) (Qiu & Leslie, 1994). Inflammatory cytokines have been shown to induce cPLA₂, resulting in high levels of eicosanoids in airway epithelial cells (Wu *et al.*, 1997), rheumatoid synovial fibroblasts (Hulkower *et al.*, 1994) or mouse osteoblasts (Chen *et al.*, 1997).

Group II sPLA₂ can act as a signalling agent that mediates cell growth induced by interleukin-1 β (IL-1 β) (Wada *et al.*, 1997). In addition, it has a role in cell activation and contributes to the inflammatory response. sPLA₂ activation

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may participate in signal transduction events such as CD11b/CD18 (MAC-1) expression on the surface of activated human neutrophils, and adhesion or degranulation (Takasaki *et al.*, 1996; Jacobson & Schrier, 1993). This enzyme activity secreted at inflammatory sites becomes associated with cell surfaces and hydrolyzes phospholipids, thus releasing arachidonic acid, which enters the cell and participate in the increased generation of inflammatory lipid mediators (Pfeilschifter *et al.*, 1993; Miyake *et al.*, 1994). In fact, administration of different types of sPLA₂ can induce or amplify inflammatory responses in animals (Vishwanath *et al.*, 1988; Tanaka *et al.*, 1995; Cirino *et al.*, 1994). Interestingly, inflammatory cytokines increase group II PLA₂ synthesis and secretion by rheumatoid synovial fibroblasts and other cell types (Pfeilschifter *et al.*, 1993; Bomalaski & Clark, 1993). Thus, IL-1 β induces an increase in group II sPLA₂ gene expression, but does not increase cPLA₂ gene expression or activity, and it provokes a parallel increase in prostaglandin E₂ (PGE₂) production by rabbit articular chondrocytes (Jacques *et al.*, 1997). Group II sPLA₂ has been reported to release arachidonic acid in some systems and may provide the substrate for both cyclooxygenase (COX) and 5-lipoxygenase (5-LO) product formation in mouse bone marrow-derived mast cells (Fonteh *et al.*, 1994). In contrast, PLA₂ secreted by guinea-pig peritoneal macrophages does not participate in the synthesis of PGE₂ accumulating in the media (Marshall *et al.*, 1994).

On the other hand, exocytosis of sPLA₂ could modulate the activity of cPLA₂ by initiating the formation of leukotriene B₄ (LTB₄), which after release stimulates its own receptor, thus leading to activation of cPLA₂ in neutrophils (Wijkander *et al.*, 1995). Exogenously added group I PLA₂ is also believed to be involved in arachidonic acid release (Hara *et al.*, 1991), in some cases accompanied by induction of group II PLA₂, and recently a group V sPLA₂ has been reported to participate in immediate prostanoid generation in the mouse macrophage cell line P388D₁ (Balboa *et al.*, 1996).

Arachidonic acid mobilization can be dependent on both types of PLA₂ in some systems, as in the case of delayed PGD₂ generation by COX-2 in rat peritoneal macrophages stimulated by lipopolysaccharide (LPS) (Naraba *et al.*, 1998), as well as in receptor-stimulated P388D₁ macrophages (Balsinde & Dennis, 1996), and human umbilical vein endothelial cells (Murakami *et al.*, 1993). In human monocytes stimulated by ionophore or zymosan, cPLA₂ would participate preferentially in the release of arachidonic acid for prostaglandin (PG) synthesis, whereas sPLA₂ probably releases the substrate for LT synthesis (Marshall *et al.*, 1997).

Marine organisms are a rich source of molecules exhibiting PLA₂ inhibitory properties *in vitro*, mainly on secretory enzymes (for review, see Potts *et al.*, 1992). Some of these compounds have been found to reduce experimental inflammatory responses, preferentially after topical application. We have examined the PLA₂ inhibitory activity of cacospongionolide B (Figure 1), a new marine metabolite isolated from the Mediterranean sponge *Fasciospongia cavernosa*. The results of our studies demonstrate that cacospongionolide B is a potent inhibitor of sPLA₂. We have also assessed its effects on models of acute and chronic inflammation.

Methods

sPLA₂ assay

sPLA₂ was assayed by using a modification of the method of Franson *et al.* (1974). *E. coli* strain CECT 101 were seeded in

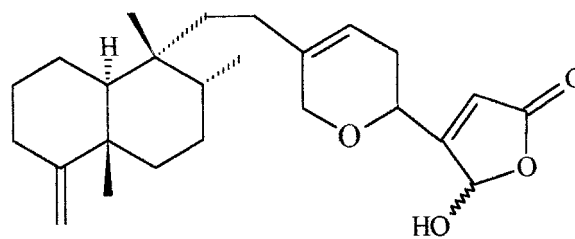


Figure 1 Chemical structure of cacospongionolide B.

medium containing 1% tryptone, 0.5% NaCl and 0.6% sodium dihydrogen orthophosphate, pH 5.0, and grown for 6–8 h at 37°C in the presence of 5 μ Ci ml⁻¹ [³H]-oleic acid (sp. act. 10 Ci mmol⁻¹). After centrifugation at 2500 $\times$ g for 10 min, the cells were washed in buffer (0.7 M Tris-HCl, 10 mM CaCl₂, 0.1% bovine serum albumin, BSA, pH 8.0), resuspended in saline and autoclaved for 30–45 min. At least 95% of the radioactivity was incorporated into phospholipids. *Naja naja* venom, porcine pancreatic, bee venom and human recombinant synovial enzymes were diluted in 10 μ l of 100 mM Tris-HCl, 1 mM CaCl₂ buffer, pH 7.5. Supernatants (10 μ l) of exudates from zymosan-injected rat air pouch (Payá *et al.*, 1996) were also used as a source of sPLA₂. Enzymes were preincubated at 37°C for 5 min with 2.5 μ l of test compound solution or its vehicle in a final volume of 250 μ l. Incubation proceeded for 15 min in the presence of 10 μ l of autoclaved oleate-labelled membranes and was terminated by addition of 100 μ l ice-cold solution of 0.25% BSA in saline to a final concentration of 0.07% w/v. After centrifugation at 2500 $\times$ g for 10 min at 4°C, the radioactivity in the supernatants was determined by liquid scintillation counting.

cPLA₂ assay

cPLA₂ was prepared from human monocytic U937 cells (Cell Collection, Department of Animal Cell Culture, C.S.I.C., Madrid, Spain) grown in the above medium which were disrupted by sonication in 10 mM HEPES buffer pH 7.4, containing 0.32 M sucrose, 100 μ M EDTA, 1 mM dithiothreitol, 2 mM phenylmethylsulphonylfluoride and 100 μ M leupeptin. The homogenated cells were centrifuged at 2000 $\times$ g for 10 min at 4°C and the resulting supernatant was further centrifuged at 100,000 $\times$ g for 100 min at 4°C to obtain the cytosolic fraction. cPLA₂ activity was measured as the release of radiolabelled arachidonic acid according to the method of Clark *et al.* (1990). 1-Palmitoyl-2-[¹⁴C]-arachidonoyl-*sn*-glycero-3-phosphocholine (57.0 mCi mmol⁻¹, 2 $\times$ 10⁶ c.p.m.) was dried under nitrogen, then suspended in 1 ml of 100 mM glycine buffer pH 9.0 containing 200 μ M Triton X-100, 10 mM CaCl₂, 0.25 mg ml⁻¹ BSA and 40% v/v glycerol. The suspension was then sonicated to form mixed micelles of phospholipid and Triton X-100. The reaction was started by adding the enzyme solution (approximately 24 μ g protein of cytosolic fraction from human monocytes) to a final volume of 100 μ l of the assay mixture which contained 1 mM CaCl₂, 2 mM 2-mercaptoethanol, 150 mM NaCl, 40% glycerol, 1 mg ml⁻¹ BSA and 50 mM HEPES pH 9.0. The substrate consisted of 5 μ l of micelles (10⁴ c.p.m.) containing dioleoyl glycerol at a molar ratio 2:1 (Kramer *et al.*, 1987). Test compounds were dissolved in methanol and added to the reaction mixture just before the addition of the enzyme solution. The final concentration of methanol in the reaction mixture was less than 1%, which showed no effect on the

enzyme activity. The reaction was stopped after a 60 min incubation period at 37°C by mixing with 0.5 ml of isopropyl alcohol/heptane/0.5 M H₂SO₄ (10:5:1). Heptane (0.7 ml) and water (0.2 ml) were then added, and the solution was vigorously mixed for 15 s. The heptane phase was mixed with 100 mg silica gel 60 (Merck, 70-230 mesh) and centrifuged, and the radioactivity in each supernatant was measured (Zhang *et al.*, 1991).

Preparation of human leukocytes

The citrated blood of healthy volunteers was centrifuged at 200 × *g* for 15 min at room temperature. The platelet-rich plasma was removed, and the leukocytes contained in the residual blood were isolated by sedimentation with 2% (w/v) dextran in 0.9% NaCl at room temperature. The supernatant was centrifuged at 1200 × *g* for 10 min at 4°C. Contaminating erythrocytes were lysed by hypotonic treatment. The pellet was resuspended in phosphate buffered saline (PBS), and Ficoll-hypaque was layered under the cell mixture. The cell gradient mixture was centrifuged at 400 × *g* for 40 min at 20°C. Neutrophils were separated and resuspended in PBS containing 1.26 mM Ca²⁺ and 0.9 mM Mg²⁺ (Bustos *et al.*, 1995). Viability was greater than 95% by the Trypan blue exclusion test. The monocyte and lymphocyte layer was removed and pelleted by centrifugation. The cell pellet was resuspended in RPMI-1640 media, pH 7.4, with 10% foetal bovine serum, 2 mM L-glutamine, 50 u ml⁻¹ penicillin and 50 µg ml⁻¹ streptomycin and was incubated at a cell density of 10⁷ ml⁻¹ in 60/15 mm tissue culture dishes. The cells were allowed to adhere for 2 h at 37°C in a 5% CO₂ atmosphere incubator. The nonadherent cells were removed by vacuum suction of media followed by two washes with 1 ml of RPMI-1640. The adherent cells resulted in a greater than 90% pure monocyte population as assessed by differential staining.

Cytotoxicity assays

The cytoplasmic marker enzyme lactate dehydrogenase (LDH) (Bergmeyer & Bernt, 1974) and the mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Gross & Levi, 1992) were used to assess the possible cytotoxic effect of cacospongionolide B on human neutrophils.

Phospholipase C (PLC)

Human neutrophils (1 × 10⁷ cells ml⁻¹) were suspended in HBSS with Ca²⁺ and Mg²⁺ and preincubated with test drugs for 5 min at 37°C, before the reaction was initiated by adding 1 µM *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) for 5 min at 37°C. Inositol triphosphate (IP3) was quantified by the method of Palmer (1989).

Synthesis of LTB₄ by high speed supernatants from human neutrophils

High speed (100,000 × *g*) supernatants from sonicated human neutrophils were obtained as previously described (Tateson *et al.*, 1988). Aliquots (50 µg of protein/tube) in PBS containing 2 mM CaCl₂ were incubated with 5 µM arachidonic acid at 37°C for 5 min, in the presence of test compounds or vehicle. The samples were then heated at 90°C for 5 min and centrifuged at 10,000 × *g* at 4°C for 30 min. The LTB₄ levels in supernatants were measured by radioimmunoassay (Moroney *et al.*, 1988).

Cyclo-oxygenase-1

Human platelets were sonicated at 4°C in an ultrasonicator at maximum potency. Microsomes were prepared by centrifugation at 2000 × *g* for 5 min at 4°C followed by centrifugation of the supernatant at 100,000 × *g* for 100 min at 4°C. Microsomes (20 µg of protein/tube) were incubated for 30 min at 37°C in 50 mM Tris HCl, pH 7.4 with 5 µM arachidonic acid and test compound or vehicle in the presence of 2 µM hematin and 1 mM L-tryptophan. The reaction was terminated boiling the samples for 5 min and PGE₂ levels were determined by radioimmunoassay (Moroney *et al.*, 1988).

Cyclo-oxygenase-2

Human monocytes cells were resuspended in RPMI-1640 culture medium containing aspirin (300 µM) and incubated at 37°C for 2 h. The cells were washed twice, resuspended in RPMI-1640 with 10% foetal bovine serum and incubated with *E. coli* lipopolysaccharide (10 µg ml⁻¹) at 37°C for 24 h (Grossman *et al.*, 1995). After centrifugation the cells were sonicated at 4°C in an ultrasonicator at maximum potency, and microsomes were prepared as above. Microsomes (40 µg of protein/tube) were used as a source of cyclo-oxygenase-2 and reactions were carried out in the same conditions as above. PGE₂ synthesis was determined by radioimmunoassay (Moroney *et al.*, 1988).

Inducible nitric oxide synthase (iNOS) assay

NOS activity was induced by i.p. injection of LPS (2 mg kg⁻¹) to rats. After 24 h the animals were killed and livers were excised and homogenated in 10 mM HEPES, pH 7.4, containing saccharose (0.32 M), EDTA (100 µM), dithiothreitol (1 mM), phenylmethylsulfonyl fluoride (1 mg ml⁻¹) and leupeptin (10 µg ml⁻¹) (Knowles *et al.*, 1990). The homogenate was centrifuged at 1200 × *g* for 10 min at 4°C, followed by centrifugation of the supernatant at 100,000 × *g* for 100 min at 4°C. NOS activity was determined in supernatants by monitoring the conversion of L-[³H]-arginine to L-[³H]-citrulline, (Mitchell *et al.*, 1991). Samples (40 µg protein) were incubated at room temperature for 60 min with 100 µl of the above buffer in the presence of NADPH (1 mM) and a mixture of unlabelled and L-[³H]-arginine (10 µM, 1 µCi ml⁻¹). Incubations were terminated by the addition of 20 mM HEPES (1 ml, pH 5.5) containing 1 mM EGTA and 1 mM EDTA. L-[³H]-citrulline was separated from arginine by adding 1.5 ml of a 1:1 suspension of Dowex (50 W) in water. Radioactivity was measured in supernatants by liquid scintillation counting.

Rat air pouch

Male Wistar rats (120–150 g) were used. Air pouches were formed as previously described (Edwards *et al.*, 1981). The animals were anaesthetized with ethyl ether and given a 20 ml injection of sterile air in the subcutaneous tissue of the back, and 3 days later 10 ml of sterile air was injected into the same cavity. After 3 days 1 ml of sterile saline (saline group), 1 ml of 1% (w/v) zymosan in saline + 10 µl ethanol (zymosan control group) or 1 ml of 1% w/v zymosan in saline + test drug (dissolved in 10 µl of ethanol: treated groups), was administered into the air pouch. After 8 h, rats were sacrificed and the exudate was collected in 1 ml of saline. Leukocytes in exudate fluids were counted by Coulter counter. After centrifugation of the exudate at 1200 × *g* at 4°C for 10 min, the supernatants were used to measure PLA₂ activity as above. Protein was

quantified by the Bradford technique (Bradford, 1976) using BSA as standard.

Mouse ear oedema

The protocols were approved by the institutional Animal Care and Use Committee. All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals. TPA (5 µg) dissolved in 20 µl of acetone was applied in 10 µl volumes to both inner and outer surfaces of the right ear of Swiss mice (20–25 g). Test compounds were applied topically in acetone before TPA administration. The left ear (control) received only acetone. The animals were killed by cervical dislocation after 4 h, and equal sections of both ears were punched out and weighed. The increase in the weight of the right ear punch over that of the left indicated the oedema (Carlson *et al.*, 1985). The ear sections were homogenized in 750 µl saline, and after centrifugation at $10,000 \times g$ for 15 min at 4°C, the myeloperoxidase activity was measured in aliquots of supernatants. The reaction mixture contained 50 µl supernatant, 150 µl phosphate buffered saline, 20 µl 0.22 M NaH_2PO_4 pH 5.4, 20 µl 0.026 (v/v) % H_2O_2 and 20 µl 18 mM tetramethylbenzidine in 8% (v/v) aqueous dimethylformamide. After 10 min reaction at 37°C, 30 µl 1.46 M sodium acetate, pH 3.0 was added and absorbance at 620 nm was read using a microtiter plate reader (De Young *et al.*, 1989).

Mouse paw oedema

Swelling was induced following a modification of the technique of Sugishita *et al.* (1981). Female Swiss mice (20–25 g) were fasted for 12 h with free access to water. Drugs or vehicle (ethanol, tween 80, distilled water: 5/5/90, v/v/v) were administered p.o. (0.5 ml) 1 h before the injection of carrageenin (0.05 ml; 3% w/v in saline) into the subplantar area of the right hind paws of groups of six animals. The volumes of injected and contralateral paws were measured at 1, 3 and 5 h after induction of oedema by using a plethysmometer (Ugo Basile, Comerio, Italy). The volume of oedema was expressed for each animal as the difference between the carrageenin-injected and contralateral paws.

Mouse air pouch

Female Swiss mice (25–30 g) were anaesthetized with ether, and 10 ml of sterile air was injected into the subcutaneous tissue of the back. Three days later, pouches were reinflated with 5 ml of sterile air. In 6 days-old air pouches, mice were administered saline (saline group), zymosan + vehicle (control group) or zymosan + test drug, as indicated in the rat air pouch method. Four hours after administration, the animals were killed by cervical dislocation, and the exudate in the pouch was collected with 1 ml of saline (Edwards *et al.*, 1981). Leukocytes present in exudates were measured using a Coulter counter. After centrifugation of exudates at $1200 \times g$ at 4°C for 10 min, the supernatants were used to measure LTB_4 and PGE_2 levels as indicated above or $\text{TNF}\alpha$ by ELISA.

Adjuvant arthritis

Adjuvant arthritis was elicited in female Lewis rats (126–150 g) by injecting 0.1 ml of *Mycobacterium butyricum* (10 mg ml^{-1}) in mineral oil into the base of the tail (Taurog *et al.*, 1988). Paw volumes were measured at the beginning of

the experiment by using a plethysmometer. Animals were housed in propylene cages with food and water *ad libitum*. The light cycle was automatically controlled (on 0700 h; off 1900 h) and the room temperature thermostatically regulated to $21 \pm 1^\circ\text{C}$. The magnitude of the inflammatory response was evaluated by measuring the volume of both paws at day 13. The oedema was calculated as the mean increase in paw volume. Animals with oedema paw volumes 0.60 ml larger than normal paws were then randomized into treatment groups. Cacospongionolide B (20 mg kg^{-1}), indomethacin (5 mg kg^{-1}) or vehicle (ethanol, tween 80, distilled water: 5/5/90, v/v/v) were administered p.o. (1.0 ml) once-daily on days 13–17. Serum was collected on the last day of the experiment (day 18) for the determination of PGE_2 , thromboxane B_2 (TXB_2) and LTB_4 . After death, paws from arthritic, treated groups and non-arthritic normal animals were amputated above the ankle and homogenized in 2.5 ml saline. After centrifugation at $10,000 \times g$ for 15 min at 4°C, supernatants were used for the determination of PGE_2 and sPLA_2 . Prior to evaluating the sPLA_2 content, a purification by acidic treatment was performed (Bolognese *et al.*, 1995). Stomachs were homogenized in 2 ml of methanol and aliquots of supernatants were used to determine the content of PGE_2 as above.

Materials

Cacospongionolide B was isolated from the sponge *Faciospongia cavernosa* following known procedures (De Rosa *et al.*, 1995). Antibody against LTB_4 was kindly provided by Zeneca Pharmaceuticals, Macclesfield, Cheshire, U.K. Human synovial recombinant PLA_2 was a gift from Dr R.M. Kramer (Lilly Research Laboratories, Indianapolis, U.S.A.). [9,10- ^3H]-oleic acid and L-3-phosphatidylcholine 1-palmitoyl-2-arachidonyl [arachidonyl-1- ^{14}C] were purchased from Du Pont, (Itisa, Madrid, Spain). [5,6,8,11,12,14,15(n)- ^3H]- PGE_2 , [5,6,8,9,11,12,14,15(n)- ^3H]- LTB_4 , [5,6,8,9,11,12,14,15(n)- ^3H]- TXB_2 , L-[^3H]-arginine, IP_3 measurement kit and the $\text{TNF}\alpha$ ELISA kit were from Amersham Iberica, (Madrid, Spain). Palmityl trifluoromethyl ketone (PTK) was purchased from Cayman Chem. (MI, U.S.A.). *M. butyricum* was obtained from Difco (MI, U.S.A.). The rest of reagents were from Sigma Chem.(MO, U.S.A.). *E. coli* strain CECT 101 was a gift from Prof Uruburu, Department of Microbiology, University of Valencia, Spain.

Statistical analysis

The results are presented as means $\pm$ s.e.mean; *n* represents the number of experiments. Inhibitory concentration 50% (IC_{50}) values were calculated from at least four significant concentrations (*n* = 6). The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons.

Results

Effect on PLA_2 and other enzyme activities in vitro

The effect of cacospongionolide B on sPLA_2 was determined using different assay systems *in vitro*. As shown in Table 1, this marine compound preferentially inhibited the synovial and pancreatic secretory enzymes, and its potency on the human synovial enzyme was comparable to that of the reference inhibitor, manoalide. Other sPLA_2 , including the enzyme

present in the inflammatory exudates of zymosan-injected rat air pouch and bee venom PLA₂, were inhibited by cacospongionolide B to a lesser extent, whereas it exerted no effect on the *Naja naja* venom enzyme. We also determined the concentration-dependent inhibition of human recombinant synovial PLA₂ of cacospongionolide B and manolide at 10 μM , without and with 100 $\mu\text{g ml}^{-1}$ BSA in reaction buffer, resulting a partial reduction in their potency (cacospongionolide B, $86.7 \pm 2.5\%$ and $60.1 \pm 1.9\%$ of inhibition without and with BSA, respectively, $n=6$, $P<0.01$ and manolide, $93.2 \pm 0.2\%$ and $53.1 \pm 5.2\%$ of inhibition without and with BSA, respectively, $n=6$, $P<0.01$). This type of non-specific action has been reported previously in manolide (Jacobson *et al.*, 1990). In contrast, cacospongionolide B had no inhibitory effects on cPLA₂, which was partially inhibited by manolide at 10 μM (Table 2). We also tested the possible influence of cacospongionolide B on other enzymes involved in the release or metabolism of arachidonic acid, such as PLC, COX-1, COX-2 and 5-LO, as well as on iNOS. Only 5-LO was slightly inhibited by cacospongionolide B in a manner similar to manolide. In contrast, this reference compound also inhibited PLC. Cacospongionolide B did not exert significant cytotoxic effects on human neutrophils at concentrations up to 50 μM , as assessed by the release of LDH and the MTT method (data not shown). Otherwise manolide showed a slight cytotoxicity effect at 50 μM , as assessed by the MTT method ($18.9 \pm 3.0\%$ of cytotoxicity, $n=6$, $P<0.01$).

To further characterize the inhibitory activity of cacospongionolide B on sPLA₂ we also studied the type of inhibition, which was apparently irreversible as assessed by the kinetic analysis of enzyme activity as a function of the human synovial PLA₂ concentration in the absence or presence of cacospongionolide B (Figure 2) because there was no significant difference in the slopes of the straight lines (Segel, 1975). This

type of inhibition has been reported for other marine compounds such as manolide (Jacobson *et al.*, 1990).

Effect on sPLA₂ in vivo

Having established the inhibitory properties of cacospongionolide B on sPLA₂ *in vitro*, we tested the effects of this marine compound on an animal model in which high levels of secretory type II PLA₂ is generated (Payá *et al.*, 1996). As shown in Table 3, cacospongionolide B administered into the air pouch dose-dependently inhibited the PLA₂ activity present

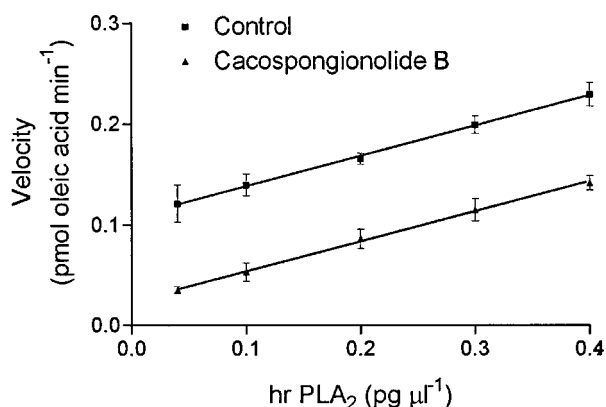


Figure 2 Activity of human synovial sPLA₂ as a function of enzyme concentration in the absence or presence of cacospongionolide B. Data are the means $\pm$ s.e. mean of $n=6$. Different enzyme concentrations were preincubated with vehicle (control) or cacospongionolide B (1 μM) for 5 min at 37°C, and after addition of substrate, incubation proceeded for 15 min.

Table 1 Effect of cacospongionolide B and manolide on different secretory PLA₂ activities

Drug (10 μM)	N. Naja venom		Pancreas		Human synovial		RAP + zymosan		Bee venom	
	%I		%I	IC ₅₀ (μM)	%I	IC ₅₀ (μM)	%I	IC ₅₀ (μM)	%I	IC ₅₀ (μM)
Cacospongionolide B	0.0 ± 0.0		$64.2 \pm 2.1^{**}$	4.0	$86.7 \pm 2.5^{**}$	4.3	$36.9 \pm 1.4^{**}$	ND	$35.4 \pm 1.2^{**}$	ND
Manolide	$17.0 \pm 1.7^*$		$32.3 \pm 2.7^{**}$	ND	$93.2 \pm 0.2^{**}$	3.9	$8.4 \pm 0.5^{**}$	ND	$62.5 \pm 3.8^{**}$	7.5

RAP: Rat air pouch. Results are the means $\pm$ s.e. mean of $n=6$. * $P<0.05$; ** $P<0.01$ with respect to the enzyme control group. IC₅₀ values were determined for the compounds that reach 50% inhibition at 10 μM . ND: not determined.

Table 2 Effect of cacospongionolide B and manolide on hr-sPLA₂, cPLA₂, PLC, 5-LO, COX-2, COX-1 and iNOS activities *in vitro*

Group	hr-sPLA ₂ (pmol OA mg ⁻¹ min ⁻¹)	cPLA ₂ (pmol AA mg ⁻¹ min ⁻¹)	PLC (nmol IP ₃ 10 ⁻⁶ cells)	5-LO (ng LTB ₄ ml ⁻¹)	COX-2 (ng PGE ₂ ml ⁻¹)	COX-1 (ng PGE ₂ ml ⁻¹)	iNOS (pmol citrulline mg ⁻¹ min ⁻¹)
Control	6093.8 ± 245.8	5.4 ± 0.5	2.4 ± 0.3	22.6 ± 2.1	1.5 ± 0.1	6.4 ± 0.6	30.9 ± 0.9
Cacospongionolide B	$782.2 \pm 174.3^{**}$	5.9 ± 0.6	2.2 ± 0.6	$13.6 \pm 0.7^{**}$	1.4 ± 0.4	5.8 ± 0.6	28.9 ± 0.6
Manolide	$358.9 \pm 200.0^{**}$	$3.3 \pm 0.5^{**}$	$1.2 \pm 0.1^*$	$14.7 \pm 1.02^{**}$	1.2 ± 0.2	6.5 ± 0.3	27.3 ± 1.5
PTK	ND	$1.4 \pm 0.5^{**}$	ND	ND	ND	ND	ND
U73122	ND	ND	$0.9 \pm 0.2^*$	ND	ND	ND	ND
ZM 230,487	ND	ND	ND	$5.4 \pm 1.3^{**}$	ND	ND	ND
NS398	ND	ND	ND	ND	$0.5 \pm 0.1^{**}$	$4.0 \pm 0.5^{**}$	ND
Indomethacin	ND	ND	ND	ND	$1.0 \pm 0.1^*$	$0.8 \pm 0.1^{**}$	ND
L-NAME	ND	ND	ND	ND	ND	ND	$10.4 \pm 1.6^{**}$

Results are the means $\pm$ s.e. mean of $n=6-12$. * $P<0.05$; ** $P<0.01$ with respect to control group. Cacospongionolide B, manolide and reference inhibitors were tested at 10 μM . ND: not determined. Drugs were incubated with hr-sPLA₂, cytosolic fraction of U937 cells (cPLA₂), human neutrophils stimulated with fMLP (PLC), high speed supernatants from human neutrophil homogenates (5-LO), microsomal fraction from human monocytes treated with LPS (COX-2), microsomal fraction from human platelets (COX-1) or cytosolic fraction of liver homogenates from LPS-injected rats (iNOS).

in the 8 h zymosan-injected rat air pouch, whereas the accumulation of leukocytes in the pouch was reduced by CB treatment only at the highest dose tested ($1 \mu\text{mol pouch}^{-1}$).

Effect on mouse ear oedema

Evaluation of the topical anti-inflammatory activity of cacospongionolide B was performed in the TPA-induced mouse ear oedema. Control animals showed an oedema of $17.8 \pm 1.0 \text{ mg}$ and myeloperoxidase levels of $0.852 \pm 0.104 \text{ O.D.}_{620}$ units 4 h after TPA administration (Table 4). Topical treatment with cacospongionolide B resulted in a dose-dependent inhibition of TPA-induced ear oedema, in addition to a decrease in myeloperoxidase levels measured in ear homogenates. Indomethacin was more effective on myeloperoxidase than on oedema, which was also attenuated, but to a lesser extent than in animals treated with cacospongionolide B.

Effect on mouse paw oedema

Oral pretreatment (1 h before carrageenin) with 5, 10 or 20 mg kg^{-1} of cacospongionolide B significantly reduced oedema (Figure 3). This inhibitory effect was observed at the three time points considered, 1, 3 and 5 h after carrageenin, for the doses of 10 and 20 mg kg^{-1} , whereas the dose of 5 mg kg^{-1} caused significant inhibition at 1 and 3 h. Interestingly, cacospongionolide B was more effective than indomethacin in this model, mainly at 3 h carrageenin administration.

Effect on the mouse air pouch

An important increase in leukocyte migration was observed in zymosan-injected animals in comparison with the saline-injected group 4 h after the induction of inflammation. Cacospongionolide B blocked cell accumulation in exudates

at the dose of $1 \mu\text{mol pouch}^{-1}$, but it was ineffective at lower doses (Figure 4a). This inflammatory response also showed high levels of PGE_2 , LTB_4 and $\text{TNF}\alpha$ in the air pouch exudates of control animals injected with zymosan (Figure 4b, c and d). Treatment with cacospongionolide B resulted in a significant decrease in PGE_2 levels at a dose as low as $1 \text{ nmol pouch}^{-1}$, whereas LTB_4 levels were significantly reduced at higher doses (0.5 and $1 \mu\text{mol pouch}^{-1}$). In this model, $\text{TNF}\alpha$ levels were very sensitive to cacospongionolide B; $1 \text{ nmol pouch}^{-1}$ of this compound achieved a significant reduction in $\text{TNF}\alpha$ and at $1 \mu\text{mol pouch}^{-1}$ the levels of this cytokine were abolished. As expected, the 5-LO inhibitor ZM 230,487 ($0.1 \mu\text{mol pouch}^{-1}$) strongly reduced LTB_4 levels ($87.3 \pm 5.8\%$ of inhibition, $n=6$, $P<0.01$) and cell migration ($36.7 \pm 3.1\%$ of inhibition, $n=6$, $P<0.01$), whereas the COX inhibitor indomethacin ($0.1 \mu\text{mol pouch}^{-1}$) decreased PGE_2 levels ($89.2 \pm 4.8\%$ of inhibition, $n=6$, $P<0.01$). Dexamethasone (2 mg kg^{-1} i.p.) inhibited cell migration ($37.8 \pm 3.3\%$ of inhibition, $n=6$, $P<0.01$) as well as PGE_2 and LTB_4 ($74.8 \pm 3.6\%$ and $82.8 \pm 4.0\%$ of inhibition, respectively, $n=6$, $P<0.01$) and $\text{TNF}\alpha$ ($75.7 \pm 7.1\%$ of inhibition, $n=6$, $P<0.01$) levels in exudates. The effects of cacospongionolide B this experimental model were also confirmed after oral administration at a single dose of 20 mg kg^{-1} , which reduced PGE_2 , LTB_4 and $\text{TNF}\alpha$ levels (58.6 ± 4.8 , 64.7 ± 8.2 and $59.5 \pm 7.1\%$ of inhibition, respectively; $n=6$, $P<0.01$) to a similar extent, and had a lower effect on cell accumulation in exudates ($38.5 \pm 4.1\%$ of inhibition, $n=6$, $P<0.01$).

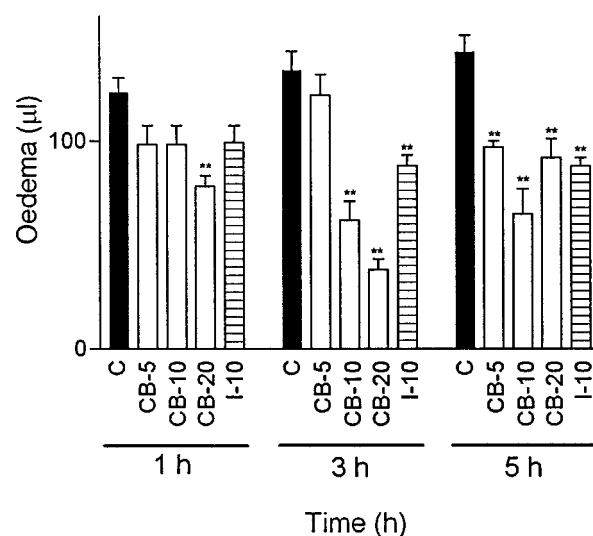


Figure 3 Effect of cacospongionolide B (CB) and indomethacin (I) on mouse paw oedema induced by carrageenin. Data represent means $\pm$ s.e. mean of $n=6$. * $P<0.05$; ** $P<0.01$, significantly different from control (C). Cacospongionolide B (5 – 20 mg kg^{-1}) and indomethacin (10 mg kg^{-1}) were administered p.o. 1 h before the injection of carrageenin.

Table 3 Effect of cacospongionolide B on cellular accumulation and secretory PLA_2 in the 8 h zymosan-injected rat air pouch

Group	Total cells ($\times 10^6 \text{ ml}^{-1}$)	sPLA ₂ (pmol OA ml^{-1})
Zymosan	144.8 ± 8.8	312.2 ± 15.8
Saline	$25.3 \pm 6.3^{**}$	$54.4 \pm 11.0^{**}$
Zymosan + cacospongionolide B		
0.01 $\mu\text{mol pouch}^{-1}$	140.2 ± 5.0	$244.3 \pm 10.2^*$
0.1 $\mu\text{mol pouch}^{-1}$	135.9 ± 10.2	$223.7 \pm 9.8^{**}$
1 $\mu\text{mol pouch}^{-1}$	$62.3 \pm 8.6^{**}$	$180.3 \pm 24.1^{**}$

Results are the means $\pm$ s.e. mean of $n=6$. * $P<0.05$; ** $P<0.01$ with respect to the zymosan control group. OA: Oleic acid.

Table 4 Topical inhibitory effect of cacospongionolide B and indomethacin on mouse ear oedema induced by application of TPA

Drug		% Oedema inhibition	% Myeloperoxidase inhibition
Cacospongionolide B	$50 \mu\text{g ear}^{-1}$	$43.4 \pm 4.8^*$	$64.3 \pm 7.2^{**}$
Cacospongionolide B	$100 \mu\text{g ear}^{-1}$	$54.6 \pm 4.5^{**}$	$70.9 \pm 6.3^{**}$
Indomethacin	$62.5 \mu\text{g ear}^{-1}$	14.0 ± 1.3	$46.6 \pm 1.7^{**}$
Indomethacin	$125 \mu\text{g ear}^{-1}$	$37.0 \pm 5.0^*$	$77.4 \pm 10.4^{**}$

Percentages of inhibition are the means $\pm$ s.e. mean of $n=6$. * $P<0.05$; ** $P<0.01$ with respect to the control group.

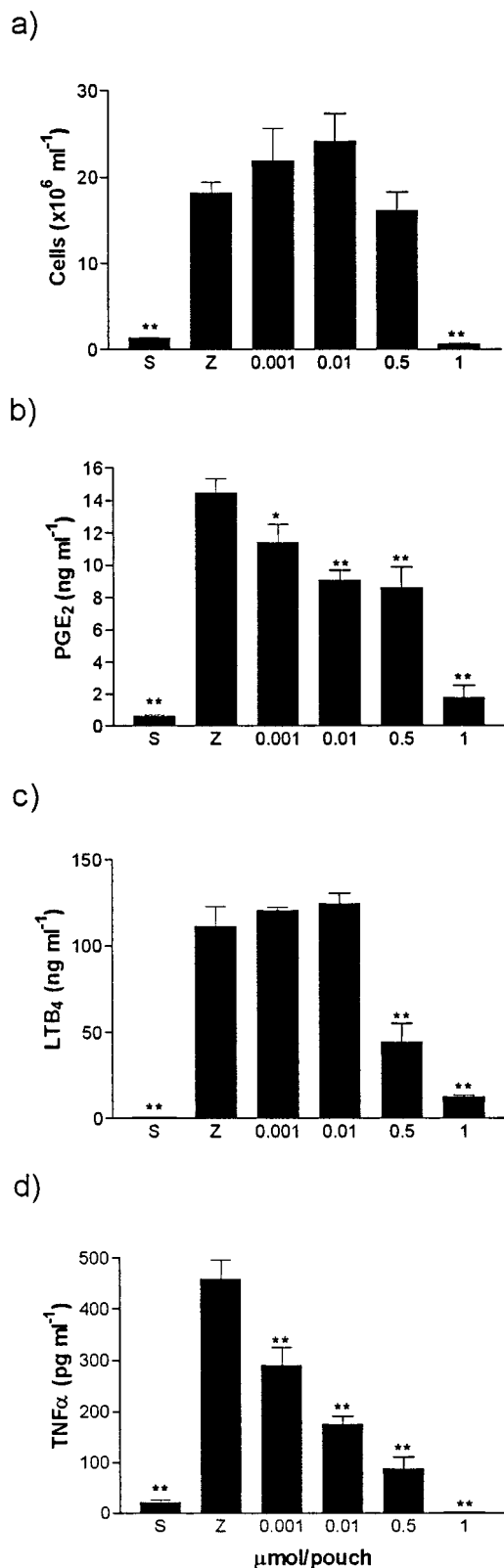


Figure 4 Effect of cacospongionolide B on the mouse air pouch injected with zymosan. Data represent means $\pm$ s.e. mean of $n=6$. * $P<0.05$; ** $P<0.01$ with respect to the zymosan control group. Cacospongionolide B was injected into the air pouch at the same time as zymosan. S=saline, Z=zymosan. (a) Number of cells present in exudates 4 h after zymosan. (b) PGE_2 levels in exudates. (c) LTB_4 levels in exudates. (d) $\text{TNF}\alpha$ levels in exudates.

Effect on adjuvant arthritis

We tested the effects of cacospongionolide B on a model of chronic inflammation, the established adjuvant-induced arthritis, to further characterize its anti-inflammatory properties. Administration of 20 mg kg^{-1} daily on days 13–17 after adjuvant injection, to animals with developed arthritis, significantly reduced mean paw oedema on the two measures performed, on days 15 and 18 after adjuvant (Figure 5). As shown in Table 5, arthritic animals showed a significant increase in eicosanoid levels in different tissues measured at the end of the experiment (day 18), with respect to the non-arthritic control. Cacospongionolide B did not modify eicosanoid levels in serum, stomach and paw homogenates. Interestingly, an increase in sPLA_2 activity was detected in paw homogenates of arthritic rats which was significantly inhibited in animals treated with this marine metabolite. Indomethacin was very effective in oedema reduction, and with it a striking inhibition of prostanoid levels was obtained in serum, stomach and paw homogenates. Nevertheless, the stomachs of the animals treated with this NSAID showed redness and perforations, and suppurative peritonitis was observed in two animals. All these toxic effects were absent in the rats treated with cacospongionolide B.

Discussion

Our results indicate that cacospongionolide B is a new inhibitor of sPLA_2 with a strong effect on human synovial PLA_2 (group II), and a somewhat milder effect on the pancreatic enzyme (group I) and bee venom PLA_2 (group III). Acting in an irreversible way, this marine metabolite showed *in vitro* a selectivity of inhibition for sPLA_2 versus cPLA_2 , and a potency on the human synovial enzyme similar to that of manolide. Several reports on the structure-activity relationship of manolide indicate that the most likely initial reaction between manolide and PLA_2 is the formation of a Schiff base between a lysine residue and the aldehyde generated upon opening of the manolide γ -lactone ring (Jacobson *et al.*, 1990;

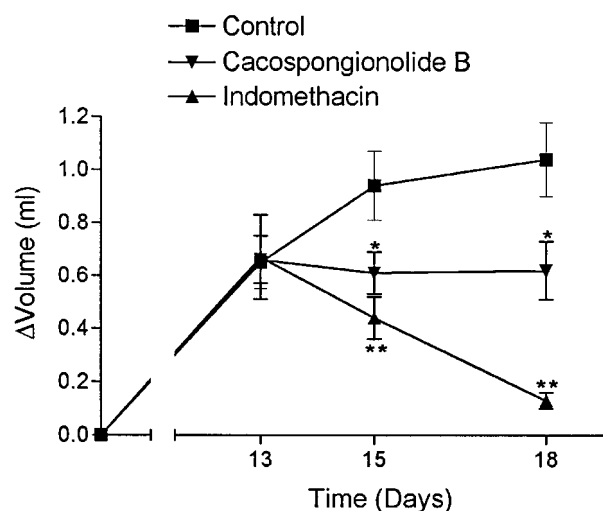


Figure 5 Effect of cacospongionolide B (20 mg kg^{-1}) p.o. and indomethacin (5 mg kg^{-1}) p.o. on the development of adjuvant-induced arthritis in female Lewis rats. Values are the means $\pm$ s.e. mean of $n=6$. * $P<0.05$; ** $P<0.01$ with respect to the vehicle-treated arthritic rats.

Table 5 Effect of cacospongionolide B and indomethacin on some parameters associated with the development of inflammatory process in adjuvant-induced arthritis

Group	Treatment	Serum eicosanoids			Stomach	Paw	
		<i>PGE</i> ₂ (ng ml ⁻¹)	<i>TXB</i> ₂ (ng ml ⁻¹)	<i>LTB</i> ₄ (ng ml ⁻¹)	<i>PGE</i> ₂ (ng ml ⁻¹)	<i>PGE</i> ₂ (ng ml ⁻¹)	<i>sPLA</i> ₂ (pmol OA mg ⁻¹ min ⁻¹)
Arthritic rats	Vehicle	5.5 ± 0.9	284.6 ± 38.9	51.7 ± 8.4	45.7 ± 5.2	96.8 ± 9.7	10.3 ± 1.1
Normal rats	Vehicle	3.7 ± 0.8	28.4 ± 4.2**	13.2 ± 2.0**	25.0 ± 2.2**	31.3 ± 3.5**	2.2 ± 0.9**
Arthritic rats	Cacospongionolide B	4.8 ± 0.4	166.8 ± 45.4	45.0 ± 3.0	34.7 ± 2.5	76.0 ± 2.2	6.6 ± 1.0*
Arthritic rats	Indomethacin	2.4 ± 0.5*	13.2 ± 8.7**	63.2 ± 10.2	15.9 ± 3.3**	16.0 ± 3.3**	12.1 ± 1.0

Results are the means ± s.e.mean of $n=6$. * $P<0.05$; ** $P<0.01$ with respect to arthritic control group. OA: Oleic acid. All the samples were collected on the last day of the experiment (day 18). Vehicle, cacospongionolide B (20 mg kg⁻¹) or indomethacin (5 mg kg⁻¹) were administered p.o. once-daily on days 13–17.

Glaser *et al.*, 1989). Manoalide and cacospongionolide B are very close chemically, differing from each other by the hydroxylic substitution in the dihydropyran ring and in the hydrophobic region of the molecule that is cyclic in cacospongionolide B structure. Sharing both compounds a non-specific action on lysine-containing proteins, the *in vitro* loss of potency observed is lower for cacospongionolide B. Interestingly, we have demonstrated that cacospongionolide B also inhibits group II sPLA₂ *in vivo*, in the 8 h zymosan-injected rat air pouch, a model sensitive to this type of inhibitor (Payá *et al.*, 1996). This effect was not due to inhibition of neutrophil accumulation in the air pouch exudate, which is the main source of this secretory enzyme (Payá *et al.*, 1996), for inhibition of this activity was also observed at doses that did not affect cell migration. The bioavailability by oral route of this compound was also confirmed in the adjuvant-induced arthritis model of chronic inflammation.

A potential protective role of group II sPLA₂ inhibitors can be inferred from studies showing that this type of enzymes participates in the inflammatory process, which leads to the generation of mediators and production of tissue injury in different pathological states. High levels of this enzyme are known to be present in synovial fluids, articular cartilage and blood from patients with rheumatic diseases (Pruzanski *et al.*, 1987; Bomalaski & Clark, 1993). In addition, recent studies suggest that group II sPLA₂ may play a broad role in tissue injury. Elevated levels of this enzyme, for example, have been associated with a poor clinical outcome in the acute respiratory distress syndrome (Arbibe *et al.*, 1997). This enzyme activity has also been detected in human atherosclerotic plaques and it could increase the atherogenicity of LDL by hydrolyzing the phospholipids present in this lipoprotein (Eckey *et al.*, 1997). A high expression of group II sPLA₂ has been demonstrated in colon biopsies from patients of ulcerative colitis (Haapamaki *et al.*, 1997), and its levels are also increased in effusions from cancer patients, associated with a high mRNA expression in carcinoma cells (Abe *et al.*, 1997).

On the other hand, sPLA₂ could also play a role in cellular defence against infection, as this enzyme activity is bactericidal against *E. coli* (Weiss *et al.*, 1994), *L. monocytogenes* (Weiss *et al.*, 1994; Harwig *et al.*, 1995) and *S. aureus* (Weinrauch *et al.*, 1996). In addition, it has been suggested that cPLA₂, sPLA₂ and diacylglycerol/monoacylglycerol lipase participate in arachidonic acid release in rat NK cells, which plays a crucial role in the lytic activity of these cells (Cifone *et al.*, 1997).

Cacospongionolide B can act as a topical anti-inflammatory agent and has also shown oral efficacy. Our data thus demonstrate that a selective inhibitor of sPLA₂ is able to decrease the inflammatory reaction in models of acute and chronic inflammation. The production of eicosanoids derived

from the COX and 5-LO pathways was reduced by cacospongionolide B in an acute inflammatory response, the mouse air pouch. This effect is probably the consequence of a reduction in substrate availability, since this compound is not an inhibitor of COX and is only a weak inhibitor of 5-LO. This weak effect would explain the slightly higher inhibition of LTB₄. In contrast, the fact that in the model of chronic inflammation, cacospongionolide B did not significantly affect the levels of eicosanoids suggests the participation of other pathways in the regulation of arachidonic acid availability. It has been suggested that inhibition of cPLA₂ interferes with cellular activation and therefore results in the control of adjuvant arthritis (Amandi-Burgermeister *et al.*, 1997). This model of chronic inflammation is a complex response involving different mediators, and therefore there is a possibility of multiple interactions, e.g. inhibition of iNOS results in the control of the inflammatory features (Connor *et al.*, 1995). Our results indicate that inhibition of sPLA₂ can also interfere with this model of chronic inflammation without producing toxic effects.

Interestingly, cacospongionolide B inhibited TNFα levels in the mouse air pouch dose-dependently. This effect is absent in COX and 5-LO inhibitors and is similar to that of dexamethasone. The molecular mechanism of this inhibitory effect remains to be established. Inflammatory cytokines induce the enzymes responsible for arachidonic acid release and metabolism, thus leading to increased levels of eicosanoids, and they are involved in the chronification of the inflammatory response. Eicosanoids, in turn, may regulate in part the synthesis of inflammatory cytokines, although there are differences depending on the cell type and the experimental conditions. In certain cases, LTB₄ can increase the generation of IL-1β (Rola-Pleszczynski & Lemaire, 1985), whereas PGE₂ inhibits the production of TNFα and IL-1β in human monocytes (Caughey *et al.*, 1997). In contrast, thromboxane A₂ (TXA₂) facilitates cytokine production in these cells, and the balance between different eicosanoids may determine the resulting effect on cytokines (Caughey *et al.*, 1997).

Of the anti-inflammatory drugs in use, NSAID can not prevent the progression of chronic inflammation. This group is able to affect cytokine synthesis by human monocytes stimulated by TPA at high concentrations only (Jiang *et al.*, 1998), and in addition, the inhibition of PG synthesis can potentiate the expression of TNFα induced by LPS (Bondeson & Sundler, 1996). Glucocorticoids are potent agents for the treatment of chronic inflammatory diseases, although severe side effects limit the long term administration required in chronic disorders. These drugs exert complex effects on inflammation, with inhibition of G protein-dependent activation of cPLA₂ activity (Croxtall *et al.*, 1995) and the expression of cPLA₂, sPLA₂ and COX-2, thus leading to inhibition of

eicosanoid biosynthesis. These anti-inflammatory agents also inhibit the biosynthesis of other enzymes, cytokines and adhesion molecules, and act on gene regulation by different mechanisms (Goppelt-Strube, 1997). The inhibition of cytokine generation by compounds active after oral administration, such as cacospongionolide B, can offer an interesting approach to the search for new anti-inflammatory agents, since this type of drugs can have disease modifying properties (Geiger *et al.*, 1994).

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Anaphylactic bronchoconstriction in BP2 mice: interactions between serotonin and acetylcholine

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1 Immunized BP2 mice developed an acute bronchoconstriction *in vivo* and airway muscle contraction *in vitro* in response to ovalbumin (OA) and these contractions were dose dependent.

2 Methysergide or atropine inhibited OA-induced bronchoconstriction *in vivo* and airway muscle contraction *in vitro*.

3 Neostigmine potentiated the OA-induced bronchoconstriction *in vivo* and airway muscle contraction *in vitro* of BP2 mice. This potentiation was markedly reduced by the administration of methysergide or atropine and when the two antagonists were administered together, the responses were completely inhibited.

4 Neostigmine also potentiated the serotonin (5-HT)- and acetylcholine (ACh)-induced bronchoconstriction and this potentiation was significantly reversed by atropine.

5 These results indicate that OA provokes a bronchoconstriction in immunized BP2 mice by stimulating the release of 5-HT, which in turn acts *via* the cholinergic mediator, ACh.

Keywords: Anaphylactic bronchoconstriction; mice; 5-HT; atropine; methysergide; ACh; neostigmine

Abbreviations: ACh, Acetylcholine; BP2 mice, Biozzi prepared hyperreactive mice; OA, Ovalbumin; 5-HT, Serotonin

Introduction

A number of studies have shown that lungs of rats (Church *et al.*, 1972) and mice (Levitt & Mitzner, 1989) are responsive to 5-HT and ACh. In the rat, the response to 5-HT is mediated by a direct action of this agonist on airway smooth muscle (Pauwels *et al.*, 1985). In contrast, in the mouse, the effect of 5-HT may be mediated by indirect mechanisms, such as the release of ACh. In fact, Levitt & Mitzner (1989) showed that in certain inbred strains of mice, which are very responsive to 5-HT, the bronchoconstriction induced by 5-HT was blocked by atropine. These data implicated an interaction of 5-HT with the nervous system and cholinergic mechanisms.

In sensitized mice, the ovalbumin dose-dependent contractions have been reported to be associated with the release of 5-HT (Iff & Vaz, 1966; Lima, 1967). Larsen and co-workers (1994) showed that repeated airway exposure to an allergen and the development of an IgE-responsive state lead to an altered neural control of airways with an increase in ACh release from nerve terminals. These *in vitro* data supported similar observations suggesting a link between elevated IgE titers in mice and airway hyper-responsiveness to contractile agents (Saloga *et al.*, 1994; Weinmann *et al.*, 1990). These results provided evidence for the hypothesis that an ovalbumin-induced bronchoconstriction in mice may be due to the direct contractile effect on airway muscle of ACh which is released by 5-HT. However, no studies have been performed which demonstrate that this series of physiological events occurred in any one of the proposed animal models of airway hyper-reactivity. Therefore, the aim of this investigation was to examine the ovalbumin induced bronchoconstriction in a

mouse model where an elevated serum IgE titer and airway hyper-responsiveness have been reported (Eum *et al.*, 1995).

Methods

Mice and immunization procedure

Female BP2 mice were obtained from the 'Centre d'Elevage R. Janvier' (BP5, 53940 Le Genest Saint-Isle, France). Mice from 8–11 weeks were used in the experiments. These BP2 mice, a selection of Biozzi mice, produce high titers of antibodies (Biozzi *et al.*, 1979) including IgE (Eum *et al.*, 1995). These animals were derived from Swiss mice which were bred for the production of either high or low amounts of antibodies against selective antigens. After 16 generations the animals differed by 280-fold in mean agglutinin titers of antibodies produced against sheep erythrocytes (Biozzi *et al.*, 1970).

Mice were immunized by injecting 0.4 ml of an OA solution (s.c. 250 µg ml⁻¹) mixed with Al(OH)₃ (4 mg ml⁻¹) twice at an interval of 7 days. Mice were utilized after 1 week following the second injection.

Evaluation of bronchoconstriction in vivo and airway muscle contraction in vitro

Bronchoconstriction *in vivo* was measured by the methods described by Eum *et al.* (1995) and the contraction of isolated trachea *in vitro* by Norel *et al.* (1993). Briefly, in the *in vivo* experiments, immunized mice were anaesthetized with ethyl carbamate (i.p., 1.5 mg g⁻¹). The trachea was cannulated and prepared for recording of dynamic compliance and airway resistance, by adapting the equipment of the computerized pulmonary analyser (Mumed PR800 system, U.K.) to mice airways at a tidal volume of 2.10⁻² ml g⁻¹ and a frequency of

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100 breaths min^{-1} . The animals were paralysed with pancuronium bromide (Pavulon[®], 10 $\mu\text{g kg}^{-1}$ i.v.) and airway resistance was calculated from the differential pressure between the airways and pleural cavity and the airflow. Control basal values of resistance were 500 ± 20 (cm water (1 sec^{-1}) $^{-1}$) for $n = 20$ animals (mean $\pm$ s.e.mean). Depending on the protocol used, the data were expressed either as per cent increase of resistance (% increase), or as the increase above basal resistance. The per cent increase was calculated as follows: $((\text{resistance after treatment} - \text{basal resistance}) / \text{basal resistance}) \times 100\%$. In the protocols using neostigmine the data are shown as the resistance measured after treatment – basal resistance after neostigmine.

In the *in vitro* experiments, tracheal preparations were set up in the 10 ml organ baths containing Tyrode's solution (concentration in mM): NaCl 139.2, KCl 2.7, CaCl_2 1.8, MgCl_2 0.49, NaHCO_3 11.9, NaH_2PO_4 0.4 and glucose 5.5; pH 7.4; gassed with 95% O_2 /5% CO_2 under initial loads (1 g). These loads ensured that responses to contractile agonists were optimal. Isometric force displacement transducers (Narco F-60) and physiographs (Linseis) were used to record the changes in force. The tissues were allowed to equilibrate for 90 min and the bath fluid was exchanged every 15 min with fresh Tyrode's solution. After this period, all tracheal preparations were contracted with carbachol (3 μM). The tissues were washed with fresh Tyrode's solution and allowed to return passively to their resting tone. When resting tone was established, the preparations were incubated for 30 min in Tyrode's solution or Tyrode's solution containing either neostigmine (0.1 μM), atropine (1 μM) or methysergide (1 μM) and the response to an individual dose of OA or the relationship to 5-HT was determined.

Drug treatment

Drugs used to study the mechanisms of anaphylactic bronchoconstriction were injected in 0.9% NaCl (saline) through the cannulated jugular vein before OA injection. After the animals were prepared on the Mumed system (see 'Evaluation of bronchoconstriction *in vivo*'), drugs were injected in the following order at 5 min intervals; ACh (80 $\mu\text{g kg}^{-1}$), 5-HT (40 $\mu\text{g kg}^{-1}$), saline or neostigmine (10 $\mu\text{g kg}^{-1}$), 5-HT (40 $\mu\text{g kg}^{-1}$), OA (50 mg kg^{-1}) and ACh (80 $\mu\text{g kg}^{-1}$). In order to determine the effects of each drug on OA-induced bronchoconstriction, either methysergide (200 $\mu\text{g kg}^{-1}$), atropine (10 $\mu\text{g kg}^{-1}$; 200 $\mu\text{g kg}^{-1}$), or methysergide (200 $\mu\text{g kg}^{-1}$) + atropine (10 $\mu\text{g kg}^{-1}$) were injected after the injection of saline or neostigmine and prior to the second injection of 5-HT.

Materials

Ovalbumin (5 $\times$ crystallized) was from Immunobiological (Costa Mesa, U.S.A.). Aluminium hydroxide was from Merck (Darmstadt, Germany). 5-Hydroxytryptamine (5-HT), ethyl carbamate, neostigmine, atropine, carbachol and acetylcholine were from Sigma (St. Louis, MO, U.S.A.). Pancuronium bromide (Pavulon) was from Organon Teknika (France). Methysergide was from Sandoz (Bâle, Swiss).

Statistical analysis

The results of each measurement are reported as means $\pm$ s.e.mean. Values significantly different between experimental and control groups were analysed by the Student's *t*-test (unpaired) and *P* values of less than 0.05 were considered to be significant.

Results

OA-induced bronchoconstriction and contractions of isolated trachea in immunized BP2 mice

Intravenous challenge with OA in immunized BP2 mice induced a dose-dependent augmentation of bronchial resistance, the maximal effect (approximately 160% augmentation) was reached at 50 mg kg^{-1} (Figure 1a). Saline-challenged mice (controls) showed no significant augmentation in bronchial resistance. The addition of OA to the organ bath containing trachea from immunized BP2 mice also induced dose-dependent contractions, which were approximately 40% of the carbachol effect (carbachol, 1 μM : 1.70 ± 0.11 g, $n = 45$; Figure 1b).

Drug modulation of anaphylactic bronchoconstriction in vivo and on OA contractions of isolated trachea in vitro

Anaphylactic bronchoconstriction was markedly attenuated by methysergide (200 $\mu\text{g kg}^{-1}$) and atropine (10 $\mu\text{g kg}^{-1}$; Figure 2a). In addition, a higher dose of atropine (200 $\mu\text{g kg}^{-1}$) completely abolished the OA response in these animals ($n = 3$; data not shown). Neostigmine significantly potentiated the OA-induced bronchoconstriction (Figure 3). This potentiation was blocked by methysergide or atropine (10 $\mu\text{g kg}^{-1}$) co-administered with neostigmine (Figure 3). When the drug combination of methysergide and atropine (10 $\mu\text{g kg}^{-1}$) was administered, the anaphylactic bronchoconstriction in the presence of neostigmine was suppressed (data not shown). In isolated trachea, the OA-induced contraction was also inhibited by either methysergide or atropine (Figure 2b). This contraction was potentiated by neostigmine (approximately 100%, $n = 8$) and the combined injection of methysergide and atropine inhibited this contraction ($n = 3$; data not shown).

Intravenous injection of neostigmine also potentiated the 5-HT-induced bronchoconstriction. This augmentation was blocked by either methysergide or atropine (Figure 3) and the simultaneous injection of both antagonists blocked the responses to 5-HT (data not shown). The ACh-induced bronchoconstriction was also increased by neostigmine and this was blocked by atropine, but not by methysergide (Figure 3). In isolated trachea, the 5-HT-induced contraction was also increased by neostigmine, but was not significantly inhibited by atropine (Figure 4).

Discussion

OA produced a dose-dependent contraction of airway muscle both *in vivo* and *in vitro* (present report) in immunized BP2 mice. These airway muscle responses were blocked by treating the animals or tissues with methysergide or atropine, suggesting that 5-HT and ACh were involved in the contraction. In addition, neostigmine potentiated the bronchoconstriction to OA, 5-HT and ACh, an effect markedly inhibited in each case by atropine. These data support the hypothesis that ovalbumin provoked a bronchoconstriction by stimulating the release of 5-HT and the direct contractile effect on airway smooth muscle was due to ACh. Thus the present study shows that in hyper-responsive BP2 mice, antigen-induced anaphylactic bronchoconstriction has a dual mediator release profile. While 5-HT and ACh measurements were not performed, the antagonist data indirectly support the involvement of both agents.

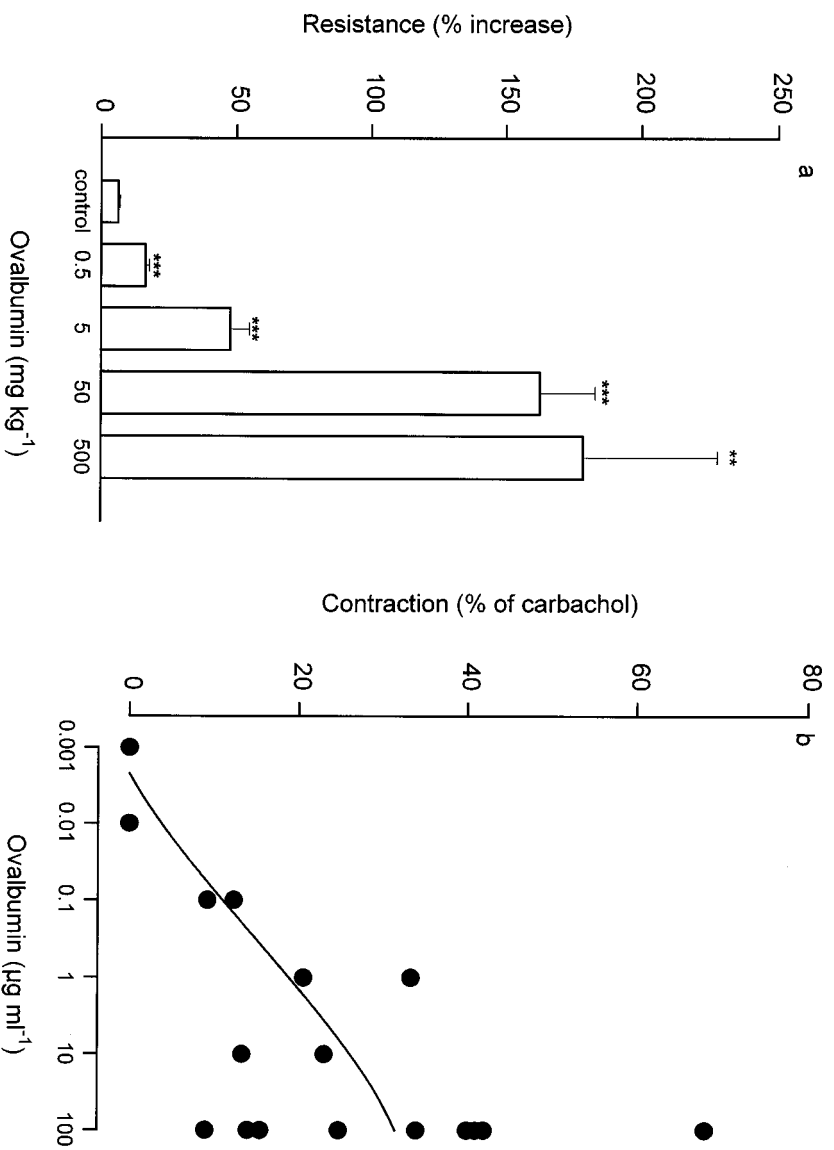


Figure 1 In immunized BP2 mice OA-induced bronchoconstriction *in vivo* (a) and airway muscle contraction *in vitro* (b). The values are in (a) means $\pm$ s.e. mean derived from 4–20 mice whereas in (b) data were from individual animals. ** $P < 0.01$, *** $P < 0.001$ compared with the control group (saline challenged mice).

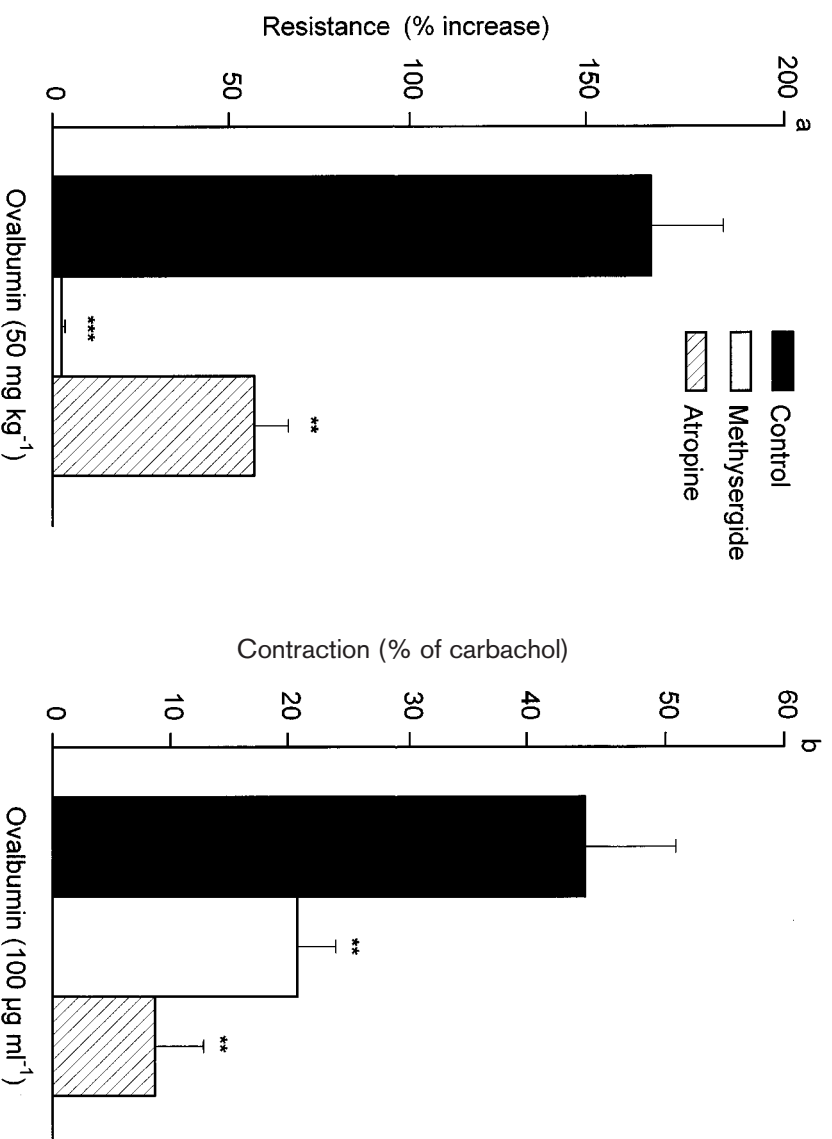


Figure 2 The effects of methysergide (200 μ g kg⁻¹) and atropine (10 μ g kg⁻¹) on ovalbumin induced bronchoconstriction *in vivo* (a) and on airway muscle contraction *in vitro* (b) in immunized BP2 mice. The values are means $\pm$ s.e. mean derived from 5–10 mice. ** $P < 0.01$, *** $P < 0.001$ compared with controls (OA challenged).

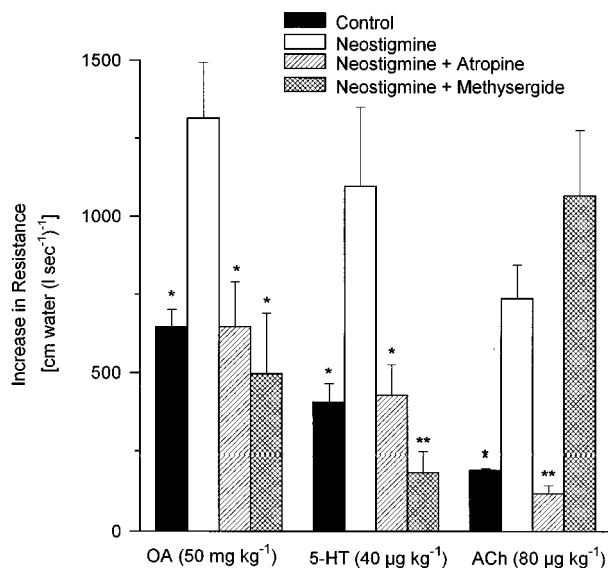


Figure 3 The effect of atropine and methysergide in combination with neostigmine on bronchoconstriction *in vivo* induced by different agents in immunized BP2 mice. Bronchoconstriction (control: agonist in absence of drugs) and in presence of neostigmine ($10 \mu\text{g kg}^{-1}$) or neostigmine ($10 \mu\text{g kg}^{-1}$)+atropine ($10 \mu\text{g kg}^{-1}$) or neostigmine ($10 \mu\text{g kg}^{-1}$)+methysergide ($200 \mu\text{g kg}^{-1}$) are shown. The agonist challenges were: acetylcholine (ACh), serotonin (5-HT) and ovalbumin (OA). Values are means $\pm$ s.e. mean derived from 4–6 mice. * $P < 0.05$, ** $P < 0.01$ compared with neostigmine group.

5-HT is released from mast cells (Kitamura, 1989) upon activation by antigen-specific IgE or by antigen binding T cell factors in mice. In rats 5-HT is the major mediator of antigen-induced anaphylactic bronchoconstriction (Church *et al.*, 1972) and airway smooth muscle contraction (Nagase *et al.*, 1995). In this study, methysergide suppressed the OA-induced bronchoconstriction, indicating that 5-HT is also a major mediator of OA-induced anaphylactic bronchoconstriction in BP2 mice. This observation is in agreement with that of Church *et al.* (1972) who also showed that antigen-induced bronchoconstriction is suppressed by methysergide in the rat. In BP2 mice (present report) methysergide also inhibited the OA-induced tracheal contractions *in vitro*. Church *et al.* (1972) initially demonstrated that atropine is inactive against the antigen-induced bronchoconstriction in the rat suggesting a direct action of this amine on the airway smooth muscle. OA-induced bronchoconstriction *in vivo* (present report) was significantly inhibited by atropine. Furthermore, atropine at a higher dose completely abolished the OA-induced constriction *in vivo*. These data suggest a cholinergic participation in the airway anaphylactic responses in BP2 mice. Previous reports have demonstrated that 5-HT facilitates cholinergic nerve-mediated contraction of the airways in a number of species including the rat (Aas, 1983; Szarek *et al.*, 1993), mouse (Van-Oosterhout *et al.*, 1991), the guinea-pig (Rizzo *et al.*, 1993), and man (Takahashi *et al.*, 1995). In mice, atropine inhibited the airway response to 5-HT (Levitt & Mitzner, 1989). These data suggest that 5-HT acts *via* a cholinergic pathway.

In the isolated trachea from Swiss mice, contractions were potentiated during electrical field stimulation, after the addition of 5-HT and this potentiation was suppressed by atropine (Van-Oosterhout *et al.*, 1991). These results suggested that ACh was released by stimulation of 5-HT receptors in trachea from these mice, specifically since 5-HT failed to affect

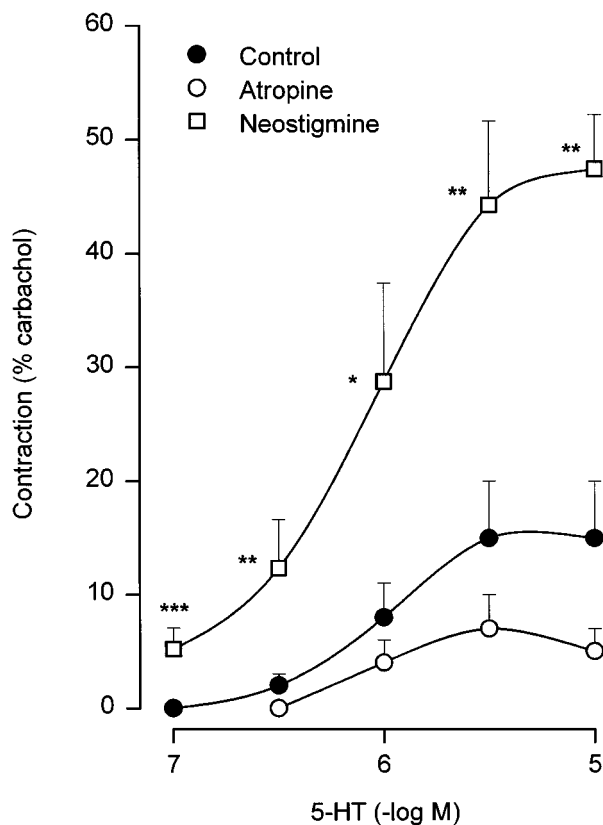


Figure 4 The 5-HT contractions in presence of either Tyrode's solution (Control), neostigmine ($0.1 \mu\text{M}$) or atropine ($1 \mu\text{M}$) in airway muscle derived from immunized BP2 mice. The values are means $\pm$ s.e. mean derived from 4–10 tracheal strips. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control group.

the response to exogenously applied ACh. A similar phenomenon has also been observed in human and guinea-pig airways by Takahashi *et al.* (1995). Such data strengthen the observation (present report) that OA-induced contractions of trachea were inhibited by atropine. Two observations further support a cholinergic pathway. First, neostigmine, a cholinesterase inhibitor, potentiated the anaphylactic bronchoconstriction of BP2 mice *in vivo* and *in vitro*, and this potentiation was markedly reduced by atropine. Secondly, neostigmine also potentiated 5-HT-induced bronchoconstriction and tracheal contraction in these mice, supporting ACh release upon stimulation of 5-HT.

Increased release of ACh induced by antigen challenge from airway parasympathetic nerve endings can contribute to increases in airway responsiveness (Larsen *et al.*, 1994). These investigators measured the release of ACh in electrically stimulated tracheal preparation derived from sensitized mice. The quantities detected were markedly different than results observed in unsensitized mice. This increased release of ACh may be due either to the absence of inhibitory prejunctional M_2 receptors or the presence of a 5-HT prejunctional facilitatory receptor on the parasympathetic neuronal fibres. Another possible explanation may be a decrease in cholinesterase activity. Previous studies have shown a modification of cholinesterase activity in dog (Mitchell *et al.*, 1991) and pig (Taisne *et al.*, 1997) airways related to the anaphylactic release of mediators. The findings (present report) suggest that ovalbumin-induced bronchoconstriction in BP2 mice may be due to the direct contractile effect on airway smooth muscle of ACh which is released by 5-HT.

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Studies of the role of endothelium-dependent nitric oxide release in the sustained vasodilator effects of corticotrophin releasing factor and sauvagine

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1 The mechanisms of the sustained vasodilator actions of corticotrophin-releasing factor (CRF) and sauvagine (SVG) were studied using rings of endothelium de-nuded rat thoracic aorta (RTA) and the isolated perfused rat superior mesenteric arterial vasculature (SMA).

2 SVG was ≈ 50 fold more potent than CRF on RTA (EC₄₀: 0.9 ± 0.2 and 44 ± 9 nM respectively, $P < 0.05$), and ≈ 10 fold more active in the perfused SMA (ED₄₀: 0.05 ± 0.02 and 0.6 ± 0.1 nmol respectively, $P < 0.05$). Single bolus injections of CRF (100 pmol) or SVG (15 pmol) in the perfused SMA caused reductions in perfusion pressure of 23 ± 1 and $24 \pm 2\%$ that lasted more than 20 min.

3 Removal of the endothelium in the perfused SMA with deoxycholic acid attenuated the vasodilatation and revealed two phases to the response; a short lasting direct action, and a sustained phase which was fully inhibited.

4 Inhibition of nitric oxide synthase with L-NAME (100 μ M) L-NMMA (100 μ M) or 2-ethyl-2-thiopseudourea (ETPU, 100 μ M) had similar effects on the vasodilator responses to CRF as removal of the endothelium, suggesting a pivotal role for nitric oxide. However the selective guanylate cyclase inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, 10 μ M) did not affect the response to CRF.

5 High potassium (60 mM) completely inhibited the vasodilator response to CRF in the perfused SMA, indicating a role for K⁺ channels in this response.

6 Compared to other vasodilator agents acting *via* the release of NO, the actions of CRF and SVG are strikingly long-lasting, suggesting a novel mechanism of prolonged activation of nitric oxide synthase.

Keywords: Corticotrophin-releasing factor; sauvagine; nitric oxide; endothelium; adenylyate cyclase; guanylate cyclase; K⁺ channels; vasodilatation; CRF receptor

Abbreviations: CRF, corticotrophin-releasing factor; EPTU, 2 ethyl-2-thiopseudourea; RTA, rat thoracic aorta; SMA, superior mesenteric artery vasculature; SVG, sauvagine; UI, urotensin I

Introduction

Corticotrophin-releasing factor (CRF) was originally identified as the principle hypothalamic regulator of corticotrophin (ACTH) secretion from the anterior pituitary gland (Vale *et al.*, 1981). During the same period two further peptides with structural homology and similar biological actions to CRF were isolated and characterized – namely, sauvagine (SVG), from the skin of the South American frog *Phyllomedusa Sauvagei* (Montecucchi *et al.*, 1980), and urotensin I (UI) from the urophysis of the teleost fish *Catostomus commersoni* (Lederis & Medakovic, 1974; Lederis *et al.*, 1982). Subsequent studies have shown both amphibians and teleost fish to possess their own CRF molecules (Morley *et al.*, 1991; Stenzel-Poore *et al.*, 1992). The cloning from rat brain of urocortin, a peptide with greater structural homology to UI than to CRF (Vaughan *et al.*, 1995), has shown that mammals also have more than one CRF-like peptide. This indicates that some physiological functions ascribed to CRF may involve other endogenous members of this family of peptides.

Current evidence suggests important peripheral actions of CRF or a structurally homologous peptide from this family. Intravenous injection of CRF results in an increase in blood glucose levels (Brown *et al.*, 1982a,b), and inhibition of both gastric emptying and intestinal transit, whilst increasing colonic transport (Sheldon *et al.*, 1990; Williams *et al.*, 1987). Systemic administration of CRF also causes a pronounced reduction in blood pressure with a transient increase in heart rate in a number of species including rat, dog and primates (Gardiner *et al.*, 1988; Corder *et al.*, 1992; Lenz *et al.*, 1985; MacCannell *et al.*, 1982; Schurmeyer *et al.*, 1985; Udelsman *et al.*, 1986). Two distinct CRF receptors have been cloned, one of which is found in the heart and skeletal muscle and displays higher affinity for SVG and UI (Chang *et al.*, 1993; Kishimoto *et al.*, 1995). This is consistent with the greater potency of these peptides, compared to CRF, at decreasing blood pressure and dilating the mesenteric vasculature (MacCannell *et al.*, 1982; Lenz *et al.*, 1985).

The hypotensive action of these peptides is primarily due to widespread vasodilatation, including the mesenteric vasculature and hindquarters (Gardiner *et al.*, 1988; MacCannell *et al.*, 1982), and is thought to be a direct action of CRF on blood vessels. Studies with mesenteric resistance arteries (Lei *et al.*, 1993) and the isolated heart (Grunt *et al.*, 1993) support this, as does work showing the binding of [¹²⁵I]-Tyr-CRF to rabbit

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aorta (Dashwood *et al.*, 1987). However the exact mechanism of action remains unclear, with evidence for a direct action on vascular smooth muscle mediated by increased cyclic AMP levels (Lei *et al.*, 1993; Gerritsen & Lederis, 1979), or an indirect action for instance in the the coronary circulation where the response is partly endothelium-dependent and attenuated by L-N^G-monomethylarginine (L-NMMA) and indomethacin (Grunt *et al.*, 1993). Consistent with our initial investigations (Barker & Corder, 1995), recent studies in the rat aorta have reported a partial inhibition of the response by removal of the endothelium, L-N^G-nitroarginine methyl ester (L-NAME), or LY83583, an inhibitor of guanylate cyclase (Jain *et al.*, 1997).

We have investigated the vasodilator response to CRF using the isolated perfused superior mesenteric arterial vasculature (SMA) of the rat, and compared its actions with those of SVG. The role of the endothelium in the vasodilator action was determined by its removal with the detergent deoxycholic acid, selective inhibition of nitric oxide synthase (NOS) or inhibition of guanylate cyclase with 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, Moro *et al.*, 1996). The involvement of potassium channels in the vasodilator mechanism was evaluated using high extracellular potassium chloride, the non-selective potassium channel blocker tetraethylammonium (TEA), and the ATP-sensitive potassium channel blocker glibenclamide (Langton *et al.*, 1991; Edwards & Weston, 1993). Direct vasodilator effects of CRF and SVG were compared using rings of rat thoracic aorta (RTA) where the endothelium had been removed.

Methods

Isolated perfused rat superior mesenteric arterial vasculature

Male Wistar rats (A. Tuck & Son, Battlebridge, U.K.) weighing 200–400 g were anaesthetized with pentobarbitone (60 mg kg⁻¹, i.p.). A midline incision was made and the superior mesenteric artery was located, cleared of surrounding tissue, cannulated and perfused with heparinized Krebs solution (100 U ml⁻¹) at 2.5 ml min⁻¹. The superior mesenteric arterial vascular bed was separated from the intestines and transferred to the perfusion apparatus where it was perfused at 5 ml min⁻¹ with Krebs solution (mM): NaCl 118, NaHCO₃ 25, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 0.7, CaCl₂ 2.5 and glucose 5.6, warmed to 37°C and gassed with 95% O₂: 5% CO₂. Perfusion pressure was recorded on a Gould RS3400 recording system, *via* Gould P23XL pressure transducers. A 15 min stabilization period was allowed, after which an infusion of methoxamine (final concentration 50 µM) was started. A further stabilization period was allowed before testing the endothelium-dependent and endothelium-independent vasodilator responses by administration of single bolus doses of acetylcholine (ACh, 100 pmol), sodium nitroprusside (SNP, 1 nmol), and isoprenaline (1 nmol, for evaluating the effects of detergent and L-NAME only).

Dose response curves to CRF or SVG were constructed using doses in the range 10 pmol–1 nmol. For subsequent investigations single bolus doses of CRF (100 pmol) or SVG (15 pmol) were used, as these gave comparable reductions in perfusion pressure (PP). The vasodilator responses to single bolus doses of CRF and SVG were measured for 20 min. Due to the sustained action of CRF and SVG, for studies evaluating the mechanism of the vasodilatation each SMA preparation was used to measure only one response.

The role of the endothelium

The effect of removal of the endothelium was studied using the detergent deoxycholic acid. After the initial verification of vasodilator responses with ACh, SNP and isoprenaline, methoxamine infusion was stopped and deoxycholic acid (5 mM) was infused for 20 s. After a period of recovery (approximately 30 min) the methoxamine infusion was restarted, and once perfusion pressure was stable, the responses to ACh, SNP and isoprenaline were re-tested. This was followed by a single bolus dose of CRF (100 pmol) or SVG (15 pmol).

The role of nitric oxide

The role of endothelial NOS (eNOS) derived nitric oxide (NO) was investigated in separate experiments, using the NOS inhibitors L-NAME (100 µM), L-NMMA (100 µM) or 2-ethyl-2-thiopseudourea (ETPU, 100 µM; Southan *et al.*, 1995), or the inactive isomer D-NAME (100 µM). The effect of inhibiting guanylate cyclase was studied using ODQ (10 µM).

The initial doses of ACh and SNP were followed by an infusion of one of the above compounds. The perfusion pressure was allowed to stabilize, after which the doses of ACh and SNP were repeated. A single bolus dose of CRF (100 pmol) or SVG (15 pmol; L-NAME experiments only) was then administered and the response recorded as above.

The role of NO was also investigated using L-NAME to reverse the vasodilator response to CRF. The endothelium-dependent and -independent responses were verified with ACh and SNP as above. One of three treatments was employed in each set of tissues, with all responses being measured for 35 min. In the control group basal pressure was recorded for 20 min, followed by an infusion of 100 µM L-NAME for 15 min. A second group was given a single bolus of 500 pmol CRF, followed 20 min later by an infusion of L-NAME (100 µM). The third group received an infusion of isoprenaline (8 nmol min⁻¹; 1.6 µM), which gave a vasodilatation of similar magnitude to the CRF. After 20 min L-NAME (100 µM) was also infused, and the response recorded for a further 15 min.

The role of prostaglandins

The influence of products of cyclo-oxygenase on the vasodilator response was investigated by infusion of indomethacin (3 µM) after the initial doses of ACh and SNP. Further doses of ACh and SNP were then given followed by a single dose of CRF (100 pmol).

The role of potassium channels

The involvement of potassium channels in the vasodilator response to CRF was investigated by constricting the SMA with a high concentration of potassium. After the initial doses of ACh and SNP, the infusion of methoxamine was terminated and an infusion of potassium chloride (60 mM) was commenced. Once the perfusion pressure had stabilized, further doses of ACh and SNP were given followed by CRF (100 pmol).

The role of specific potassium channels in the response to CRF was studied using the non-selective potassium channel blocker, TEA, and the selective blocker of ATP-sensitive potassium channels, glibenclamide. After the initial doses of ACh and SNP an infusion of TEA (3 or 10 mM) or glibenclamide (10 µM) was started. Further doses of ACh and

SNP were given followed by a single dose of CRF (100 pmol).

Rings of rat thoracic aorta

Male Wistar rats were killed with an overdose of anaesthetic (sodium pentobarbitone, 120 mg kg⁻¹, i.p.). The thoracic aorta was removed, cleared of surrounding tissue and cut into rings 3–4 mm in length. The endothelium was removed by gently scraping the inside of each ring with the tip of a pair of forceps. Rings were mounted in organ baths containing Krebs solution (formula as above) warmed to 37°C and gassed with 95%O₂:5%CO₂, and gradually placed under 2.5 g tension. Changes in tension were measured on an isometric Siegestab K30 force transducer, and recorded on a Graphtec Linearcorder Mark VI. Tissues were contracted with 50 µM methoxamine, and the response to ACh (10⁻⁷–10⁻⁵ M) was evaluated to ensure the absence of endothelium-dependent vasodilator responses. Any tissues that responded to ACh were discarded. After washing the tissues and reapplication of methoxamine, concentration response curves to either CRF or SVG (10⁻¹⁰–10⁻⁷ M) were constructed. Due to the long lasting effect of CRF and SVG each RTA preparation was used for only one concentration response curve.

Materials

SVG was obtained from Bachem, U.K. Rat CRF was a gift from Professor N. Ling, Neurocrine (La Jolla, CA, U.S.A.). Peptides were dissolved in 10 mM sodium bicarbonate and dilutions were made in Krebs solution containing 0.1% bovine serum albumin. ODQ was obtained from Calbiochem. All other compounds were purchased from Sigma. Compounds were dissolved and diluted in Krebs solution except for indomethacin which was dissolved in 0.6 M sodium bicarbonate, and ODQ and glibenclamide which were dissolved in DMSO. For each a dilution of 1:1000 into Krebs was followed by a further 1:30 dilution on entry into the perfusion system, where appropriate vehicle controls were carried out.

Data analysis

Results shown as means ± s.e.mean. were compared by analysis of variance or Student's *t*-test as appropriate, with statistically significant differences accepted at *P* < 0.05. Basal perfusion pressures (PP, mmHg) are given in figure legends. To facilitate comparison of vasodilator responses during different treatments, values were expressed as a percentage of PP prior to administration of each agent. Comparison of relative potencies was made using EC40 (the concentration causing a 40% reduction in tension) and ED40 (the dose causing a 40% reduction in PP). Changes in perfusion pressure are indicated by ΔPP expressed as a percentage.

Results

In the perfused SMA SVG was 10 fold more potent than CRF (Figure 1a), with ED40 values of 0.05 ± 0.02 nmol (*n* = 8) and 0.6 ± 0.1 nmol (*n* = 7) respectively (*P* < 0.05). On the RTA SVG was also the most potent of the two peptides, and ≈ 50 fold more active than CRF. The threshold concentration for vasodilatation of the RTA was 0.3 nM for SVG (*n* = 8) and 10 nM for CRF (*n* = 7), and EC40 values for these peptides were 0.9 ± 0.2 and 44 ± 9 nM respectively (Figure 1b).

A single bolus injection of either CRF or SVG produced a long lasting vasodilatation in the SMA (Figure 2). CRF (100 pmol, *n* = 10) produced a maximum fall in perfusion pressure of 23 ± 2% at 3 min. This response was sustained with perfusion pressure still reduced by 15 ± 2% at 20 min. Similarly, SVG (15 pmol, *n* = 8) caused a maximum fall in perfusion pressure of 24 ± 2% at 4 min, with a reduction in perfusion pressure of 17 ± 4% still present at 20 min.

Deoxycholic acid

Infusion of detergent inhibited endothelium-dependent but not endothelium-independent responses. There was no vasodilator response to ACh (100 pmol), compared with a pre-deoxycholic acid fall in perfusion pressure of 48 ± 1%. The magnitude of the SNP induced vasodilatation was increased markedly (ΔPP -18 ± 5%, control; ΔPP -34 ± 5%, detergent treated; *P* < 0.05), but the response to isoprenaline was unchanged (ΔPP -15 ± 4%, control; ΔPP -13 ± 3%, detergent treated). The response to a single bolus dose of CRF (100 pmol) after deoxycholic acid infusion (*n* = 6) was reduced in both size and duration (Figure 3a). The initial fall in perfusion pressure was

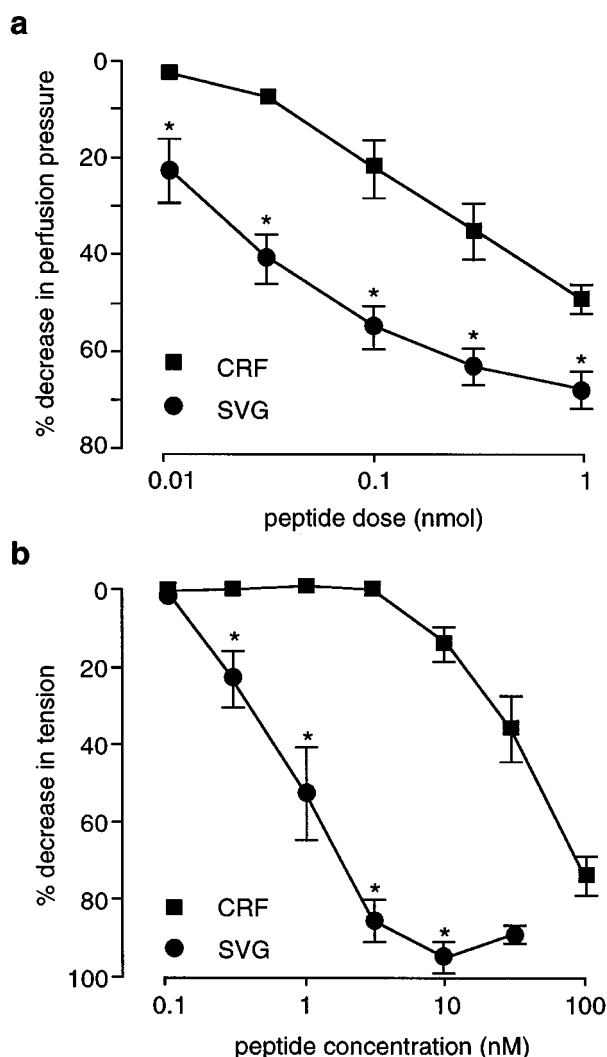


Figure 1 Dose response curves to CRF and SVG in (a) perfused SMA (CRF, *n* = 7, basal PP 116 ± 9 mmHg; SVG, *n* = 8; basal PP, 88 ± 9 mmHg), and (b) endothelium de-nuded RTA (CRF, *n* = 5; SVG, *n* = 6). **P* < 0.05 compared to CRF.

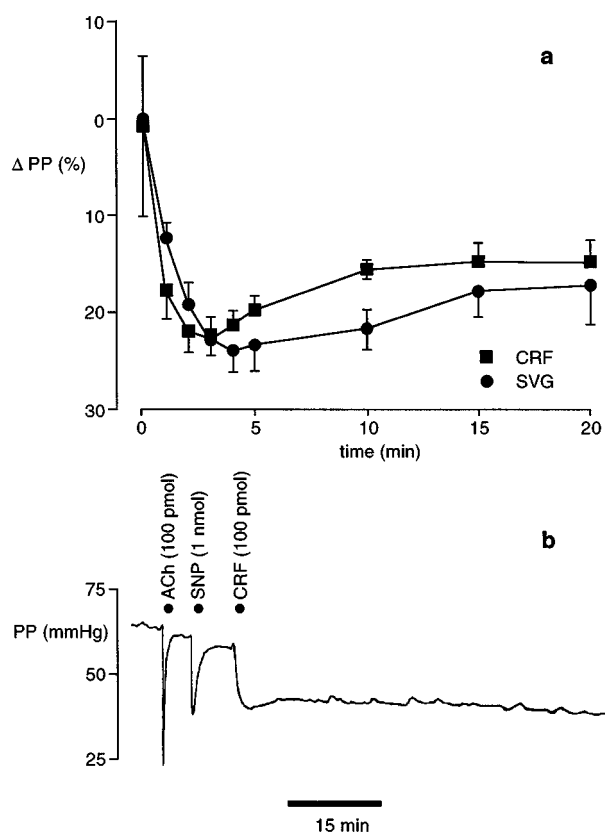


Figure 2 Effect of a single bolus dose of CRF or SVG in the perfused SMA. (a) Vasodilator responses to 100 pmol CRF ($n=10$, basal PP 79 ± 7 mmHg) and 15 pmol SVG ($n=7$, basal PP 104 ± 10 mmHg). (b) Typical vasodilator response to ACh (100 pmol), SNP (1 nmol) and CRF (100 pmol).

only $9 \pm 2\%$, compared to a fall of $23 \pm 2\%$ in the control preparations ($P < 0.05$). In contrast to the sustained response in control tissues, after removal of the endothelium perfusion pressure returned to pre-CRF levels within 5 min. Deoxycholic acid also attenuated the response to SVG (Table 1). Perfusion pressure in detergent treated tissues ($n=6$) fell by $9 \pm 3\%$, compared to a decrease of $24 \pm 2\%$ in control tissues ($P < 0.05$). In comparison with CRF, a small vasodilator response to SVG persisted for 10 min but it was substantially less than the response in control preparations.

NOS inhibition

Infusion of L-NAME reduced the ACh-induced vasodilatation ($\Delta PP -48 \pm 1\%$, control; $\Delta PP -25 \pm 4\%$, L-NAME; $P < 0.05$), but increased the response to SNP ($\Delta PP -18 \pm 5\%$, control; $\Delta PP -39 \pm 5\%$, L-NAME; $P < 0.05$). The response to isoprenaline was unaffected ($\Delta PP -15 \pm 4\%$, control; $\Delta PP -16 \pm 3\%$, L-NAME; $P > 0.05$).

L-NAME had a similar effect on the CRF induced vasodilatation in the SMA (Figure 3b) to the removal of the endothelium with detergent. The initial fall in perfusion pressure of $14 \pm 3\%$ ($n=6$) was significantly less than the control response ($P < 0.05$). At 5 min the perfusion pressure in the L-NAME treated preparations had almost returned to pre-CRF levels, and by 10 min the CRF response was completely inhibited. The response to SVG was also attenuated by L-NAME (Table 1). The initial vasodilatation ($\Delta PP -12 \pm 3\%$) was smaller during L-NAME infusion ($n=6$) compared to the

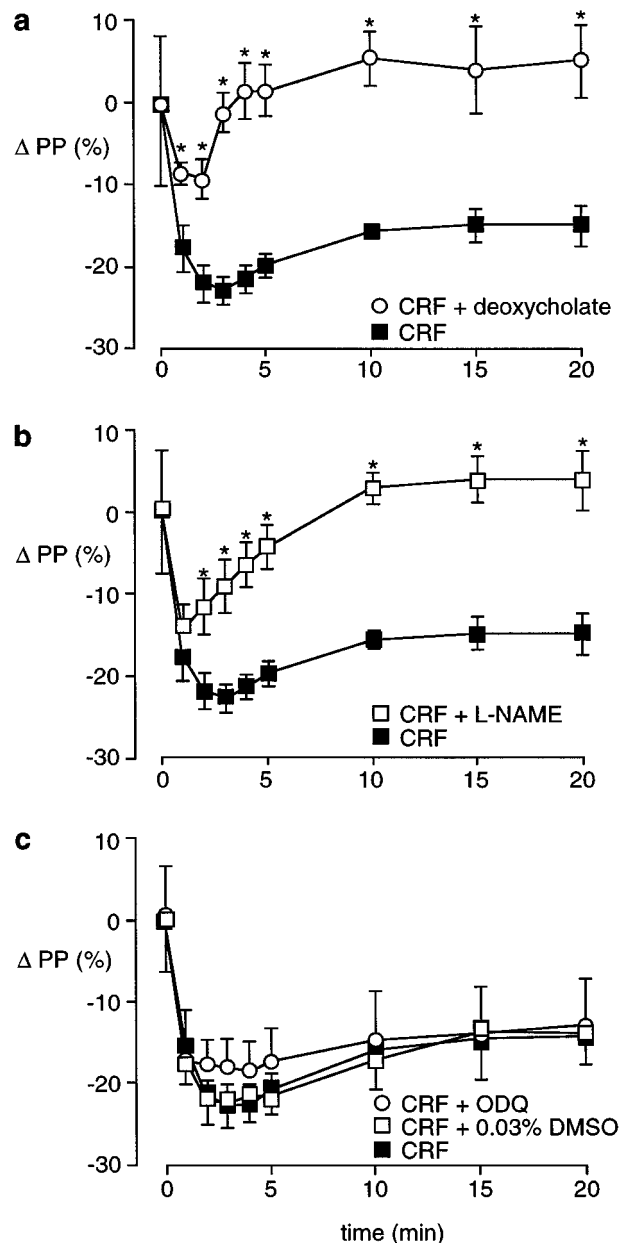


Figure 3 Effect of different treatments on the vasodilator response to CRF ($n=10$, basal PP 79 ± 7 mmHg) in the perfused SMA. (a) 5 mM deoxycholic acid for 20 s ($n=6$, basal PP 83 ± 9 mmHg); (b) 100 μM L-NAME ($n=6$, basal PP 165 ± 14 mmHg); (c) 10 μM ODQ ($n=8$, basal PP 138 ± 10 mmHg), or 0.03% DMSO control ($n=7$, basal PP 87 ± 7 mmHg). * $P < 0.05$ compared to control response.

control tissues ($P < 0.05$), and perfusion pressure had returned to baseline 10 min after SVG administration.

L-NMMA attenuated the fall in perfusion pressure to ACh ($\Delta PP -48 \pm 1\%$, control; $\Delta PP -38 \pm 4\%$, L-NMMA; $P < 0.05$) but increased the size of the response to SNP ($\Delta PP -18 \pm 5\%$, control; $\Delta PP -37 \pm 4\%$, L-NMMA; $P < 0.05$). Although the magnitude of the initial response to CRF was not decreased, L-NMMA ($n=6$) attenuated the response to CRF with significant inhibition of the response at 5 min ($P < 0.05$), and a return to pre-CRF perfusion pressure by 10 min (Table 2).

In common with L-NAME and L-NMMA, ETPU significantly reduced the ACh-induced fall in perfusion pressure ($\Delta PP -48 \pm 1\%$, control; $\Delta PP -23 \pm 5\%$, ETPU; $P < 0.05$) whilst increasing the SNP-induced vasodilatation (ΔPP

Table 1 Effect of deoxycholic acid (5 mM for 20 s) and L-NAME (100 μ M) on SVG (15 pmol)-induced vasodilatation of the perfused SMA

		Basal PP (mm Hg)	Maximum fall in PP	5 min	% Change in perfusion pressure 10 min	15 min	20 min
SVG control	<i>n</i> = 7	104 $\pm$ 10	-24 $\pm$ 2	-23 $\pm$ 3	-22 $\pm$ 3	-18 $\pm$ 3	-17 $\pm$ 4
SVG + deoxycholic acid	<i>n</i> = 6	82 $\pm$ 10	-9 $\pm$ 2*	-9 $\pm$ 3*	-6 $\pm$ 2*	-4 $\pm$ 2*	-2 $\pm$ 2*
SVG + L-NAME	<i>n</i> = 6	150 $\pm$ 12	-12 $\pm$ 3*	-10 $\pm$ 4*	-10 $\pm$ 4*	7 $\pm$ 4*	10 $\pm$ 5*

P* < 0.05 compared to control response.Table 2** Effect of L-NMMA, ETPU and D-NAME on CRF (100 -pmol)-induced vasodilatation of the perfused SMA

		Basal PP (mm Hg)	Maximum fall in PP	% Change in perfusion pressure			
				5 min	10 min	15 min	20 min
CRF control	<i>n</i> = 10	79 $\pm$ 7	-23 $\pm$ 3	-21 $\pm$ 2	-16 $\pm$ 1	-14 $\pm$ 3	-14 $\pm$ 3
CRF + L-NMMA (100 μ M)	<i>n</i> = 6	141 $\pm$ 7	-23 $\pm$ 3	-13 $\pm$ 3*	-7 $\pm$ 2*	-4 $\pm$ 2*	-4 $\pm$ 2*
CRF + ETPU (100 μ M)	<i>n</i> = 4	120 $\pm$ 9	-14 $\pm$ 6	0 $\pm$ 6*	13 $\pm$ 10*	16 $\pm$ 10*	6 $\pm$ 8*
CRF + D-NAME (100 μ M)	<i>n</i> = 6	75 $\pm$ 4	-21 $\pm$ 2	-17 $\pm$ 2	-15 $\pm$ 3	-11 $\pm$ 3	-9 $\pm$ 4

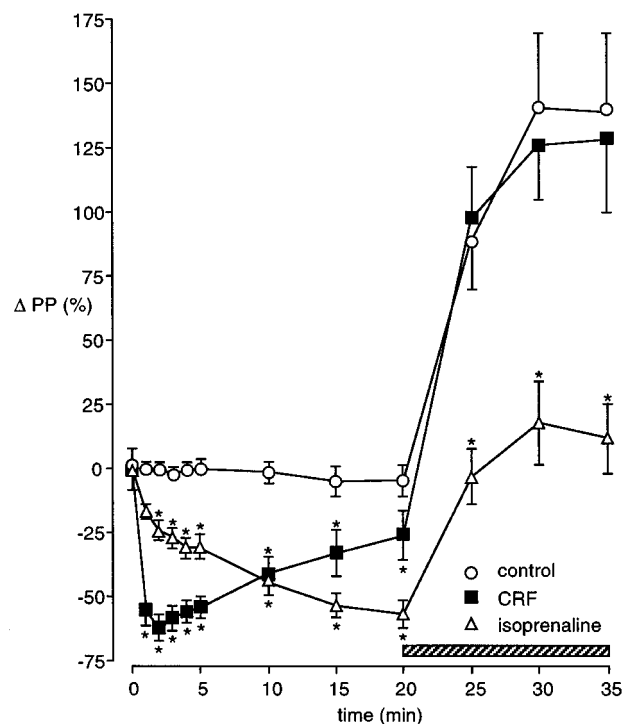
**P* < 0.05 compared to control response.

-18 $\pm$ 5%, control; Δ PP -43 $\pm$ 7%, ETPU; *P* < 0.05). ETPU (*n* = 4) also inhibited the response to CRF reducing the maximum fall in perfusion pressure and the duration of the response (Table 2).

In contrast D-NAME had no significant effect on either the ACh-induced fall in perfusion pressure (Δ PP -48 $\pm$ 1%, control; Δ PP -46 $\pm$ 5%, D-NAME) or the reduction in perfusion pressure to SNP (Δ PP -18 $\pm$ 5%, control; Δ PP -18 $\pm$ 2%, D-NAME). There was also no significant effect on either the magnitude or the length of the CRF-induced vasodilator response (*n* = 6, Table 2).

DMSO vehicle (0.03%) had no effect on responses to ACh (Δ PP -48 $\pm$ 1%, control; Δ PP -51 $\pm$ 2%, DMSO), SNP (Δ PP -18 $\pm$ 5%, control; Δ PP -20 $\pm$ 1%, DMSO), or CRF (*n* = 7, Figure 3c). Infusion of ODQ (10 μ M) significantly reduced the fall in perfusion pressure to ACh (Δ PP -48 $\pm$ 1%, control; Δ PP -33 $\pm$ 3%, ODQ) and completely abolished the SNP response. However ODQ (*n* = 6) did not inhibit either the size of the response to CRF or its duration of action (Figure 3c).

In order to evaluate the effect of eNOS inhibition on already established vasodilator responses a second set of experiments with L-NAME was performed (Figure 4). Both CRF and isoprenaline showed large vasodilator effects, with the maximum fall in perfusion pressure (Δ PP -62 $\pm$ 5%) to the bolus of 500 pmol CRF being recorded at 2 min (*n* = 6). The largest reduction in perfusion pressure during the isoprenaline infusion was 57 $\pm$ 6% at 20 min (*n* = 6). In control tissues the perfusion pressure remained stable throughout the initial 20 min (*n* = 6), and L-NAME infusion increased perfusion pressure by 141 $\pm$ 33% above the basal level. In tissues where CRF was administered, perfusion pressure was still 26 $\pm$ 10% below the basal level 20 min after bolus administration. L-NAME infusion reversed the effect of CRF and perfusion pressure increased to 128 $\pm$ 28% above the baseline level. This increase was not significantly different from the response to L-NAME in control preparations. In isoprenaline treated tissues, L-NAME infusion increased perfusion pressure to 16 $\pm$ 16% above the baseline level which was significantly less than the L-NAME-induced increase in control and CRF-treated preparations (*P* < 0.05).

**Figure 4** Effect of infusing L-NAME (100 μ M) during the vasodilator response to CRF (500 pmol) or isoprenaline (8 nmol min⁻¹) in the perfused SMA. The hatched bar shows the period of L-NAME infusion. Control L-NAME infusion, basal PP 46 $\pm$ 7 mmHg; L-NAME during the CRF response, basal PP 48 $\pm$ 8 mmHg; L-NAME during isoprenaline infusion, basal PP 51 $\pm$ 6 mmHg. *n* = 6 for each, **P* < 0.05 compared to control L-NAME response.

The role of prostaglandins

Indomethacin infusion (3 μ M) did not significantly affect the fall in perfusion pressure to ACh (Δ PP -48 $\pm$ 2%, control Δ PP -39 $\pm$ 5%, indomethacin) or SNP (Δ PP -28 $\pm$ 2%,

control; ΔPP $-23 \pm 3\%$, indomethacin). The initial fall in perfusion pressure to CRF was slightly but significantly increased (ΔPP $-25 \pm 3\%$, control; $-34 \pm 5\%$, indomethacin; $n=6$, $P<0.05$), but there were no significant differences at any other time.

Potassium channel antagonists

Infusion of 60 mM KCl inhibited the fall in perfusion pressure to ACh (ΔPP $-48 \pm 1\%$, control; ΔPP $-17 \pm 3\%$, 60 mM KCl; $P<0.05$), but did not significantly alter the SNP-induced fall in perfusion pressure (ΔPP $-18 \pm 5\%$, control; ΔPP $-11 \pm 1\%$, 60 mM KCl). The response to CRF was completely inhibited at all time points ($n=6$, Figure 5a).

The non-selective potassium channel antagonist TEA (3 and 10 mM) significantly inhibited ACh-induced decreases in

perfusion pressure (ΔPP $-48 \pm 1\%$, control; ΔPP $-20 \pm 6\%$, 3 mM TEA; ΔPP $-20 \pm 3\%$, 10 mM TEA; $P<0.05$) but had no effect on the fall in perfusion pressure to SNP (ΔPP $-18 \pm 5\%$, control; ΔPP $-23 \pm 1\%$, 3 mM TEA; ΔPP $-26 \pm 4\%$, 10 mM TEA). However neither 3 mM ($n=4$) nor 10 mM ($n=6$) TEA had any effect on the vasodilator response to CRF (Figure 5b).

In contrast to TEA, glibenclamide, a selective blocker of ATP-sensitive potassium channels did not inhibit either the fall in perfusion pressure to ACh (ΔPP $-48 \pm 1\%$, control; ΔPP $-54 \pm 3\%$, glibenclamide) or SNP (ΔPP $-18 \pm 5\%$, control; ΔPP $-20 \pm 1\%$, glibenclamide), but significantly reduced the response to CRF (Figure 5c). Although the initial response was not affected (ΔPP $-22 \pm 2\%$), 10 min after CRF the vasodilator response was partially inhibited and by 15 min perfusion pressure had returned to basal levels.

Discussion

CRF and SVG produced dose-dependent vasodilations of endothelium de-nuded RTA and the perfused SMA. A single bolus dose of CRF or SVG causes a long lasting vasodilatation of the perfused SMA. In agreement with *in vivo* studies that have compared the two peptides, SVG was more potent than CRF in both vascular preparations (Brown *et al.*, 1982a; Lenz *et al.*, 1985). Earlier studies of the vasodilator action of CRF have reported the effect to be either a direct vascular smooth muscle effect (Lei *et al.*, 1993), or partly endothelium-dependent (Grunt *et al.*, 1993; Jain *et al.*, 1997). Here using the RTA from which the endothelium had been removed CRF and SVG produced concentration-dependent relaxation of vascular smooth muscle by a direct action. But of greater interest, and of more relevance to the effect of systemic administration of these peptides on blood pressure, studies of the perfused SMA showed these peptides to have two components to their vasodilator action; a short direct action on vascular smooth muscle, followed by a sustained endothelium-dependent vasodilatation. This was demonstrated firstly by the removal of the endothelium with deoxycholic acid and secondly using three different eNOS inhibitors. From these results it can be concluded that the long-lasting endothelium-dependent vasodilator response is due to the sustained activation of eNOS and a prolonged release of NO.

The role of NO in the prolonged vasodilator response to CRF was also demonstrated by infusing L-NAME during the prolonged phase of CRF-induced vasodilatation. This increased the perfusion pressure to a level that was not significantly different to L-NAME infusion alone. Hence the vasodilatation at the point when L-NAME is infused is due solely to NO, because any direct effect on vascular smooth muscle, or release of other endothelial vasodilator molecules would be expected to attenuate the rise in perfusion pressure. This is clearly seen with the infusion of isoprenaline, where there is a small increase in perfusion pressure, due to the inhibition of basal NO synthesis, but it remains below that seen during L-NAME infusion in control- and CRF-treated preparations. Some studies indicate a role for NO in the vasodilator actions of isoprenaline (Graves & Poston, 1993), but here the response to a single bolus dose of isoprenaline was unaffected by either deoxycholic acid or L-NAME, indicating a vasodilator effect which is largely independent of any action on the endothelium. Inhibition by indomethacin of the vasodilator response to CRF in the isolated heart (Grunt *et al.*, 1993) suggests that prostaglandins play a role in the endothelium-dependent vasodilatation, but no evidence for CRF-induced

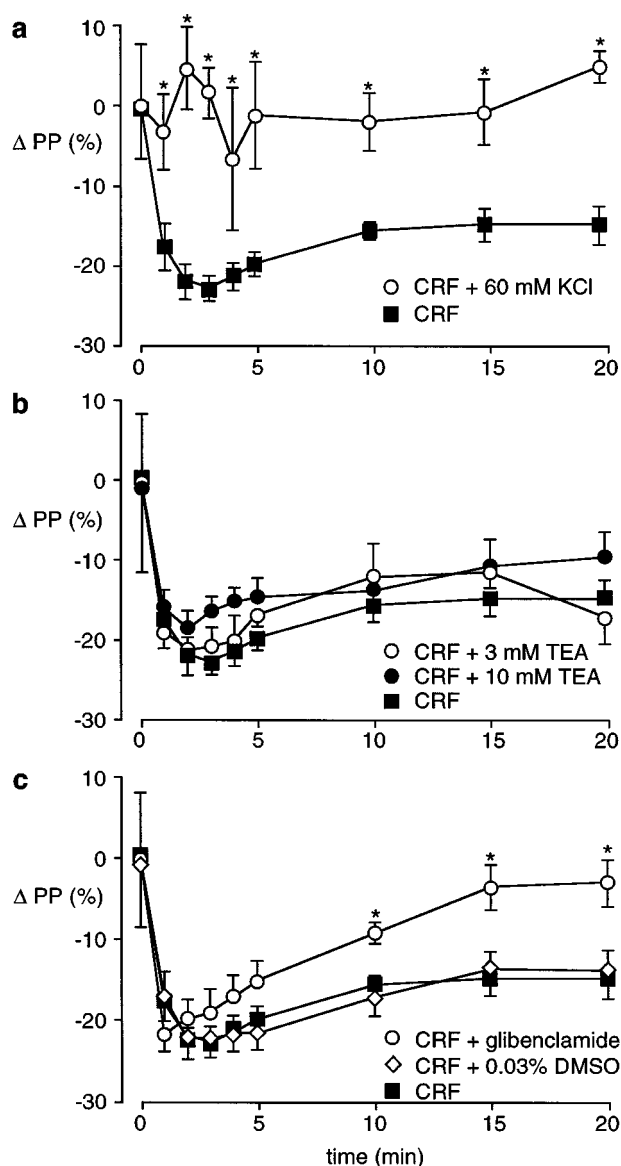


Figure 5 Effect of inhibiting potassium channel function on the vasodilator response to 100 pmol CRF in the perfused SMA (CRF $n=10$, basal PP 79 ± 7 mmHg). (a) 60 mM KCl ($n=4$, basal PP 81 ± 6 mmHg); (b) 3 mM TEA ($n=4$, basal PP 68 ± 8 mmHg), 10 mM TEA ($n=6$, basal PP 90 ± 7 mmHg); (c) 10 μ M glibenclamide ($n=9$, basal PP 81 ± 7 mmHg), 0.03% DMSO ($n=7$, basal PP 84 ± 7 mmHg). * $P<0.05$ compared to control response.

synthesis of vasodilator prostanoids was observed in the perfused SMA.

In agreement with the pivotal role of the endothelium in the vasodilator responses to CRF or SVG, autoradiographic studies of rabbit aorta have shown the endothelium as well as the underlying vascular smooth muscle to bind [^{125}]-Tyr-CRF (Dashwood *et al.*, 1987). Direct effects of this family of peptides on vascular smooth muscle have been observed through measurement of increased cyclic AMP in response to UI (Gerritsen & Lederis, 1979). Consistent with the results described here, the effect of UI on cyclic AMP levels in vascular smooth muscle is short lasting (Gerritsen & Lederis, 1979). A peripheral CRF receptor (CRF-R2 β) has been cloned which is G-protein coupled to adenylate cyclase (Kishimoto *et al.*, 1995). This receptor has been identified in blood vessels (Lovenberg *et al.*, 1995), but its localization to endothelial or vascular smooth muscle cells has yet to be confirmed. In previous studies of the vasodilator effect of CRF in intact rat mesenteric arteries it was suggested that the CRF-R2 β receptor was involved in the response (Rohde *et al.*, 1996). In the studies of direct vasodilator effects on the endothelium de-nuded RTA described here, the EC₄₀ values for SVG and CRF, and the 50 fold difference in potency of these peptides are very similar to values reported for the induction of cyclic AMP synthesis by the human CRF-R2 β receptor (Liaw *et al.*, 1997). Hence, the CRF-R2 β receptor may well mediate the direct vasodilator effects of CRF on vascular smooth muscle, but it is unclear whether the endothelium-dependent effects are also *via* this receptor subtype.

A surprising aspect of these studies was the mechanism of the CRF-induced NO-dependent vasodilatation. Although it is generally accepted that NO produces relaxation of vascular smooth muscle primarily by activation of guanylate cyclase and an increase in cyclic GMP, the response to CRF in the perfused SMA was not altered by the selective guanylate cyclase inhibitor ODQ at concentrations which inhibited completely SNP-induced vasodilatation. This contrasts with results obtained in studies of CRF on the rat thoracic aorta using LY83583 which is also described as a guanylate cyclase inhibitor (Jain *et al.*, 1997). However, LY83583 also inhibits NOS, and generates free radicals that scavenge NO, thus preventing the actions of NO independently of any inhibitory effect on guanylate cyclase (Kontos & Wei, 1993; Kannan & Johnson, 1995; Luo *et al.*, 1995). When compared with the lack of effect of ODQ described here, it seems likely that LY83583 inhibited CRF-induced vasodilatation by a mechanism unrelated to guanylate cyclase inhibition.

An alternative mechanism of action for NO has been described, involving the opening of potassium channels in vascular smooth muscle to produce vasodilatation (Bolotina *et al.*, 1994). The inhibition of both CRF and ACh-induced vasodilator effects by high extracellular potassium indicates a role for potassium channels in their vasodilator responses. However, comparison of the effects of TEA and glibenclamide on the actions of ACh and CRF suggest the involvement of different potassium channels in their mechanisms. TEA attenuated the ACh response at a concentration (3 mM) which shows selectivity for inhibition of calcium-activated potassium channels (Langton *et al.*, 1991), without altering the response to CRF. Conversely the NO-dependent phase of the response to CRF was attenuated by glibenclamide, a selective inhibitor of

ATP-sensitive potassium channels (Edwards & Weston, 1993), even though it did not alter the response to ACh. High concentrations of TEA (10 mM) are reported to cause a non-selective blockade of potassium channels (Langton *et al.*, 1991), but no effect on the vasodilator action of CRF was observed.

It has been proposed that endothelium-derived hyperpolarizing factor (EDHF) is more important than NO in mediating the endothelium-dependent response to ACh in the small resistance vessels of the mesenteric vasculature (Garland & McPherson, 1992; Parsons *et al.*, 1994). However the complete inhibition of the sustained phase of CRF vasodilatation with inhibitors of eNOS indicates that EDHF is not involved. In the mesenteric vasculature and isolated perfused kidney of the rat, EDHF appears to act *via* calcium-activated potassium channels (McPherson & Angus 1991; Rapacon *et al.*, 1996), but the hyperpolarizing response to NO is mediated by ATP-sensitive channels (Garland & McPherson, 1992). The precise role of ATP-sensitive potassium channels in the NO-dependent vasodilatation induced by CRF is not evident at this time.

The ability of a single bolus of CRF or SVG to produce a long lasting, NO-mediated vasodilatation is of particular interest given that other vasodilator responses involving the release of NO tend to be of short duration. For example ACh produces a large endothelium-dependent vasodilatation lasting <1 min. In some respects the cascade superfusion technique using cultured endothelial cells (Gryglewski *et al.*, 1986) is similar to that used here as the agents applied are rapidly washed through. This has shown that bradykinin, another peptide mediator causing endothelium-dependent vasodilatation induces NO release for <5 min. In comparison, the calcium ionophore, A23187, can cause a longer lasting output of NO (Gryglewski *et al.*, 1986), indicating that a lengthy influx of Ca²⁺ causes a sustained activation of eNOS. Hence, a prolonged receptor mediated influx of Ca²⁺ could play a role in the endothelium-dependent vasodilator effect of CRF.

However, physiologically, the most notable factor causing a sustained NO-dependent vasodilatation is shear stress (Frangos *et al.*, 1996). Hence, the mechanism underlying shear stress-induced NO synthesis may be of more relevance to the response to CRF. NO release stimulated by constant shear involves both calcium-dependent and ATP-sensitive potassium channels (Hutcheson & Griffith, 1994). Indeed, the inhibitory effect of glibenclamide on the response to CRF may well be due to an effect on NO release (Hutcheson & Griffith, 1994), thus indicating similarities in the mechanisms of shear stress and CRF-induced NO release. Interestingly, heat shock protein 90 (Hsp90) has recently been shown to modulate the activity of eNOS during stimulation with agonists or shear stress (García-Cardena *et al.*, 1998). Based on these findings, the sustained NO-dependent vasodilator effect of CRF could involve the induction of eNOS-Hsp90 heterocomplexes with a resultant sensitization of the endothelium to shear stress induced NO release in the perfused SMA. In conclusion, further work is required for a full appreciation of this unusual and potentially important mechanism of eNOS activation by the CRF-family of peptides.

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The effects of inflammation and inflammatory mediators on nociceptive behaviour induced by ATP analogues in the rat

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1 We have studied the behavioural effects of intraplantar injections of adenosine 5'-triphosphate (ATP) and related compounds in freely moving rats and investigated whether these nociceptive effects are augmented in the presence of inflammatory mediators.

2 We find that in normal animals ATP and analogues produce dose-dependent nocifensive behaviour (seen as bursts of elevation of the treated hindpaw), and localized thermal hyperalgesia. The rank order of potency was: α,β -methyleneadenosine 5'-triphosphate (α,β -methylene ATP) > 2-methylthioadenosine triphosphate (2-methylthio ATP) > ATP. After neonatal treatment with capsaicin, to destroy small calibre primary sensory neurones, nocifensive behaviour was largely absent.

3 The effects of ATP analogues were assessed in three models of peripheral sensitization: 2 h after dilute intraplantar carrageenan (0.25% w v⁻¹); 24 h after irradiation of the hindpaw with ultraviolet (U.V.) B; immediately following prostaglandin E₂ (PGE₂) treatment. In all models the effect of α,β -methylene ATP was greatly augmented. After carrageenan, significant hindpaw-lifting behaviour activity was induced by injection of only 0.05 nmol of α,β -methylene ATP, some 100 times less than necessary in normal skin.

4 Our data suggest that it is much more likely that endogenous levels of ATP will reach levels capable of exciting nociceptors in inflamed versus normal skin. Our data also suggest the involvement of P2X₃ receptor subunits in ATP-induced nociception.

Keywords: Purinoreceptors; pain; nociceptor; sensitization; hyperalgesia

Abbreviations: α,β -methylene ATP, α,β -methyleneadenosine 5'-triphosphate; ADP, adenosine diphosphate; ATP, adenosine 5'-triphosphate; 2-methylthio ATP, 2-methylthioadenosine triphosphate; PBS, phosphate buffered saline; U.V.B., ultraviolet B

Introduction

ATP is well recognized as an energy source and modulator of cellular function, operating ubiquitously within the body. In the nervous system, ATP's role as a neurotransmitter has been established following studies demonstrating that ATP elicits fast excitatory potentials when applied to dorsal horn neurones (Jahr & Jessel, 1983; Fyffe & Perl, 1984; Salter & Henry, 1985) and dorsal root ganglion cells (e.g. Bean, 1990, see Brake & Julius, 1996 for review). In addition to its neurotransmitter role, there has been repeated speculation, dating back to the work of Holton (1959), that ATP might serve as mediator of pain. The discovery of a receptor for ATP, P2X₃, which is exclusively expressed by small diameter sensory neurones, presumed to be nociceptors (Chen *et al.*, 1995; Lewis *et al.*, 1995; Bradbury *et al.*, 1998), further suggests a nociceptive role for ATP. In a tissue culture system, Cook *et al.* (1997) reported that ATP analogues activated nociceptive neurones. The pharmacology of the responses generated was suggestive of P2X₃ receptor involvement.

Another compelling line of evidence for the role of ATP in pain relates to the algogenic effects of ATP in humans. ATP is reported to act consistently as a painful stimulus when applied to human blister bases (Bleehen & Keele, 1977), when injected intradermally, or when applied iontophoretically (unpublished

observations). The pain elicited is modest in magnitude and limited in duration to only a few minutes. This last feature may well result from the rapid biochemical degradation of ATP *in vivo*.

α,β -methylene ATP is a relatively stable ATP agonist due to its resistance to ATP-endonucleotidase degradation. It has been used in the only two studies to have examined pain-related behaviour induced by ATP in experimental animals. Bland-Ward & Humphrey (1997) gave rats intraplantar injections of ATP and related P2X agonists. They found that these agonists induced nocifensive behaviour—lifting and licking of the injected hindpaw. This behaviour, like the pain reported in human volunteers, persisted for a relatively brief period, measured in minutes and was dose-dependent. The same authors also reported that pre-treatment of the animals with bupivacaine or capsaicin abolished the nociceptive response, indicating that the lifting response was a consequence of hindpaw sensory nerve stimulation, in particular C or A δ fibres. The second behavioural study of the effects of ATP was performed by Sawynok & Reed (1997). These authors found that intraplantar injections of ATP facilitated the well-described nocifensive behaviour induced by intraplantar formalin injections. However, they did not see overt hindpaw-lifting or hindpaw-licking activity (greater than that seen with a saline injection), in response to either free ATP (50 or 500 nmol) or α,β -methylene ATP (50 nmol). Therefore, the first aim of the current work was to study the behavioural consequences of intraplantar ATP injection in an attempt to resolve these reports.

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A surprising feature of the published behavioural studies is that high doses of agonists have been required to induce effects—threshold doses for hindpaw-lifting behaviour were 100 nmol for α,β -methylene ATP and 1000 nmol for free ATP in the study by Bland-Ward & Humphrey (1997) and 50 nmol of ATP for potentiation of formalin-induced behaviour (Sawynok & Reid, 1997). It is not clear whether such high doses are likely to be realized by endogenous release of ATP, especially since the endogenous ligand, unlike some of the exogenously used analogues, is subject to rapid degradation. A second aim of the current work was to investigate whether ATP's effects might be enhanced under pathophysiological conditions. The rationale is that tissue injury is known to induce peripheral sensitization of nociceptors—that is, increase their sensitivity to a variety of stimuli including noxious heating, mechanical stimulation and several chemical agents such as bradykinin (Meyer *et al.*, 1994). We therefore tested the hypothesis that the nociceptive system might show an increased state of responsiveness to ATP and its analogues in the presence of experimental inflammation, and thus be active at low concentrations of ATP, such as those that might be present endogenously.

Methods

Animals used

Male Wistar rats weighing 225–325 g were housed in groups of five and maintained on a 12:12 h light/dark cycle at $22 \pm 2^\circ\text{C}$. Food and water were freely available. Each hindpaw of a particular animal was tested only once, and if both hindpaws were used, an interval of a week was left between successive tests. Animals tested with experimental inflammation models were used only once.

Responses to ATP analogues in normal animals

Groups of at least five normal rats were injected with a particular dose of a test ATP analogue. Prior to receiving an injection the animal was wrapped in cloth so as to expose only its hindpaw. Once calm, 100 μl of the test analogue was injected intradermally on the plantar surface of one hindpaw, using a 30G needle. One hundred μl of vehicle (phosphate buffered saline (PBS) pH 7.4) alone was injected as a control. Following the injection the animal was immediately placed in a Perspex box and a stop clock started. The hindpaw-lifting behaviour and responsiveness to noxious thermal stimuli were then assessed. The total time an animal spent with its hindpaw elevated clear of the box floor was measured in 2 min time bins following injection for a total of 6 min or 10 min in different experiments, depending whether thermal hyperalgesia was measured. Pilot experiments showed that more than 90% of hindpaw-lifting behaviour occurred within 6 min under all experimental conditions.

The hindpaw withdrawal time to noxious thermal stimulation of the plantar surface of the treated and untreated (contralateral) hindpaw was measured by the method of Hargreaves *et al.* (1988), using a commercial device (Model 7371, Ugo Basile, Italy). Baseline recordings were taken before and then 6 min following the injection. At both of these time points, three pairs of recordings were taken of the withdrawal latencies for each hindpaw, and then averaged.

Carrageenan inflammation

Fifty μl of 0.25% w v⁻¹ carrageenan was injected into the plantar surface of the rat's hindpaw. This dose is less than traditionally used to induce inflammation, but nonetheless produces a thermal hyperalgesia 2 h later (see Results). The sensitivity of the inflamed hindpaw to ATP analogues was assessed at this time in a similar fashion to that used for naïve animals (described above). The only difference was that the volume of ATP or vehicle solution injected was 50 μl (rather than 100:1), in order to minimize the nociceptive effects of distension of inflamed tissue.

PGE₂

The effects of the inflammatory mediator PGE₂ on the responses to ATP analogues were tested in other animals. PGE₂ (either 0.7 or 7 μmoles) was co-injected with α,β -methylene ATP into the plantar surface of one hindpaw (total volume of 100 μl). PGE₂ was also injected alone as a control. Hindpaw lifting behaviour was measured as before.

U.V.-irradiation

Hyperalgesia was induced by exposure of the left and right hindpaws to an ultraviolet B (U.V.B) light source (TL01, Phillips). Groups of five rats were anaesthetized using pentobarbitone at a dose 40 mg kg⁻¹. The plantar surface of hindpaws were then exposed to 1500 mJ cm⁻² of U.V.B with a wavelength of 311 nm. All other parts of the rat's body were protected from irradiation with aluminium foil and cloth. The 1500 mJ cm⁻² of U.V.B (equivalent approximately to twice the Minimal Erythemic Dose—MED) was delivered over approximately 14 min.

Six hours after the irradiation, the animals were tested with injections of ATP analogues (100 μl) into the one hindpaw, as above, and hindpaw-lifting time monitored for 6 min. This was repeated 24 h after the initial irradiation on the other irradiated hind hindpaw. Other animals were irradiated as above and tested with vehicle (PBS) injections.

Neonatal capsaicin treatment

In order to destroy the large majority of primary afferent nociceptors, litters of rats were given systemic injections of 50 mg kg⁻¹ capsaicin (10 g l⁻¹ solution dissolved in 10% v v⁻¹ ethyl alcohol, 10% v v⁻¹ tween 50, in saline) at 24 h and 72 h after birth (Nagy *et al.*, 1983). Groups of these rats were used 4 months later. They received the same treatments as the naïve animals. Female and male rats were used.

Compounds used

Adenosine 5'-triphosphate lithium salt and α,β -methylene ATP lithium salt were obtained from Sigma. 2-methylthioadenosine triphosphate tetrasodium was obtained from Tocris. All compounds were dissolved and diluted to the relevant molarity in phosphate buffered saline (PBS). The pH of each aliquot was checked and if found to be acidic (a feature of concentrated solutions) adjusted with a sodium hydroxide (40% w v⁻¹). Aliquots were stored at -70°C . On the day of use they were thawed and kept on ice prior to the injection. Prostaglandin E₂ (I.C.N.) was initially made up in ethanol (1 mg ml⁻¹) and subsequently diluted in PBS. Carrageenan (Sigma, type IV) was dissolved in saline at 0.25% w v⁻¹ and aliquots stored at -70°C until use.

Statistical analysis

The total hindpaw-lifting time exhibited by each animal was computed. Data are given here as group averages $\pm$ standard error, unless indicated otherwise. The responses of different groups of animals were compared by ANOVA followed by Dunnett or Dunn *post hoc* tests where appropriate. The Mann-Whitney Rank Sum Test was used to compare the lifting response in capsaicin treated animals with that observed in naïve animals. The withdrawal latencies before and after injection of PBS in naïve animals were compared using a paired *t*-test. The lifting response induced by α,β -methylene ATP alone was compared with that induced by co-administration of the same dose with PGE₂ using a *t*-test, as shown in Figure 6. A *t*-test was also carried out to compare the lifting response induced by α,β -methylene ATP plus PGE₂ with the hypothetical additive response calculated by adding the results of administering the compounds separately. A level of 5% was taken as evidence of statistical significance.

Results

Responses in normal rats

In normal rats, α,β -methylene ATP consistently induced periods of intermittent hindpaw-lifting behaviour which began within 10 or 20 s of injection. The animals also frequently orientated towards the injected hindpaw, and additionally showed episodes of licking and biting of the treated hindpaw. As illustrated in Figure 1, a 50 nmol dose of α,β -methylene ATP induced hindpaw-lifting behaviour that occupies about 50% of time for the first 4 min, but then subsides rapidly, with very little nocifensive behaviour present beyond 6 min. Injections of vehicle (PBS) into the hindpaw were associated with only a very limited amount of hindpaw-lifting and licking behaviour, Figure 1. For comparisons of different compounds and doses, the total hindpaw-lifting time exhibited by each animal in the 6 min after injection was calculated, Figure 2. The relative

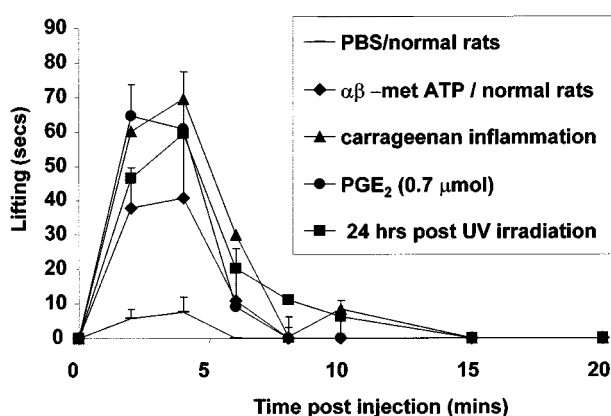


Figure 1 The time spent (in 2 min time bins) in hindpaw-lifting in rats given an intraplantar injection of α,β -methylene ATP or PBS. The ATP was given to normal rats or rats treated as indicated in the legend. The pattern of nocifensive behaviour was the same in normal rats and in the presence of inflammatory mediators, with maximal hindpaw-lifting occurring within the first 5 min and rapidly diminishing thereafter. Negligible hindpaw-lifting was observed in rats injected with equal volume of phosphate-buffered saline vehicle. $n=5$ animals in all groups. Ten nmol of α,β -methylene ATP was given, except for carrageenan-treated rats, which received 5 nmol. Values are means $\pm$ s.e.mean.

potencies of free ATP, α,β -methylene ATP were investigated in normal animals. *In vivo*, ATP is quickly degraded by extracellular ectonucleotidases. α,β -methylene ATP is a specific P2X agonist and has the advantage of being less susceptible to degradation than ATP. 2-methylthio ATP is a non-specific P2-receptor agonist. Like ATP it is vulnerable to degradation by extracellular ectonucleotidases. All analogues elicited a dose related lifting response ($P<0.05$ in all cases, ANOVA, $n=5-10$ per group). α,β -methylene ATP was found to be more potent than free ATP or 2-methylthio ATP. A dose of 100 nmol of α,β -methylene ATP elicited twice as much lifting as 2-methylthio ATP, whilst the same dose of ATP elicited a negligible amount of lifting (Figure 2). One hundred μ l of the vehicle (PBS), injected alone, also induced minimal hindpaw-lifting (13.3 ± 6.4 s, $n=10$).

Hyperalgesia in normal animals

Using the method of Hargreaves, we observed a reduction in the withdrawal latencies to noxious thermal stimuli of hindpaws treated with ATP, α,β -methylene ATP or 2-methylthio ATP, as shown in Figure 3. However, only the changes produced by α,β -methylene ATP reached statistical significance ($P<0.01$, ANOVA, $n=5-10$ per group). Injection of vehicle produced no significant change in thermal threshold, tested in the same way ($P>0.2$, paired *t*-test, $n=10$).

Neonatal capsaicin treatment

To investigate the nature of the sensory neurones being stimulated by ATP and its analogues, experiments were carried out on rats treated neonatally with 1% w v⁻¹ capsaicin. Figure 4 shows that virtually all nociceptive behaviour was abolished following injections of free ATP (100 nmol) and α,β -methylene ATP (100 nmol and 10 nmol). Only in one group of animals (those tested with 1000 nmol of 2-methylthio ATP) was appreciable hindpaw-lifting behaviour still present, amounting to about 25% of that seen in normal animals. For all compounds the lifting response was significantly reduced in capsaicin treated animals compared to naïve animals ($P<0.02$ in each case, Mann-Whitney Rank Sum Test, $n=3-5$ per group).

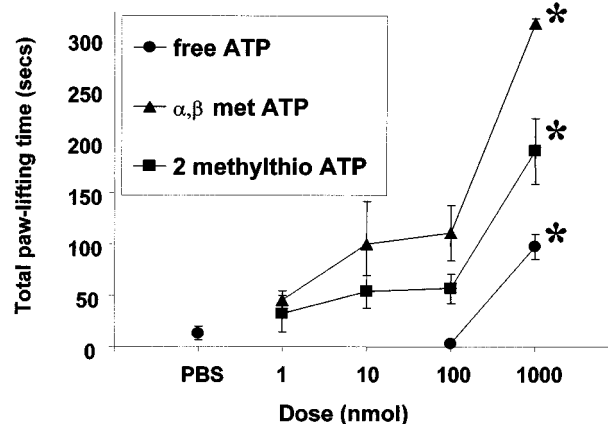


Figure 2 Total hindpaw-lifting time occurring in the 6 min following intraplantar injections of different doses of free ATP, α,β -methylene ATP and 2-methylthio-ATP, injected in a volume of 100 μ l. $n=5-10$ for each drug/dose. Values are means $\pm$ s.e.mean. All compounds induce significant hindpaw lifting ($P<0.05$, ANOVA). The asterisks indicate which individual groups at a particular dose differ significantly from PBS (Dunnett's *post hoc* test).

Effects of ATP analogues in inflammatory models

Carrageenan inflammation A 50 μ l dose 50:1 of 0.25% carrageenan produced a modest thermal hyperalgesia of the treated paw 2 h later, as reflected by a 22% reduction in the withdrawal latency at this time ($P < 0.05$, paired t -test, comparing pre to post treatment). To reduce the nocifensive actions of tissue distension, only 50 μ l of test solutions were injected into carrageenan-inflamed rats. Figure 5 shows that hindpaw-lifting behaviour induced by α,β -methylene ATP was greatly augmented in carrageenan-inflamed skin ($P < 0.05$, two-way ANOVA, $n = 5$ per group). At a dose of 50 nmol, α,β -methylene ATP induced 311.6 ± 35.2 s ($n = 5$) of lifting compared to 81.8 ± 22.1 s ($n = 5$) in naïve. Perhaps more interestingly, significant hindpaw-lifting activity was induced by only 0.05 nmol of α,β -methylene ATP, some 100 times less than that necessary in normal skin. The time course of ATP-induced hindpaw-lifting was slightly prolonged in carrageenan-inflamed skin, Figure 1.

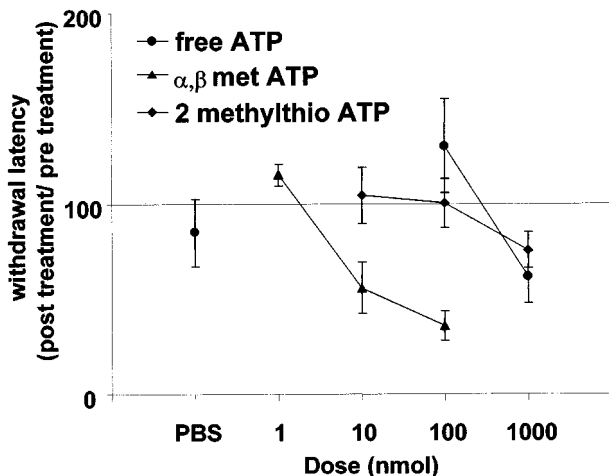


Figure 3 Thermal sensitivity of hindpaws determined 6 min after intraplantar injections of different doses of ATP, α,β -methylene ATP and 2-methylthio ATP, expressed as a percentage of the hindpaw withdrawal latency determined prior to intraplantar injection. Values less than 100 therefore indicate hyperalgesia. $n = 5-10$ for each drug/dose. Values are means $\pm$ s.e.mean. The effects of α,β -methylene ATP are statistically significant ($P < 0.05$, ANOVA).

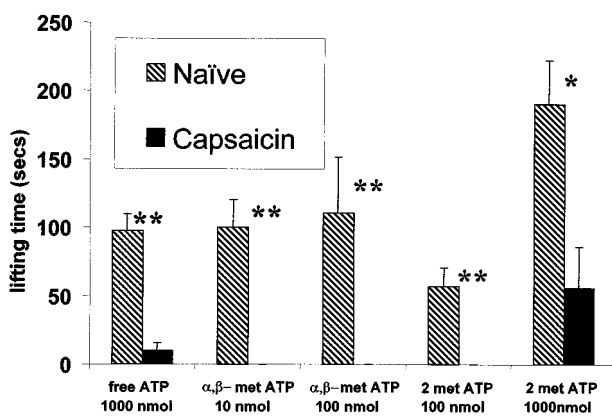


Figure 4 Total hindpaw-lifting time in the 6 min following intraplantar injections of ATP, α,β -methylene ATP and 2-methylthio ATP, in naïve rats and in rats treated neonatally with capsaicin. $n = 3-5$ per drug per dose. The reduction in hindpaw-lifting time in capsaicin-treated rats is highly significant in all cases (single asterisk indicates $P < 0.05$; double asterisk indicates $P < 0.01$, Mann-Whitney Rank Sum t -test).

Co-administration with PGE₂ Pilot experiments were carried out to determine the effects of intraplantar injection of PGE₂ alone on hindpaw-lifting behaviour. Figure 6 shows that a dose of 7 μ moles induced 73.8 ± 17.3 s ($n = 5$) of hindpaw-lifting while 0.7 μ moles induced only 31.0 ± 10.2 s ($n = 5$). α,β -methylene ATP (1 nmol and 10 nmol) when co-injected with PGE₂ induced far greater nociceptive responses than in normal rats. Figure 6 shows the data for a dose of 1 nmol. At both doses of PGE₂, the lifting times were significantly greater than the additive lifting times induced by the two substances separately ($P < 0.05$, unpaired t -test, $n = 5$ per group). With

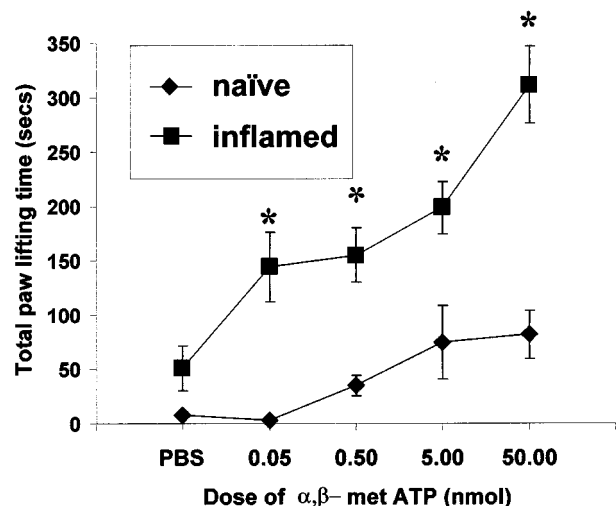


Figure 5 Nocifensive behaviour induced by intraplantar injections of α,β -methylene ATP or vehicle (50 μ l) into a hindpaw inflamed with carrageenan (50 μ l of 0.25%) 2 h previously. $n = 5$ per group. The total hindpaw-lifting time in the 10 min period following intraplantar injection is plotted. The increases seen in carrageenan-inflamed skin are significant ($P < 0.05$, ANOVA). Asterisks indicate significant differences ($P < 0.05$) between inflamed and normal animals (Dunnett's *post hoc* test).

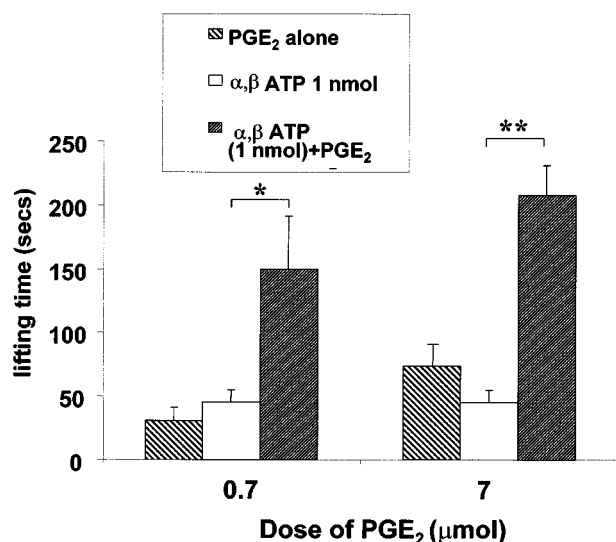


Figure 6 Effect of PGE₂ on lifting behaviour induced by 1 nmol of α,β -methylene ATP. PGE₂ was given at a dose of 0.7 or 7 μ mol either alone or mixed with 1 nmol of α,β -methylene ATP, and the ensuing hindpaw-lifting time accumulated over the following 10 min $n = 5$ for each group. PGE₂ potentiates ATP-induced hindpaw-lifting behaviour. Asterisks indicate a significant difference between groups (single asterisk $P < 0.05$, double asterisk $P < 0.01$, Mann-Whitney Rank Sum t -test).

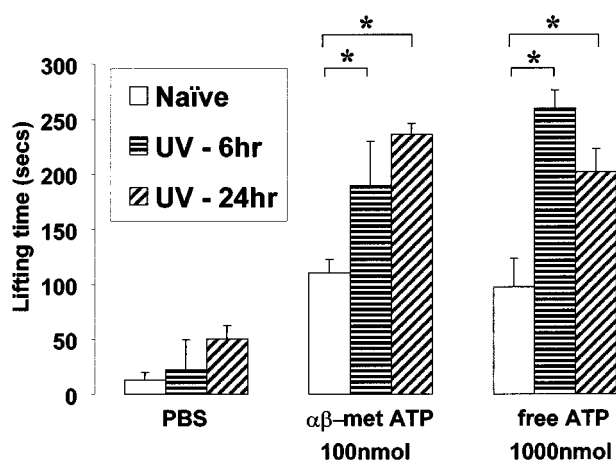


Figure 7 Effect of U.V. inflammation on hindpaw-lifting behaviour occurring in the 6 min after intraplantar injections of 1000 nmol of ATP, 100 nmol of α,β -methylene ATP or vehicle. The dose of U.V.B irradiation given was 1500 mJ cm^{-2} . U.V. inflammation potentiates the nocifensive activity of ATP analogues. The paw lifting behaviour was significantly augmented for both compounds ($P < 0.05$, ANOVA). The asterisks indicate significant difference between individual groups at a particular dose ($P < 0.05$, Dunnett's *post hoc* test).

0.7 μmoles of PGE_2 , α,β -methylene ATP (10 nmol) induced $134.6 \pm 29.5 \text{ s}$, ($n = 5$) of hindpaw-lifting compared to a calculated additive response of 84.9 s. With a higher dose of PGE_2 , 7 μmoles α,β -methylene ATP (10 nmol) induced almost double the projected lifting response. Thus, the effect of α,β -methylene ATP is potentiated in the presence of PGE_2 .

U.V. irradiation Initial experiments showed that the dose of U.V.B used produced a thermal hyperalgesia of the treated skin. Withdrawal latencies to noxious thermal stimuli decreased from $10.2 \pm 1.5 \text{ s}$ to $9.2 \pm 0.7 \text{ s}$ at 6 h and $8.5 \pm 0.9 \text{ s}$ at 24 h. The change at 24 h was statistically significant ($P < 0.05$ *t*-test, $n = 5$ per group). Animals were tested 6 and 24 h following irradiation with α,β -methylene ATP (100 nmol) and free ATP (1000 nmol). The total hindpaw-lifting behaviour was significantly augmented in the presence of U.V. inflammation for both compounds ($P < 0.05$, ANOVA, $n = 5$ per group), Figure 7. The hindpaw-lifting behaviour to vehicle was not significantly ($P > 0.06$, ANOVA $n = 8$ per group) increased in U.V. inflamed skin.

Discussion

Behavioural responses in naïve animals

The behavioural responses induced by the intraplantar injections of ATP and its related agonists α,β -methylene ATP and 2-methylthio ATP strongly suggest these compounds elicit a brief period of pain or local irritation. The lack of such responses in animals treated neonatally with capsaicin also indicates that ATP responses were pain-related, since this treatment selectively destroys small calibre sensory nerve fibres, more than 90% of which are known to be nociceptors (see Snider & McMahon, 1998). The very limited behavioural response remaining in some of the animals probably reflects the fact that destruction of nociceptors is usually incomplete—typically 10% or more fibres survive (Nagy *et al.*, 1983). Local (primary) thermal hyperalgesia was also apparent with some stimuli—those producing the greatest nocifensive behaviour—suggesting that ATP provokes thermal sensitization of

nociceptive terminals in skin. Similar sensitization is seen with other chemical algogens such as bradykinin (see Meyer *et al.*, 1994).

All three compounds induced hindpaw-lifting behaviour with the same time course, suggesting a common mechanism of action. The dose-dependence of the nociceptive response is consistent with a receptor-mediated event. Only the ipsilateral hindpaw responded to the injections; the lack of response in the contralateral hindpaw implies a peripherally mediated mechanism. In normal animals, our findings are very similar to those reported by Bland-Ward & Humphrey (1997), in terms of the qualitative nature of responses, including time-course, dose-dependence, and the relative potencies of α,β -methylene ATP versus free ATP. While Sawynok & Reid (1997) reported pro-nociceptive effects of ATP and α,β -methylene ATP (potentiation of formalin second phase response), they did not see any overt nocifensive behaviour attributable to either of these agonists, at doses we here find effective. It is not clear what methodological or other differences might account for the different findings. One salient feature, however, might be that ATP-induced pain in humans is quite modest in intensity (unpublished observations). This, coupled with the short duration of effect of ATP, might render the behavioural responses susceptible to stress-induced analgesia associated, for example, with handling and injection.

Behavioural responses in models of peripheral sensitization

We report here that nociceptive actions of ATP are markedly augmented in the presence of inflammation or inflammatory mediators. We saw both an increase in nocifensive behaviour to ATP analogues and a striking reduction in threshold doses. The most obvious functional implication of this result is that it is much more likely that extracellular levels of ATP will reach levels capable of activating nociceptors in inflamed tissues. Doses of only 0.05 nmol were effective in the pathophysiological states, approximately 100 times less than in normal tissue. It is interesting that the two human psychophysical studies in the literature also show different threshold doses at which ATP produces pain: when injected in normal skin, Coutts *et al.* (1981) found that doses of about 250 nmol of ATP were necessary to produce pain, but on blister bases pain was reported with doses of 0.2 to 0.6 nmol (calculated from data in Bleehen & Keele, 1977). It may be that the injury associated with the creation of a blister base is sufficient to sensitize nociceptors.

There are several ways in which the enhanced responses seen here might arise. One possibility is that changes in the pH of the extracellular environment might account for the augmented behaviour, since there is ample evidence that pH can modify responses of P2X receptors when studied in culture (Wildman *et al.*, 1997; Li *et al.*, 1997). Inflammation can also be associated with tissue acidosis (see Steen *et al.*, 1992). However, this cannot account for the changes seen with PGE_2 because these occurred in normal skin. Whilst carrageenan and U.V. treated skin might become acidic, the ATP analogues used in our studies were delivered in buffered vehicle. One hallmark of inflamed tissue is, of course, increased tenderness to tactile stimuli. However, the augmentation seen here cannot be due to this factor, because we controlled for the effects of volume *per se* using vehicle. Changes in CNS excitability (so-called central sensitization—McMahon *et al.*, 1993) are not features of PGE_2 or U.V. inflammation, and are therefore unlikely contributors to changes seen here.

Another possibility is that inflammatory stimuli might up-regulate receptor levels in nociceptors—for instance as is reported for bradykinin receptors (Petersen *et al.*, 1996). While this might be a feasible explanation for the altered responses seen after U.V. irradiation, the effects seen with carrageenan and PGE₂ are too rapid to reflect altered gene expression. It is more likely that some modification of the receptor, such as its state of phosphorylation, could modify its sensitivity, perhaps by altering the rate at which it desensitizes. Calcium-dependent dephosphorylation appears to be a common mechanism for desensitization of various ligand-gated ion channels (see Yakel, 1997).

The inflammatory stimuli used here are known to activate second messenger systems within primary afferent nociceptors (Pitchford & Levine, 1991) and produce a peripherally mediated hyperalgesia (Moncada *et al.*, 1975; Hargreaves *et al.*, 1988; Urban *et al.*, 1993).

Nature of receptors mediating nociceptive responses to ATP

Circumstantial evidence suggests that the nociceptive effects of ATP are likely to involve P2X₃ receptors, for the following reasons: (i) α,β -methylene ATP, the most potent ligand in the present study, is known to be relatively selective for P2X₁ and P2X₃ receptors (Rongen *et al.*, 1997; Fredholm *et al.*, 1997), and little P2X₁ mRNA is found in dorsal root ganglia (Lewis *et al.*, 1995; Kidd *et al.*, 1995; Collo *et al.*, 1996); P2X₃ is selectively expressed in small neurones, known to be

predominantly nociceptive in nature (Chen *et al.*, 1995; Lewis *et al.*, 1995); (ii) Artificial assembly of P2X₃ and P2X₂ subunits results in a channel with a unique phenotype which produces a current resembling that induced by ATP in the native channel expressed by nociceptors (Cook *et al.*, 1997). (iii) Adenosine and ADP, the main degradation products of ATP, do not elicit substantial hindpaw-lifting following intraplantar injections into the rat hindpaw (Bland-Ward & Humphrey, 1997), making it unlikely that P1 or P2Y receptor activation contributes to the responses seen.

The lack of specific tools (e.g. selective agonists or antagonists of different receptors subtypes, knock-out animals etc.) make a more precise assessment difficult.

Together our results provide further evidence that ATP may function as a peripheral mediator of pain. In particular, it may be important in inflammatory or other pathophysiological conditions, since at concentrations that are likely to be physiologically relevant, ATP generates a nociceptive response only in the presence of inflammatory mediators. This latter finding may also explain why some workers have failed to see effects of ATP in intact dorsal root ganglion cells (Stebbing *et al.*, 1998), where the conditions used may have been associated with sub-optimal receptor sensitivity.

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Mechanisms involved in the metabotropic glutamate receptor-enhancement of NMDA-mediated motoneurone responses in frog spinal cord

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1 The metabotropic glutamate receptor (mGluR) agonist *trans*-(±)-1-amino-1,3-cyclopentanedicarboxylic acid (*trans*-ACPD) (10–100 µM) depolarized isolated frog spinal cord motoneurons, a process sensitive to kynurenate (1.0 mM) and tetrodotoxin (TTX) (0.783 µM).

2 In the presence of NMDA open channel blockers [Mg²⁺; (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine hydrogen maleate (MK801); 3,5-dimethyl-1-adamantanamine hydrochloride (memantine)] and TTX, *trans*-ACPD significantly potentiated NMDA-induced motoneurone depolarizations, but not α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA)- or kainate-induced depolarizations.

3 NMDA potentiation was blocked by (RS)-α-methyl-4-carboxyphenylglycine (MCPG) (240 µM), but not by α-methyl-(2*S*,3*S*,4*S*)-α-(carboxycyclopropyl)-glycine (MCCG) (290 µM) or by α-methyl-(*S*)-2-amino-4-phosphonobutyrate (L-MAP4) (250 µM), and was mimicked by 3,5-dihydroxyphenylglycine (DHPG) (30 µM), but not by L-(+)-2-amino-4-phosphonobutyrate (L-AP4) (100 µM). Therefore, *trans*-ACPD's facilitatory effects appear to involve group I mGluRs.

4 Potentiation was prevented by the G-protein decoupling agent pertussis toxin (3–6 ng ml⁻¹, 36 h preincubation). The protein kinase C inhibitors staurosporine (2.0 µM) and *N*-(2-aminoethyl)-5-isoquinolinesulphonamide HCl (H9) (77 µM) did not significantly reduce enhanced NMDA responses. Protein kinase C activation with phorbol-12-myristate 13-acetate (5.0 µM) had no effect.

5 Intracellular Ca²⁺ depletion with thapsigargin (0.1 µM) (which inhibits Ca²⁺/ATPase), 1,2-bis(*O*-aminophenoxy)ethane-*N,N,N',N'*-tetracetic acid acetyl methyl ester (BAPTA-AM) (50 µM) (which buffers elevations of [Ca²⁺]_i), and bathing spinal cords in nominally Ca²⁺-free medium all reduced *trans*-ACPD's effects.

6 The calmodulin antagonists *N*-(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide (W7) (100 µM) and chlorpromazine (100 µM) diminished the potentiation.

7 In summary, group I mGluRs selectively facilitate NMDA-depolarization of frog motoneurons via a G-protein, a rise in [Ca²⁺]_i from the presumed generation of phosphoinositides, binding of Ca²⁺ to calmodulin, and lessening of the Mg²⁺-produced channel block of the NMDA receptor.

Keywords: *N*-methyl-D-aspartate receptors; frog; spinal cord motoneurons; *trans*-(±)-1-amino-1,3-cyclopentanedicarboxylic acid; metabotropic glutamate receptors; G-proteins; open channel block; Mg²⁺ ions; Ca²⁺ ions; calmodulin

Abbreviations: 1*S*,3*R*-ACPD, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; BAPTA-AM, 1,2-bis(*O*-aminophenoxy)ethane-*N,N,N',N'*-tetracetic acid acetyl methyl ester; 8-bromo-cyclic AMP, 8-bromo-3',5'-cyclic adenosine monophosphate; cyclic AMP, 3',5'-cyclic adenosine monophosphate; DAG, diacylglycerol; DHPG, (RS)-3,5-dihydroxyphenylglycine; DMSO, dimethyl sulphoxide; DR, dorsal root; DR-VRP, dorsal root-ventral root potential; G-protein, guanosine triphosphate-binding protein; H9, *N*-(2-aminoethyl)-5-isoquinolinesulphonamide HCl; IBMX, 3-isobutyl-1-methylxanthine; iGluR, ionotropic glutamate receptor; IP₃, inositol 1,4,5-triphosphate; KA, kainate; KYN, kynurenate; L-AP4, L-(+)-2-amino-4-phosphonobutyric acid; L-MAP4, α-methyl-(*S*)-2-amino-4-phosphonobutyrate; MCCG, α-methyl-(2*S*,3*S*,4*S*)-α-(carboxycyclopropyl)-glycine; MCPG, (RS)-α-methyl-4-carboxyphenylglycine; MEM, memantine, 3,5-dimethyl-1-adamantanamine hydrochloride; mGluR, metabotropic glutamate receptor; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine hydrogen maleate; NMDA, *N*-methyl-D-aspartate; PMA, phorbol-12-myristate 13-acetate; PTX, pertussis toxin; *trans*-ACPD, (±)-1-amino-*trans*-1,3-cyclopentane-dicarboxylic acid; TTX, tetrodotoxin; VR, ventral root; W7, *N*-(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide.

Introduction

Receptors for the excitatory amino acid neurotransmitter L-glutamate fall into two classes: ionotropic glutamate receptors

(iGluRs) and metabotropic glutamate receptors (mGluRs). iGluRs are cation-specific ion channel complexes that share fundamental features with other ligand-gated channels. The efficacy of different iGluR agonists has been used to classify iGluRs into three types: *N*-methyl-D-aspartate (NMDA); α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and kainate (Collingridge & Lester, 1989). L-glutamate, acting

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at iGluRs, functions as the major excitatory transmitter at synapses in the spinal cord of both mammals and amphibians (Davies *et al.*, 1982). mGluRs consist of a family of excitatory amino acid receptors that use G-proteins and/or various intracellular signal transduction pathways to modify neurotransmission and ion conductances (Pin & Duvoisin, 1995; Conn & Pin, 1997). In contrast to our understanding of iGluRs, how mGluRs function physiologically in the spinal cord is still in dispute. mGluRs may regulate excitatory synaptic transmission mediated by iGluRs in the spinal cord, but data addressing this issue are inconsistent. Some data show, for example, that mGluRs depress monosynaptic excitation of rat spinal motoneurons (an effect believed to be presynaptic and to involve a decrease in the release of L-glutamate from primary afferent terminals), and variably affect polysynaptic excitation produced by activation of C-fibres (Thompson *et al.*, 1992; Boxall *et al.*, 1996; King & Liu, 1997). Other studies report that activation of mGluRs either does not affect, or else increases, NMDA-, AMPA-, or kainate-induced postsynaptic excitations of spinal cord neurones (Cerne & Randic, 1992; Neugebauer *et al.*, 1994; Ugolini *et al.*, 1997; Budai & Larson, 1997).

The present investigations were designed to further our understanding of mGluRs, namely by characterizing the effects on motoneurons in the frog spinal cord when mGluRs are activated. For this purpose we used the isolated frog spinal cord with sucrose gap recording from ventral roots to monitor changes in motoneurone membrane potential. Our results indicate that activation of spinal mGluRs produces changes in evoked reflex activity, in the membrane potential of motoneurons, and in the responses of motoneurons to activation of NMDA receptors. Some of the results have appeared in preliminary form (Hackman *et al.*, 1992, 1994).

Methods

Experiments were performed on adult grass frogs (*Rana pipiens*, 30–55 g) anaesthetized to the point of unresponsiveness by cooling on crushed ice. After decapitation the brain was immediately destroyed by pithing and a laminectomy performed to remove the spinal cord. The lumbar spinal cord was hemisected sagittally and a hemicord with attached IXth dorsal (DR) and ventral roots (VR) was transferred to a sucrose gap chamber as described previously (Davidoff & Hackman, 1980). The preparation was superfused with Ringer's solution containing (in mM): NaCl 114, KCl 2.0, CaCl₂ 1.9, NaHCO₃ 10, and glucose 5.5. In many experiments, Mg²⁺ (1.0 mM) was added to the medium. The pH was adjusted to 7.4 by bubbling the medium with 95% O₂/5% CO₂. A Peltier thermoelectric cooling device maintained the temperature at 18°C.

Drugs and agonists were bath-applied by switching the superfusing solution to one containing known concentrations of drugs and agonists. Rapid (1–2 s) solution changes were achieved by the use of a solenoid valve assembly.

For DC recording of electrotonically-conducted changes in the membrane potential of motoneurons, the IXth VR was placed across a 3 mm sucrose gap. Calomel electrodes, connected *via* agar-Ringer's bridges, measured the difference in potential between the spinal cord bath and the distal end of the VR maintained in a pool of Ringer's solution. The preparation was left ungrounded. After amplification, the signals were recorded using a rectilinear pen writer.

In some experiments, the fibres contained in the IXth dorsal root were stimulated with supramaximal rectangular pulses

(15 V, 1.0 ms) delivered *via* bipolar silver/silver chloride electrodes (Davidoff *et al.*, 1988). The ventral root potential changes evoked by DR stimulation were stored in an IBM AT computer. The integral (the area under the change in ventral root potential produced by dorsal root stimulation in mV.s) was determined by reprogrammed Asyst software, and the potentials were plotted on a laser printer.

The peak amplitude of responses to NMDA and other iGluR agonists was measured in all experiments. All data are expressed as means $\pm$ s.e.mean. The statistical significance of differences has been assessed by the use of Student's *t*-test for correlated means.

Drugs

Most drugs were dissolved in Ringer's solution shortly before use to minimize chemical degradation. However, staurosporine, forskolin, and thapsigargin were dissolved in dimethyl sulphoxide (DMSO) (<1.0%); arachidonic acid was dissolved in ethanol (<2.0%) before diluting with Ringer's solution. Control solutions containing solvent alone were tested and produced no effects. pH was adjusted when necessary. The following compounds were used in these experiments: 3,5-dimethyl-1-adamantanamine hydrochloride (memantine), *trans*-($\pm$)-1-amino-1,3-cyclopentanedicarboxylic acid (*trans*-ACPD), (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R ACPD), α -amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), (RS)-3,5-dihydroxyphenylglycine (DHPG), α -methyl-(S)-2-amino-4-phosphonobutyrate (L-MAP4), α -methyl-(2S,3S,4S)- α -(carboxycyclopropyl)-glycine (MCCG), and (RS)- α -methyl-4-carboxyphenylglycine (MCPG), all purchased from Tocris Cookson; *N*-methyl-D-aspartate (NMDA) from Cambridge Research Biochemicals; arachidonic acid, chlorpromazine, forskolin, 3-isobutyl-1-methylxanthane (IBMX), thapsigargin, staurosporine, and *N*-(2-aminoethyl)-5-isoquinolinesulphonamide HCl (H9) from Research Biochemicals International; kainate, kynurenate, 8-bromo-cyclic AMP, dimethyl sulphoxide (DMSO), and phorbol-12-myristate 13-acetate (PMA) from Sigma; and L-2-amino-4-phosphonobutyrate (L-AP4), *N*-(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide (W7), 1,2-bis(*O*-aminophenoxy) ethane-*N,N,N',N'*-tetracetic acid acetyl methyl ester (BAPTA-AM), pertussis toxin (PTX), and tetrodotoxin (TTX) from Calbiochem. (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine hydrogen maleate (MK-801) was donated by Merck.

Results

As seen in Figure 1a, short (30 s) applications of the mGluR agonist *trans*-ACPD (a racemic mixture of (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid and (1R,3S)-1-aminocyclopentane-1,3-dicarboxylic acid) (10–100 μ M) in nominally Mg²⁺-free medium produced a concentration-dependent depolarization of frog motoneurons (30 μ M, 2.9 ± 0.8 mV, $n=3$). 1S,3R ACPD also depolarized motoneurons, but was more potent than *trans*-ACPD (30 μ M, 5.0 ± 1.3 mV, $n=3$) (Figure 1b) (*cf.* Eaton *et al.*, 1993; Boxall *et al.*, 1996; Ugolini *et al.*, 1997). The *trans*-ACPD-induced depolarizations of motoneurone membranes were reversibly reduced when the spinal cord was exposed to MCPG (240 μ M) to $61.0 \pm 8.3\%$ of control values ($P < 0.01$, $n=3$) (not shown).

trans-ACPD-evoked potential changes in motoneurons might be caused by activation of iGluRs on motoneurons, produced by an indirect action of *trans*-ACPD on presynaptic

interneurons, or generated by a direct action of *trans*-ACPD on mGluRs located on motoneurone membranes. To test these hypotheses, TTX and/or kynurenate were added to the superfusate (0 mM Mg^{2+}). Application of kynurenate (1.0 mM), a broad-spectrum antagonist of iGluR-mediated excitations, significantly reduced, but did not abolish, *trans*-ACPD-motoneurone depolarizations ($30.6 \pm 6.3\%$, $P < 0.05$, $n = 3$) (Figure 2a). Similar effects were produced by TTX in a concentration (0.783 μ M) sufficient to block interneuronal firing ($34.9 \pm 10.1\%$, $P < 0.05$, $n = 3$) (Figure 2b) (*cf.* Ugolini *et al.*, 1997). Moreover, when motoneurone depolarizations generated by *trans*-ACPD were reduced by TTX, subsequent addition of kynurenate had no further effect (TTX + kynurenate, 102.2% of *trans*-ACPD-responses in TTX alone, $n = 2$).

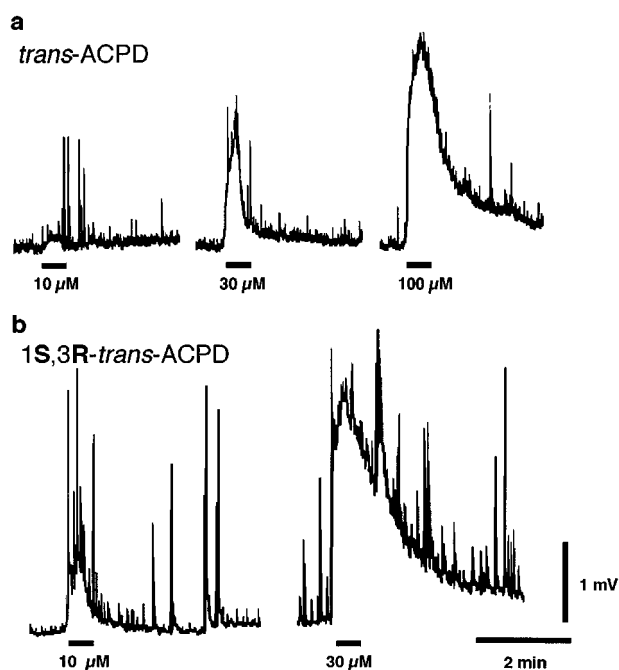


Figure 1 *trans*-ACPD and 1S,3R-ACPD depolarize frog motoneurons. (a) Changes in frog motoneurone membrane potential (electrotonically-conducted along the IXth VR) produced by three concentrations (10, 30 and 100 μ M, 30 s applications) of *trans*-ACPD. (b) Changes in frog motoneurone membrane potential produced by two concentrations (10 and 30 μ M) of 1S,3R-ACPD (30 s applications). In these, and in all subsequent records, negativity is indicated by an upward pen deflection and signifies a depolarization of motoneurons whose axons exit from the cord in the IXth VR. The upward 'spike-like' deflections of the baseline represent spontaneous ventral root potential (VRPs). Applications of *trans*-ACPD and 1S,3R-ACPD are indicated by bars below the baseline. Traces in a and b were obtained from two different spinal cords. Calibration bars apply to both a and b.

trans-ACPD and reflex responses

As seen in Figure 3, supramaximal stimulation of the dorsal root (DR) of the frog spinal cord bathed in Ringer's solution containing 1.0 mM Mg^{2+} generated early and late polysynaptic potentials in motoneurons that were electrotonically conducted along the VR (ventral root potentials, VRPs). Application of *trans*-ACPD (30 μ M) significantly potentiated polysynaptic reflex responses, particularly the longer-latency components ($239.6 \pm 38.9\%$ of control VRP area, $P < 0.05$, $n = 5$). The effects of *trans*-ACPD were partially reversible upon returning the cord to *trans*-ACPD-free Ringer's solution.

Activation of mGluRs and NMDA-induced motoneurone depolarizations: the role of NMDA channel blockers

We next sought to discern whether or not changes in the postsynaptic responsiveness of spinal neurones to excitatory amino acid agonists might account for some of *trans*-ACPD's facilitatory actions on reflex transmission. How *trans*-ACPD might affect the sensitivity of postsynaptic motoneuronal membranes to endogenously released excitatory amino acid transmitters that activate iGluRs was evaluated by determin-

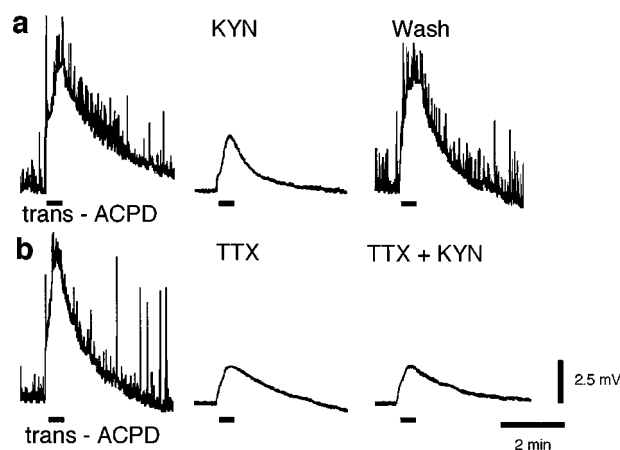


Figure 2 *trans*-ACPD-induced motoneurone depolarizations are blocked by kynurenate (KYN) and tetrodotoxin (TTX). (a) Application of *trans*-ACPD (30 μ M, 30 s) before, during, and after addition of kynurenate (1.0 mM, 30 min) to Ringer's solution. (b) Depolarization produced by *trans*-ACPD (30 μ M, 30 s) is diminished by TTX (0.783 μ M). Subsequent addition of kynurenate (1.0 μ M) had no further effect. Applications of *trans*-ACPD are indicated by bars below the baseline. Traces in a and b were obtained from two different spinal cords. Calibration bars apply to both a and b.

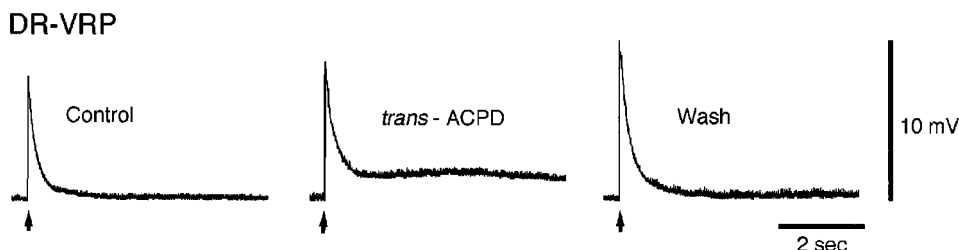


Figure 3 *trans*-ACPD potentiates polysynaptic reflexes in the frog spinal cord. Traces are ventral root potentials (VRPs) produced by single supramaximal shocks (arrows) applied to the afferent fibres in the sciatic nerve. Control VRP, VRP recorded during exposure to *trans*-ACPD (30 μ M), and VRP obtained after return to normal Ringer's solution are shown. The experiment was carried out in Ringer's solution containing 1.0 mM Mg^{2+} .

ing the effects of *trans*-ACPD on motoneurone depolarizations evoked by short (5–10 s) applications of the selective iGluR agonists NMDA (100 μ M), AMPA (10 μ M), and kainate (30 μ M). This group of experiments (and all following experiments) were conducted in medium containing 0.783 μ M TTX to reduce indirect actions of ACPD.

As seen in Figure 4a, *trans*-ACPD (30 μ M) had no effect on the depolarizations produced by iGluR agonists in nominally Mg^{2+} -free Ringer's solution (NMDA: $102.3 \pm 3.0\%$ of control, $n=6$; AMPA: $98.6 \pm 3.5\%$, $n=3$; kainate: $99.2 \pm 3.3\%$, $n=3$) (cf. Harvey & Collingridge, 1993). In contrast, in the presence of a 'physiological' concentration of Mg^{2+} (1.0 mM in amphibians) (Davidoff *et al.*, 1988), *trans*-ACPD (30 μ M) significantly potentiated NMDA-induced motoneurone depolarizations (100 μ M NMDA: $183.5 \pm 22.8\%$ of control, $P < 0.05$, $n=6$; 300 μ M NMDA: $174.3 \pm 25.5\%$, $P < 0.05$, $n=6$) (Figure 4b). Maximal potentiation of NMDA responses was seen within 1 min after beginning perfusion with Mg^{2+} -Ringer's solution containing *trans*-ACPD; the potentiation lasted for the duration of the *trans*-ACPD application. The effect of *trans*-ACPD was slowly reversible after return to normal medium. The presence of Mg^{2+} in the Ringer's solution did not change the inability of *trans*-ACPD (30 μ M) to facilitate motoneurone depolarizations evoked by application of AMPA (10 μ M, 100%, $n=2$) or kainate (30 μ M, 98.5%, $n=2$).

The effects of various concentrations of extracellular Mg^{2+} on the facilitation of NMDA-responses by *trans*-ACPD was examined. In the frog spinal cord *trans*-ACPD did not facilitate NMDA-responses until a threshold Mg^{2+} concentration of 100 μ M was reached. In some cords, a threshold concentration of Mg^{2+} added to the Ringer's solution (100–250 μ M) reduced NMDA responses ($79.5 \pm 7.8\%$ of control values in 0 mM Mg^{2+} , $n=3$), and in these cords *trans*-ACPD (30 μ M) potentiated NMDA (100 μ M) responses ($138.3 \pm 23.4\%$ of control values in Mg^{2+} , $n=3$) (Figure 5a). In contrast, in other cords these same concentrations of Mg^{2+}

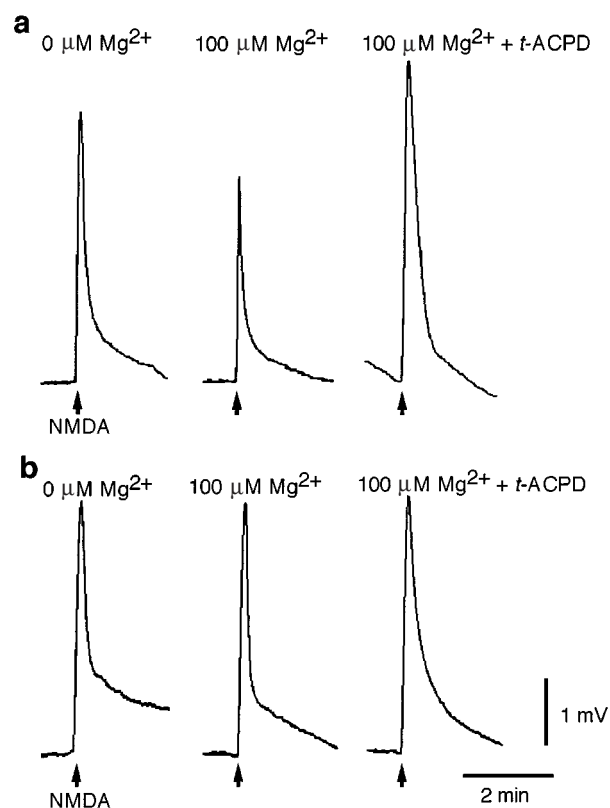


Figure 5 *trans*-ACPD potentiates NMDA responses if the concentration of extracellular Mg^{2+} is sufficient to produce channel block. (a) *trans*-ACPD (*t*-ACPD) (30 μ M) potentiates NMDA responses in a cord exposed to 100 μ M Mg^{2+} . Note the decreased amplitude of the NMDA response when the Ringer's contained 100 μ M Mg^{2+} . (b) *trans*-ACPD (30 μ M) had no effect on NMDA-induced motoneurone depolarization in a second spinal cord exposed to 100 μ M Mg^{2+} ; nor did 100 μ M Mg^{2+} affect NMDA-responses. Both experiments were performed in Ringer's solution containing TTX (0.783 μ M). Calibration bars apply to both a and b.

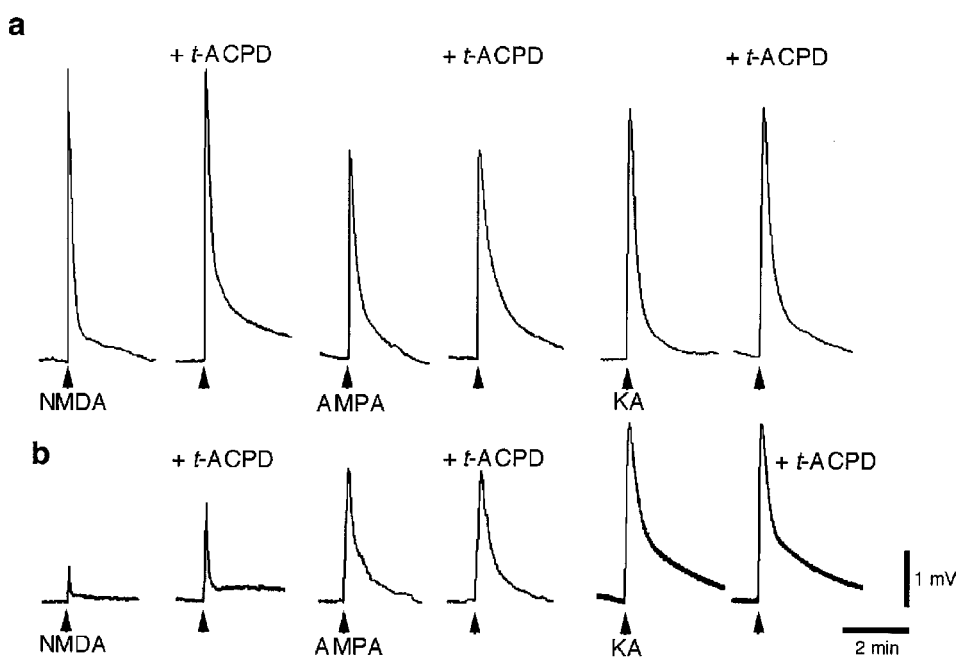


Figure 4 *trans*-ACPD facilitates NMDA-evoked motoneurone depolarizations in Ringer's solution containing Mg^{2+} . (a) Applications of NMDA (100 μ M, 10 s), AMPA (10 μ M, 10 s), and kainate (KA) (30 μ M, 10 s) in nominally Mg^{2+} -free Ringer's solution before and after exposure to *trans*-ACPD (30 μ M). (b) Same experiment carried out in another spinal cord in Ringer's solution containing 1.0 mM Mg^{2+} . Both experiments were performed in Ringer's solution containing TTX (0.783 μ M). Applications of iGluR agonists are indicated by arrows below the baseline. Calibration bars apply to both a and b.

did not diminish NMDA responses ($98.5 \pm 1.4\%$ of control values in 0 mM Mg^{2+} , $n=4$); nor was facilitation seen ($97.9 \pm 2.7\%$ of control values in Mg^{2+} , $n=4$) (Figure 5b).

Mg^{2+} binds to the NMDA receptor channel opened by activation of the receptor (MacDonald & Nowak, 1990). In the presence of other NMDA open channel blockers – MK-801 ($3\text{--}6 \mu\text{M}$) and memantine ($10\text{--}20 \mu\text{M}$) – *trans*-ACPD also significantly potentiated NMDA responses, but the potentiation was not as marked as with Mg^{2+} (Figure 6 and Table 1). It should be noted that depolarization (1.5 mV) of motoneurons by elevation of extracellular K^+ (2.5 mM) did not mimic the facilitatory effects of *trans*-ACPD on NMDA responses ($88.2\% \pm 4.0$; $n=4$). Therefore, the *trans*-ACPD-induced depolarization of motoneurons bathed in Ringer's solution containing 1.0 mM Mg^{2+} is presumably not responsible for the facilitation of NMDA effects.

mGluR subtypes and potentiation of NMDA-induced responses

To determine which mGluR subtypes mediated the potentiation of NMDA responses, the effects of several selective agonists and antagonists of mGluRs were examined in spinal cords superfused with medium containing 1.0 mM Mg^{2+} . In a manner similar to that of *trans*-ACPD, the selective group I

mGluR agonist DHPG potentiated NMDA responses, while the group III agonist L-AP4 was without effect (Table 2). The potentiation of NMDA-motoneurone responses by *trans*-ACPD ($30 \mu\text{M}$) was significantly blocked by the non-selective mGluR antagonist MCPG (Table 3). In contrast, the group II antagonist MCCG and the group III antagonist L-MAP4 did not significantly alter the actions of *trans*-ACPD on NMDA responses (Table 3).

G-proteins and *trans*-ACPD-induced effects on NMDA responses

The premise that a G-protein is involved in *trans*-ACPD's actions on NMDA responses was investigated by incubating hemisected frog spinal cords in standard Ringer's solution either with or without pertussis toxin (PTX) ($3\text{--}6 \text{ ng ml}^{-1}$) for 36 h at 4°C . In spinal cords incubated with PTX, the ability of *trans*-ACPD ($30 \mu\text{M}$) to facilitate NMDA responses in the presence of 1.0 mM Mg^{2+} was significantly reduced

Table 1 Effects of NMDA channel blockers on *trans*-ACPD-induced potentiation of NMDA responses

Channel blocker	NMDA responses (% of control)
Mg^{2+} (1.0 mM)	183.5 ± 22.8 ($n=6$)*
MK-801 ($3\text{--}6 \mu\text{M}$)	124.5 ± 4.8 ($n=4$)*
Memantine ($10\text{--}20 \mu\text{M}$)	129.0 ± 9.6 ($n=4$)*

The effects of *trans*-ACPD on NMDA responses are expressed as a mean percentage $\pm$ s.e. mean of control NMDA responses ($100 \mu\text{M}$, 5 s applications) in the same cords. Channel blockers and TTX ($0.783 \mu\text{M}$) were present in the Ringer's solution during the entire experiment. Mg^{2+} was not added to the Ringer's solution in experiments with MK-801 and memantine. * $P < 0.05$ (Student's paired *t*-test).

Table 2 Effects of mGluR agonists on NMDA responses

mGluR agonists	NMDA responses (% of control)
<i>trans</i> -ACPD ($30 \mu\text{M}$)	183.5 ± 22.8 ($n=6$)*
DHPG ($30 \mu\text{M}$)	141.5 ± 14.6 ($n=3$)*
L-AP4 ($100 \mu\text{M}$)	98.0 ± 2.0 ($n=3$)

NMDA-induced motoneurone depolarizations after exposure to mGluR agonists are expressed as a mean percentage $\pm$ s.e. mean of the control NMDA ($100 \mu\text{M}$, 5 s) responses in the same cords. Mg^{2+} (1.0 mM), and TTX ($0.783 \mu\text{M}$) were present in the Ringer's solution during the entire experiment. * $P < 0.05$ (Student's paired *t*-test).

Table 3 Effects of mGluR antagonists on *trans*-ACPD-induced potentiation of NMDA responses

mGluR antagonists	<i>trans</i> -ACPD-facilitation of NMDA responses (% of control)
MCPG ($240 \mu\text{M}$)	83.3 ± 2.9 ($n=3$)
MCCG ($290 \mu\text{M}$)	157.3 ± 6.9 ($n=4$)*
L-MAP4 ($250 \mu\text{M}$)	134.5 ± 12.4 ($n=3$)*

The effects of *trans*-ACPD on NMDA responses after exposure to mGluR antagonists are expressed as a mean percentage $\pm$ s.e. mean of NMDA ($100 \mu\text{M}$, 5 s) responses in the presence of antagonist alone in the same cords. Antagonists, Mg^{2+} (1.0 mM), and TTX ($0.783 \mu\text{M}$) were present in the Ringer's solution during the entire experiment. * $P < 0.05$ (Student's paired *t*-test).

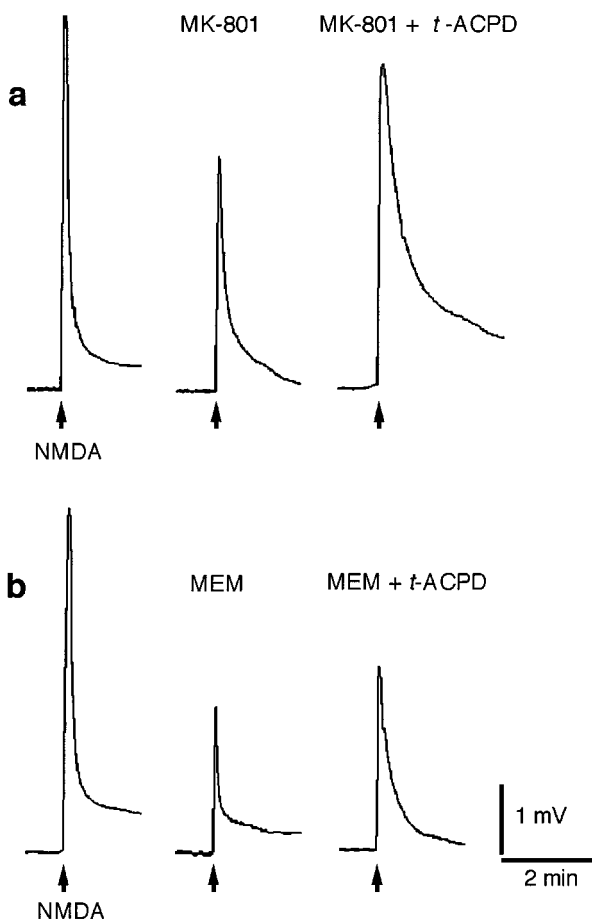


Figure 6 *trans*-ACPD-induced potentiation of NMDA responses is present in spinal cords exposed to NMDA receptor channel blockers. (a) Spinal cord exposed to MK-801 ($6 \mu\text{M}$). (b) Spinal cord exposed to memantine (MEM) ($10 \mu\text{M}$). Both experiments were performed in Ringer's solution containing TTX ($0.783 \mu\text{M}$). Applications of NMDA ($100 \mu\text{M}$, 5 s) are indicated by arrows below the baseline. Calibration bars apply to both a and b.

(94.5 ± 7.8%, $n = 3$) when compared to spinal cords incubated in Ringer's solution without PTX (141.1 ± 9.3%, $P < 0.01$, $n = 4$).

Second messengers, internal Ca^{2+} ($[\text{Ca}^{2+}]_i$), and the facilitatory actions of trans-ACPD

Activation of group I mGluRs is reported to lead to the activation of protein kinase C and the formation of phosphoinositides. To evaluate the possible involvement of protein kinase C in the potentiation of NMDA by trans-ACPD, we used two potent inhibitors of protein kinase C – staurosporine (2.0 μM) and H9 (77 μM) (Hidaka & Kobayashi, 1992) – and a potent activator of protein kinase C, PMA (phorbol-12-myristate 13-acetate, 5.0 μM). Staurosporine and H9 did not significantly affect the facilitatory effect of trans-ACPD (30 μM) on NMDA responses, and PMA (applied for 30 min) neither mimicked, nor reduced, the effect of trans-ACPD (Table 4).

The production of phosphoinositides results in the release of Ca^{2+} from the endoplasmic reticulum and the influx of Ca^{2+} from extracellular medium. To investigate whether the release of Ca^{2+} from intracellular loci and a subsequent increase in $[\text{Ca}^{2+}]_i$ is required for the facilitation of NMDA responses by trans-ACPD, thapsigargin (0.1 μM) (which depletes most intracellular Ca^{2+}) and BAPTA-AM (50 μM) (which buffers elevations of $[\text{Ca}^{2+}]_i$) were applied to frog spinal cords in different experiments. While neither compound had a direct effect on NMDA responses, both of them prevented the potentiation of NMDA responses by trans-ACPD (Table 4). They had no significant effect on NMDA responses. Further evidence that a Ca^{2+} -dependent process was involved in the facilitation of NMDA responses was obtained by exposing

spinal cords to nominally Ca^{2+} -free medium (containing 1.0 mM Mg^{2+}). This treatment did not affect NMDA responses (92.6 ± 4.6% of control responses in 1.9 mM Ca^{2+} , $n = 3$) but blocked potentiation of NMDA responses by trans-ACPD (30 μM) (Table 4).

Ca^{2+} ions possess numerous intracellular targets including the Ca^{2+} -binding protein calmodulin. The facilitation of NMDA responses by trans-ACPD (30 μM) was significantly reduced by the calmodulin antagonists W7 (100 μM) and chlorpromazine (100 μM) (Table 4).

mGluRs use intracellular signal transduction pathways other than the one involving phosphoinositides to modify neurotransmission and ion conductances (Conn & Pin, 1997). To determine whether or not the facilitation of NMDA-depolarizations of motoneurons caused by mGluR activation was linked to cyclic AMP-coupled mechanisms, we evaluated the effects of agents known to increase the effective intracellular concentration of cyclic AMP. As Table 5 indicates, high concentrations of 8-bromo-cyclic AMP, forskolin and IBMX did not potentiate NMDA-induced motoneurone depolarizations. Arachidonic acid was also without effect on NMDA responses (Table 5).

Discussion

Polysynaptic reflexes in the frog spinal cord are presumed to be mediated by the synaptic release of excitatory amino acids and the subsequent activation of iGluRs, including NMDA receptors, on interneurons and motoneurons (Davies *et al.*, 1982). The present study provides evidence that activation of specific mGluRs augments reflex effects on frog spinal motoneurons. In addition, the consequences of mGluR activation on reflexes appear to be mediated, at least in part, by a postsynaptic action at NMDA receptors.

Our results show that trans-ACPD – which activates mGluRs – facilitated the polysynaptic components of segmental spinal reflexes evoked by supramaximal stimulation of afferent fibres in the frog spinal cord when the cords are bathed in Ringer's solution containing Mg^{2+} ions. The same concentrations of trans-ACPD that significantly increase reflex activity selectively potentiate the motoneurone responses produced by applications of NMDA. And in contrast to previous reports in both *in vivo* and *in vitro* mammalian spinal cord preparations, our results show that activation of mGluRs in the frog spinal cord had no effect on motoneurone depolarizations mediated by AMPA and kainate (*cf.* Cerne & Randic, 1992; Neugebauer *et al.*, 1994; Ugolini *et al.*, 1997). The action of trans-ACPD on NMDA responses, therefore, appears to be selective. A second site of action for trans-ACPD at a presynaptic locus on afferent fibre terminals and/or interneurons to block the release of excitatory amino acids is not, however, precluded by our observations.

The effects of trans-ACPD on NMDA receptors appear to depend upon activation of specific mGluRs. There are at least eight major mGluRs (mGluR1-8) divisible into three groups (I, II and III) on the basis of sequence homology, pharmacological characteristics, and coupling to intracellular transduction mechanisms (Conn & Pin, 1997). In the present experiments, activation of group I mGluRs appear to cause trans-ACPD's effects on NMDA responses on frog motoneurons. This idea is supported by the observations that trans-ACPD's actions on NMDA-depolarizations were significantly blocked by the non-selective antagonist MCPG (Kemp *et al.*, 1994), but were not affected either by the selective group II antagonist MCCG (Jane *et al.*, 1994) or by the selective group III antagonist

Table 4 Effects of various treatments on trans-ACPD-induced potentiation of NMDA responses

Treatment	NMDA responses (% of control)
Staurosporine (2.0 μM)	127.9 ± 3.9 ($n = 3$)*
H-9 (77 μM)	180.0 ± 36.1 ($n = 5$)*
Thapsigargin (0.1 μM)	94.4 ± 3.1 ($n = 3$)
BAPTA-AM (65 μM)	99.4 ± 0.3 ($n = 3$)
Ca^{2+} (0 mM)	85.0 ± 2.6 ($n = 3$)
W7 (100 μM)	95.8 ± 4.2 ($n = 3$)
Chlorpromazine (100 μM)	88.4 ± 11.6 ($n = 3$)

The effects of treatments on the ability of trans-ACPD to potentiate NMDA responses are expressed as a mean percentage ± s.e. mean of control NMDA (100 μM , 5 s) responses obtained in Ringer's solution containing the appropriate agent. Agents, Mg^{2+} (1.0 mM), and TTX (0.783 μM) were present in the Ringer's solution during the entire experiment. * $P < 0.05$ (Student's paired *t*-test).

Table 5 Effects of various treatments on NMDA responses

Treatment	NMDA responses (% of control)
PMA (5.0 μM)	111.0 ± 11.7 ($n = 4$)
8-Bromo-cyclic AMP (100 μM)	85.5 ± 3.7 ($n = 7$)
Forskolin (50 μM)	73.9 ± 2.9 ($n = 4$)
IBMX (1.0 mM)	78.8 ± 11.4 ($n = 5$)
Arachidonic acid (3.0 μM)	99.7 ± 2.3 ($n = 3$)

The effects of treatments are expressed as a mean percentage ± s.e. mean of control NMDA (100 μM , 5 s) responses obtained in Ringer's solution containing Mg^{2+} (1.0 mM) and TTX (0.783 μM).

MAP4 (Jane *et al.*, 1994). Moreover, the selective group I mGluR agonist DHPG (Ito *et al.*, 1992) facilitated NMDA-evoked motoneurone depolarizations, while L-AP4, an agonist selective for group III mGluRs had no effect on NMDA-motoneurone responses (Jane *et al.*, 1994).

G-Proteins and potentiation of NMDA responses by trans-ACPD

Previous studies have shown the effects of activating group I mGluRs to be both insensitive and sensitive to the actions of PTX (Pin & Duvoisin, 1995). PTX prevents the interaction of receptors with several G-proteins (including G_o and G_i) by ADP-ribosylating their α subunits (Gilman, 1987). PTX blockade of a receptor-mediated event suggests that a G-protein (either G_o or G_i) is involved in mediation of the receptor's actions. In the present investigation, activation of mGluRs by *trans*-ACPD causing facilitation of NMDA responses was found to be sensitive to PTX. The data, therefore, support the role of a G-protein as a mediator of the actions of *trans*-ACPD in the facilitation of NMDA-responses in frog motoneurons.

Second messengers, $[Ca^{2+}]_i$ and the potentiating actions of trans-ACPD

In most systems, group I mGluRs stimulate phospholipase C to cleave membrane phospholipids yielding inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Conn & Pin, 1997). IP_3 causes a rise in $[Ca^{2+}]_i$ by mobilizing Ca^{2+} from non-mitochondrial internal stores and by increasing Ca^{2+} influx from extracellular medium (Mayer & Miller, 1990; Meldolesi *et al.*, 1991). In the present experiments, three treatments which would be expected either to reduce $[Ca^{2+}]_i$ or to prevent elevations of $[Ca^{2+}]_i$ were tested for their effects on *trans*-ACPD facilitation of NMDA responses. Thapsigargin depletes most intracellular Ca^{2+} by inhibiting Ca^{2+} /ATPase in intracellular compartments and preventing refilling of intracellular stores (Thastrup *et al.*, 1990). BAPTA-AM (50 μ M) is a membrane-permeable Ca^{2+} chelator that acts to buffer elevations of $[Ca^{2+}]_i$ (Niesen *et al.*, 1991). Reduction of extracellular Ca^{2+} ($[Ca^{2+}]_o$) presumably depletes $[Ca^{2+}]_i$ and prevents Ca^{2+} influx. Because thapsigargin, BAPTA-AM, and perfusion with nominally Ca^{2+} -free Ringer's solution prevented *trans*-ACPD from facilitating NMDA responses, an elevation of $[Ca^{2+}]_i$ appears necessary for the facilitatory actions of mGluRs in frog spinal cord. Alternatively, because neurotransmitter-activated phosphoinositide hydrolysis is reduced by low $[Ca^{2+}]_i$ procedures which reduce $[Ca^{2+}]_i$, also affect the actions of *trans*-ACPD by preventing the formation of IP_3 (Kendall & Nahorski, 1984).

How changes in $[Ca^{2+}]_i$ determine NMDA responses is unclear. Several studies indicate that NMDA receptors are transiently inactivated by increased $[Ca^{2+}]_i$ (Legendre *et al.*, 1993; Rosenmund *et al.*, 1995). In contrast, elevation of $[Ca^{2+}]_i$ resulting from the formation of inositol phosphate derivatives has been postulated to underlie the potentiation of NMDA responses produced by several neurotransmitters (Markram & Segal, 1992; Kinney & Slater, 1993; Rahman & Neuman, 1996a). Analysis is complicated by findings that intracellular Ca^{2+} does not consist of a single homogeneous pool. In neurones there are at least two pharmacologically distinguishable pools of readily releasable Ca^{2+} : the IP_3 -releasable stores and the Ca^{2+} -induced Ca^{2+} stores (Henzi & McDermott, 1992). It may well be that different pools of intracellular Ca^{2+} affect NMDA receptors in dissimilar ways. In this regard, we

have previously shown that caffeine – which induces release of Ca^{2+} from the Ca^{2+} -induced release stores – does in fact reduce NMDA responses in frog motoneurons (Hackman *et al.*, 1994).

The effects of changing Ca^{2+} concentrations of Ca^{2+} ions on frog motoneurone-NMDA responses may involve the ubiquitous, multifunctional Ca^{2+} -binding protein calmodulin. Both W-7 and chlorpromazine, two potent calmodulin inhibitors reduced *trans*-ACPD-potentiation of frog motoneurone NMDA responses (Tanaka *et al.*, 1982; Weiss *et al.*, 1982). Calmodulin binds directly to the NR1 subunit of the NMDA receptor complex in a Ca^{2+} -dependent manner (Ehlers *et al.*, 1996). Reports vary, however, on how calmodulin affects the NMDA receptor. There are findings that calmodulin binding to the NMDA receptor reduces the probability that the NMDA channel will open (Ehlers *et al.*, 1996). CaM-Kinase II (type II calcium/calmodulin-dependent protein kinase), however, phosphorylates both recombinant and native glutamate receptors in several *in vitro* systems thereby potentiating responses to NMDA in spinal cord neurones (McGlade-McCulloh *et al.*, 1993; Kolaj *et al.*, 1994).

In addition to generating IP_3 when group I mGluRs stimulate phospholipase C, the process yields DAG, a substance known to activate protein kinase C. Protein kinase C may play a role in mGluR-mediated facilitation of NMDA responses, although reports differ (*cf.* Kelso *et al.*, 1992; Harvey & Collingridge, 1993; Kinney & Slater, 1993; Rahman & Neuman, 1996a). Our studies found that the protein kinase C antagonists staurosporine and H9 did not block the potentiation of NMDA responses by *trans*-ACPD and PMA did not mimic the effects of *trans*-ACPD. These results argue against a mandatory role for protein kinase C in mediating the facilitatory actions of *trans*-ACPD on NMDA responses in frog motoneurons. The possibility exists, however, that some of *trans*-ACPD's actions on frog motoneurons result from activation of staurosporine- and H9-resistant isoforms of protein kinase C.

mGluRs have the potential to initiate several other biochemical responses in target neurones; among these are changes in cyclic AMP and arachidonic acid levels (Conn *et al.*, 1997). Moreover, cyclic AMP and arachidonic acid are reported to augment NMDA responses (Miller *et al.*, 1992; Cerne *et al.*, 1993). Our results, however, do not find changes in the concentration of these compounds causative in *trans*-ACPD-facilitated NMDA responses. Rather, we found that elevation of intracellular levels of cyclic AMP by 8-bromo-cyclic AMP (a membrane-permeant cyclic AMP analogue which elevates levels of cyclic AMP), forskolin (a direct activator of the catalytic subunit of adenylyl cyclase), or IBMX (an inhibitor of cyclic nucleotide phosphodiesterase activity) did not mimic the facilitatory effects of *trans*-ACPD on NMDA responses. Similarly, arachidonic acid was without effect on NMDA responses. These results agree with reports concerning hippocampal and neocortical neurones (Harvey & Collingridge, 1993; Rahman & Neuman, 1996a).

NMDA channel block and facilitation of NMDA responses by trans-ACPD

Although activation of mGluRs has been reported to potentiate NMDA responses in neurones bathed in nominally Mg^{2+} -free medium (Cerne & Randic, 1992; Harvey & Collingridge, 1993), the *trans*-ACPD potentiation of NMDA-induced responses in frog motoneurone required a concentration of at least 100 μ M Mg^{2+} ions in the Ringer's solution. Mg^{2+} ions are not ordinarily added to frog Ringer's

solution, but frog cerebrospinal fluid contains the cation in a concentration of 0.92 mM (Davidoff *et al.*, 1988). We assume that the interstitial fluid surrounding frog neurones *in vivo* contains Mg^{2+} in approximately that concentration as well.

One might posit that the ineffectiveness of *trans*-ACPD in Mg^{2+} -free Ringer's solution reflects the G-protein-coupled receptor's need for cytosolic Mg^{2+} ions in order to function effectively (El-Beheiry & Puil, 1990; Rahman & Neuman, 1996b). But, in the present experiments the NMDA channel blockers memantine and MK-801 were able to substitute in large measure for Mg^{2+} ions. Mg^{2+} , MK-801, and memantine all limit functioning of the NMDA receptors by binding to sites within the open ion channel operated by activation of the NMDA receptor (Huettnner & Bean, 1988; MacDonald & Nowak, 1990; Blanpied *et al.*, 1997). Our data are compatible with the hypothesis that *trans*-ACPD potentiates NMDA responses in frog motoneurons by reducing channel block of the NMDA receptor.

Activation of mGluRs and motoneurone depolarizations

It has been previously demonstrated that *trans*-ACPD depolarizes motoneurons in the rat spinal cord (Jane *et al.*, 1994; King & Liu, 1997). This is also the case in the frog where we found the depolarization was significantly reduced, but not eliminated, by either TTX (in a concentration sufficient to eliminate regenerative activity and firing of spinal interneurons and primary afferent fibres) or by the non-specific iGluR antagonist kynureate (in a concentration sufficient to block responses mediated by iGluRs). Moreover, the ability of TTX and kynureate to reduce *trans*-ACPD-induced depolariza-

tions was not additive. These findings suggest that a proportion of the *trans*-ACPD-depolarization occurs indirectly, depends upon the discharge of interneurons and/or primary afferent fibres, and may be caused by the release of L-glutamate and the subsequent activation of iGluRs. In part, the *trans*-ACPD-induced depolarization appears to result from direct effects of the agonist on motoneurone membranes. In other systems, membrane depolarization caused by activation of mGluRs appears to be the consequence either of activation of a non-specific cationic conductance or of inhibition of various different K^+ conductances (Charpak *et al.*, 1990; Crépel *et al.*, 1994; Guérineau *et al.*, 1994). In the frog spinal cord, however, we cannot yet say precisely how *trans*-ACPD produces the direct component of motoneurone depolarization.

Taken together, the results reported here suggest that the facilitation of NMDA-induced depolarizations of frog motoneurons by *trans*-ACPD is caused by a mechanism that encompasses: (1) activation of group I mGluRs; (2) activation of a G-protein; (3) a rise in $[Ca^{2+}]_i$ presumably resulting from production of phosphoinositides; (4) binding of Ca^{2+} to calmodulin and (5) reduction of the open channel block of the NMDA receptor produced by physiological concentrations of Mg^{2+} ions.

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Effects of pyrogallol, hydroquinone and duroquinone on responses to nitrgic nerve stimulation and NO in the rat anococcygeus muscle

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1 The hypothesis that endogenous superoxide dismutase (SOD) protects the nitrgic transmitter from inactivation by superoxide and that this explains the lack of sensitivity of the transmitter to superoxide generators was tested in the rat isolated anococcygeus muscle.

2 Responses to nitrgic nerve stimulation or to NO were not significantly affected by exogenous SOD or by the Cu/Zn SOD inhibitor diethyldithiocarbamic acid (DETCA).

3 Hydroquinone produced a concentration-dependent reduction of responses to NO with an IC₅₀ of 27 μ M, and higher concentrations reduced relaxant responses to nitrgic nerve stimulation with an IC₅₀ of 612 μ M. The effects of hydroquinone were only slightly reversed by SOD, so it does not appear to be acting as a superoxide generator.

4 Pyrogallol produced a concentration-dependent reduction in responses to NO with an IC₅₀ value of 39 μ M and this effect was reversed by SOD (100–1000 u ml⁻¹). Pyrogallol did not affect responses to nitrgic nerve stimulation. Treatment with DETCA did not alter the differentiating action of pyrogallol.

5 Duroquinone produced a concentration-dependent reduction of relaxations to NO with an IC₅₀ value of 240 μ M and 100 μ M slightly decreased nitrgic relaxations. After treatment with DETCA, duroquinone produced greater reductions of relaxant responses to NO and to nitrgic stimulation, the IC₅₀ values being 8.5 μ M for NO and 40 μ M for nitrgic nerve stimulation; these reductions were reversed by SOD.

6 The findings do not support the hypothesis that the presence of Cu/Zn SOD explains the greater susceptibility of NO than the nitrgic transmitter to the superoxide generator pyrogallol, but suggest that it may play a role in the effects of duroquinone.

Keywords: Anococcygeus muscle (rat); diethyldithiocarbamic acid (DETCA); duroquinone; hydroquinone; nitrgic transmission; nitric oxide; pyrogallol; superoxide dismutase

Abbreviations: ANOVA, analysis of variance; DETCA, diethyldithiocarbamic acid; DMSO, dimethylsulphoxide; EFS, electrical field stimulation; NADPH, nicotinamide dinucleotide phosphate (reduced); NO, nitric oxide; NOS, nitric oxide synthase; SOD, superoxide dismutase

Introduction

There is indisputable evidence that the functional integrity of neuronal nitric oxide synthase (nNOS) is essential for nitrgic transmission and it is generally thought that this enzyme generates free radical nitric oxide (NO) from L-arginine; however, it is difficult to reconcile the postulate that the actual transmitter is NO since several substances differentiate between the actions of the nitrgic transmitter and exogenous NO (or at least an aqueous solution of nitric oxide gas) at a number of neuroeffector junctions (Rand & Li, 1995a,b). Thus, NO does not meet one of the essential criteria for acceptance as the transmitter, namely, that responses to the putative transmitter and those produced by nerve stimulation should be affected in comparable ways by various interacting agents.

Superoxide generators are one of the groups of differentiating agents. They rapidly inactivate NO and block responses to it and to endothelium derived relaxing factor (Rubanyi &

Vanhoutte, 1986). However, a number of superoxide generators do not affect responses to nitrgic nerve stimulation although responses to exogenous nitric oxide in the same tissues are blocked. This has been shown with pyrogallol in the rat gastric fundus (De Man *et al.*, 1996), duroquinone in the mouse anococcygeus muscle (Lilley & Gibson, 1995) and rat gastric fundus (De Man *et al.*, 1996), 7-ethoxyresorufin in the rat anococcygeus muscle (Li & Rand, 1996), LY83583 in the rat gastric fundus (Barbier & Lefebvre, 1992; Lefebvre, 1996), xanthine or hypoxanthine in the presence of xanthine oxidase in the mouse anococcygeus muscle (Gibson *et al.*, 1994) and rat gastric fundus (Lefebvre, 1996), and hydroquinone, which may act as superoxide generator or NO-scavenger depending on conditions, in the bovine retractor penis muscle (Paisley & Martin, 1996), mouse anococcygeus muscle (Hobbs *et al.*, 1991; Lilley & Gibson, 1995) and rat gastric fundus (Lefebvre, 1996).

It has been postulated that elimination of superoxide by endogenous superoxide dismutase (SOD) protects transmitter NO from inactivation since inhibition of Cu/Zn SOD with diethyldithiocarbamic acid (DETCA) brought about increases in the sensitivity of the transmitter to hydroquinone, duroquinone and LY83583 in the bovine retractor penis

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(Martin *et al.*, 1994; Paisely & Martin, 1996) and mouse anococcygeus muscles (Lilley & Gibson, 1996), to pyrogallol and duroquinone in the rat gastric fundus (DE Man *et al.*, 1996) and to pyrogallol in the rat anococcygeus muscle (Liu *et al.*, 1997). However, Lefebvre (1996) showed that although DETCA slightly increased the transmitter-blocking action of LY83583, it did not affect that of hydroquinone or hypoxanthine:xanthine oxidase on the rat gastric fundus. Furthermore, DETCA treatment did not render the nitrgic transmitter in the mouse anococcygeus sensitive to xanthine:xanthine oxidase (Lilley & Gibson, 1996).

The aim of the present study was to determine whether DETCA affected the differentiating actions of the superoxide generators pyrogallol and duroquinone in the rat anococcygeus muscle; hydroquinone was also used but turned out not to be acting as a superoxide generator.

Methods

Tissue preparation and experimental protocols

Male Sprague-Dawley rats (300–450 g) were killed by decapitation and the anococcygeus muscles were removed and set up as previously described (Gillespie, 1972; Li & Rand, 1989). Briefly, muscles were mounted under a resting tension of 1 g and equilibrated for a period of 30 min before commencing experimental intervention. After the tone was raised by guanethidine (10–30 μM), and clonidine (0.05 μM) was added to help stabilize the contraction, relaxant responses to either nitric oxide in aqueous solution (NO) or electrical field stimulation (EFS; 1 ms pulses at 1 Hz for 10 s) were obtained. Once these responses had reached a steady extent, hydroquinone, pyrogallol, duroquinone or superoxide dismutase (SOD) was added in cumulatively increasing concentrations and the effect on NO- or EFS-induced relaxations determined. Each concentration was left in contact with the tissue for at least 20 min before the addition of the next concentration.

NO (0.4, 0.8 and 2 μM) was added in successively increasing concentrations at 1–3 min intervals, during which the relaxant action of the previous concentration of NO had completely dissipated. At least two concentration-response curves to NO were obtained before the addition of other agents and at least 5 min was allowed to elapse before constructing another concentration-response curve to NO.

Parallel experiments without addition of the substances tested were carried out on a second anococcygeus muscle from the same donor rat to serve as a time control in each set of experiments.

Inhibition of superoxide dismutase with DETCA

In some experiments, the consequences of inhibition of Cu/Zn superoxide dismutase on the effects of pyrogallol or duroquinone on EFS and NO were determined. In these, the anococcygeus was incubated with DETCA (3 mM) for 1 h followed by a washout period of 30 min. Then guanethidine and clonidine were added to the organ bath to raise the tone, as in other experiments. Responses to EFS (1–5 Hz) and NO (0.4–2 μM) were obtained, and once these had stabilized, the effects of pyrogallol (100 μM) or duroquinone (10–100 μM) on them were determined. Only one frequency of stimulation was used in each preparation. Parallel experiments without DETCA were carried out on a second muscle from the same donor rat to serve as a control.

Drugs and solutions

The PSS had the following composition (mM): NaCl, 118; KCl, 4.7; NaHCO_3 , 25; MgSO_4 , 0.45; KH_2PO_4 , 1.03; CaCl_2 , 2.5; D-(+)-glucose, 11.1. The PSS was gassed with 5% CO_2 and 95% O_2 and maintained at 37°C.

The drugs used and their sources were: clonidine hydrochloride, guanethidine sulphate, diethyldithiocarbamic acid (DETCA), hydroquinone, pyrogallol, superoxide dismutase (Sigma, St. Louis, MO, U.S.A.); duroquinone (Aldrich Chemical Co., Castle Hill, NSW, Australia), nitric oxide (compressed gas; CIG, Melbourne, Victoria, Australia). Saturated aqueous solutions of NO were prepared from NO gas as previously described (Rajanayagam *et al.*, 1993). All drugs were dissolved in distilled water prior to dilution with PSS with the exception of duroquinone, which was dissolved in 100% DMSO. The maximum concentration of DMSO in the organ bath was 0.01% v/v, which was without effect on responses to NO or EFS in control experiments.

Analysis of results

Responses to NO and EFS were expressed as reductions of tension. Data are expressed as means $\pm$ s.e. means and *n* indicates the number of animals contributing tissues. Differences between means were assessed by Student's *t*-test (paired or unpaired, as appropriate) or analysis of variance (ANOVA; two-way or repeated measurements). Probability values of $P < 0.05$ were considered significant.

The IC_{50} values for duroquinone, pyrogallol and hydroquinone were calculated from the falls in tension caused by the maximal concentrations of NO used (2 μM) or the maximal frequency of EFS plotted against increasing concentration of drugs. An IC_{50} value was determined for each experiment and the mean $\pm$ s.e. of IC_{50} values was then calculated.

Ethical considerations

The experiments reported here were approved by the Animal Experimentation Ethics Committee of the Royal Melbourne Institute of Technology and conformed to the guidelines of the Australian National Health and Medical Research.

Results

SOD

Exogenous SOD (100–1000 u ml⁻¹) had no effect on the tone of the anococcygeus muscle and did not significantly affect responses to EFS or NO.

DETCA

Incubation with DETCA (3 mM) for 1 h did not significantly alter the contraction induced by guanethidine and clonidine, which was 8.7 ± 0.3 g ($n = 21$) compared with 8.7 ± 0.4 g ($n = 21$) in control untreated tissues, and did not significantly affect relaxant responses to NO or EFS.

Hydroquinone

Hydroquinone (10–100 μM) had no effect on tone but produced concentration-dependent reductions in responses to NO (Figure 1) with an IC_{50} value of 27 ± 4.7 μM ($n = 5$). A concentration of 100 μM had no effect on responses to EFS,

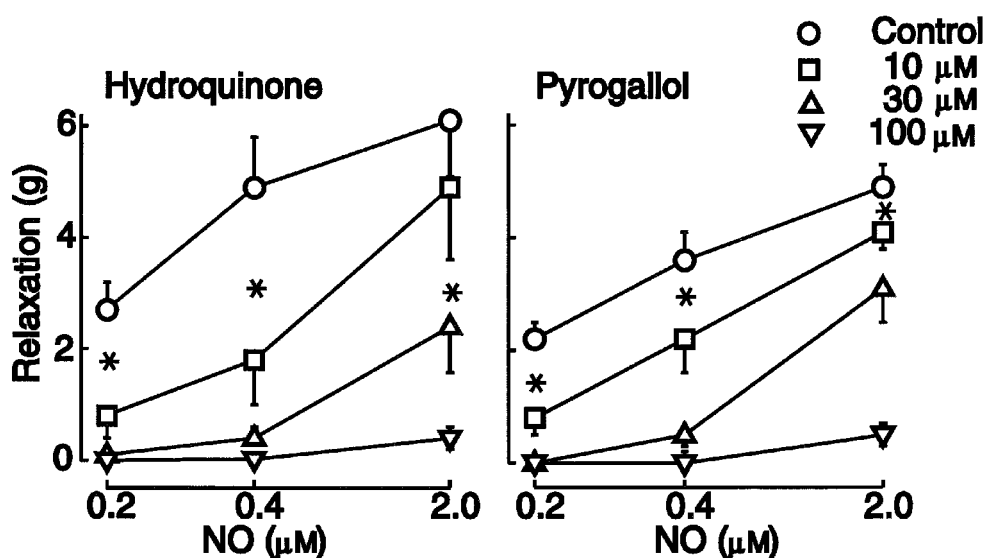


Figure 1 Effects of hydroquinone ($n=4$) and pyrogallol ($n=5$) on relaxant responses to NO (0.4 – 2 μM) in anococcygeus muscles in which tone was raised by guanethidine plus clonidine. Control responses are in the absence of pyrogallol or hydroquinone. Symbols represent means and I-bars are s.e.means. Symbols below * are significantly less than corresponding control value.

but higher concentrations (300 and 1000 μM) significantly reduced them (Figure 2), the IC_{50} value for the reduction being 612 ± 98 μM ($n=6$). Thus it had a 30 fold greater potency in discriminating against NO compared with the nitrgic transmitter.

The reduction by hydroquinone (100 μM) of responses to NO was only slightly reversed by the addition of SOD (100–1000 u ml^{-1}) and the reversal was only significant with the highest concentration of 1000 u ml^{-1} SOD (Figure 3). Thus it is unlikely to be acting predominantly as a superoxide generator in this situation.

Pyrogallol

Pyrogallol (10–100 μM) produced concentration-dependent reductions in responses to NO (Figure 1) with an IC_{50} value of 39 ± 7 μM ($n=5$) but did not affect responses to EFS (1 Hz): the responses in its absence and in the presence of 10, 30 and 100 μM pyrogallol being (in g) 3.1 ± 0.2 , 3.3 ± 0.4 , 3.5 ± 0.4 and 3.3 ± 0.3 ($n=6$), respectively. Higher concentrations of pyrogallol were not used in this series of experiments as it generally produced spontaneous activity in the muscles and altered the basal tone.

The addition of SOD (100–1000 u ml^{-1}) significantly reversed in a concentration-dependent manner the reduction by pyrogallol (100 μM) of responses to NO, and at a concentration of 1000 u ml^{-1} of SOD the responses to NO were restored to the control level (Figure 3).

Treatment with DETCA did not alter the differentiating action of pyrogallol (100 μM) in that the response to EFS (1 Hz) was not significantly changed and the response to NO (0.4 μM) was reduced to the same extent as in the absence of DETCA-pretreatment (Figure 4).

Duroquinone

In time control experiments, the solvent for duroquinone, dimethylsulphoxide, had no effect on relaxations elicited by NO or EFS. Duroquinone had no effect in low concentrations (10 and 30 μM), but the highest concentration used (100 μM)

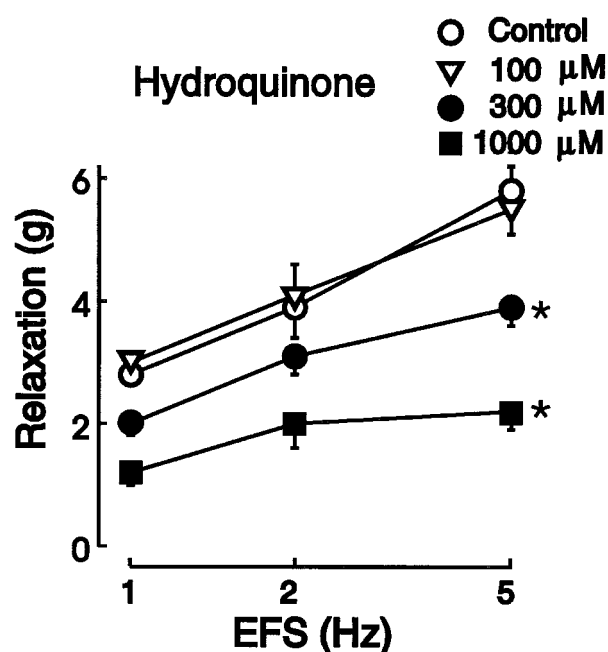


Figure 2 Effects of hydroquinone on relaxant responses to electrical field stimulation (EFS, 1–5 Hz) in rat anococcygeus muscles in which tone was raised by guanethidine plus clonidine. Symbols represent means and I-bars are s.e.means ($n=3$ – 6). *Values in curves marked thus are significantly less than in control curve (ANOVA; $P < 0.05$).

produced significant reductions of responses to NO (Figure 5a) and EFS (Figure 5b). The estimated IC_{50} of duroquinone against NO was 240 ± 56 μM ($n=4$).

Treatment with DETCA enhanced the blocking action of duroquinone on both NO (Figure 5c) and EFS (Figure 5d). The IC_{50} values were 8.5 ± 0.9 μM ($n=4$) for NO and 40 ± 7 μM ($n=5$) for EFS. The reductions of responses to NO and EFS (1 Hz) produced by duroquinone after treatment with DETCA were partially reversed by the addition of exogenous SOD (1000 u ml^{-1}) (Figure 6).

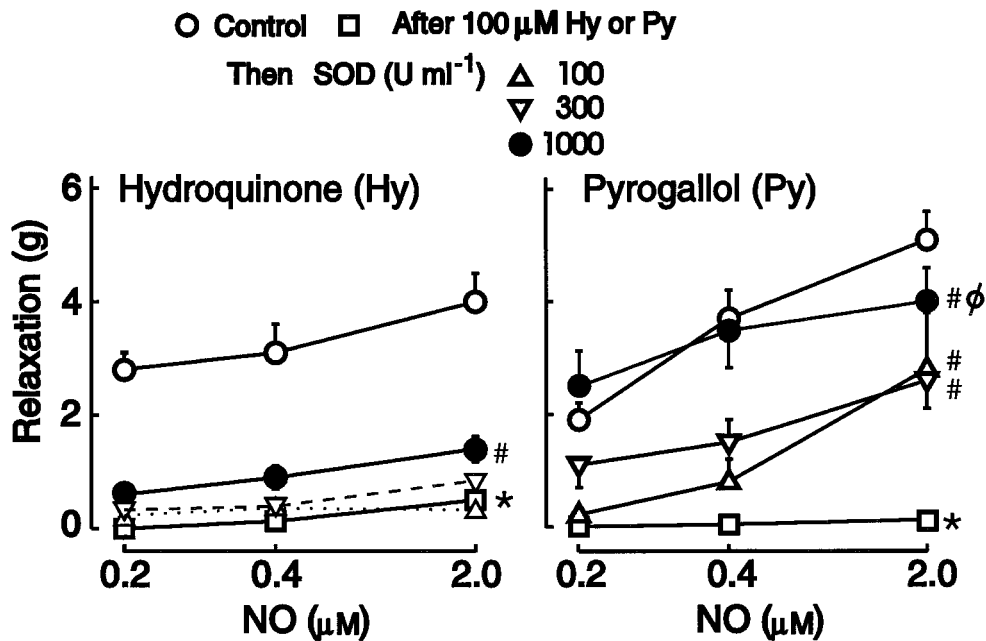


Figure 3 Relaxant responses to NO in the absence and presence of hydroquinone or pyrogallol (for each: 100 μM ; $n=4$). Then increasing concentrations of SOD were added. In the left-hand panel, the responses in the presence of 100 and 300 U ml^{-1} of SOD are indicated by broken lines (... and - - -, respectively). Symbols represent means and I-bars are s.e.means, which were smaller than the size of the symbol in some cases. *Values in these curves significantly less than control (ANOVA, $P < 0.01$). #Values in these curves significantly greater than in absence of SOD (ANOVA, $P < 0.05$). ϕ Curve not significantly different from control (ANOVA, $P > 0.5$).

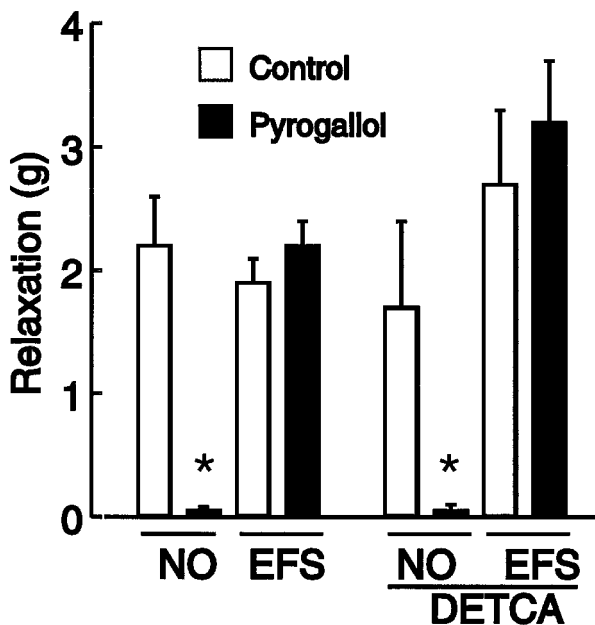


Figure 4 Effects of pyrogallol (100 μM) on relaxant responses to electrical field stimulation (EFS, 1 Hz) and NO (0.4 μM) in the absence of (left columns) and after treatment with DETCA (3 mM) (right columns). Columns represent means and I-bars are s.e.means, $n=4$. *Indicates significantly less than corresponding control (paired t -test; $P < 0.01$).

Discussion

The addition of exogenous SOD had no significant effect on responses to NO or to the nitrgic transmitter in the rat anococcygeus muscle in the present work and as reported by

Liu *et al.* (1997), or on rat gastric fundus strips (Lefebvre, 1996); therefore, if endogenous SOD does have an influence, its activity must be maximal. However, DETCA, which inhibits extracellular and intracellular endogenous Cu/Zn SOD (Kelner *et al.*, 1989) also had no effect on these responses. Liu *et al.* (1997) reported that DETCA slightly reduced EFS-induced relaxations from $61.5 \pm 10.5\%$ ($n=10$) to $51.7 \pm 10.8\%$ ($n=10$), but this difference is not statistically significant when tested by unpaired t -test ($P=0.52$). These findings suggest either that the anococcygeus muscle does not normally produce superoxide or that the endogenous Cu/Zn SOD which is present in nitrgic nerves in the rat anococcygeus muscle (Liu *et al.*, 1997) has little or no influence on exogenous NO or the nitrgic transmitter. However, in addition to Cu/Zn SOD, as De Man *et al.* (1996) pointed out, there is a mitochondrial Mn SOD that is not inhibited by DETCA and is present in the rat gastrointestinal tract muscle and nerve cells, where it is colocalized with NADPH diaphorase, a marker for NO synthase (Ceillely *et al.*, 1995; Fang & Christensen, 1995). Although the anococcygeus muscle is not part of the gastrointestinal tract, it may nevertheless contain Mn SOD and the effect of inhibiting this enzyme remains to be tested.

Hydroquinone (10–100 μM) had no effect on nitrgic transmission but significantly reduced responses to exogenous NO in a concentration-dependent manner, being slightly more potent than pyrogallol. In contrast to the finding with pyrogallol, the effect of hydroquinone was only slightly reversed by SOD, even at a high concentration. This finding is in agreement with the view of Hobbs *et al.* (1991), Lefebvre (1996) and Lilley & Gibson (1996) that hydroquinone in this situation acts mainly as a free radical scavenger rather than as a superoxide generator. For this reason, the effect of inhibiting Cu/Zn SOD with DETCA on the activity of hydroquinone was not investigated.

Pyrogallol significantly reduced responses to exogenous NO in a concentration-dependent manner and this effect was

reversed by addition of SOD, thus supporting the view that the reduction was due to the superoxide-generating activity of pyrogallol. However, pyrogallol (100 μM) had no effect on nitrgic transmission, and Liu *et al.* (1997) also found that pyrogallol (30 μM) had no effect on nitrgic relaxations of the rat anococcygeus muscle. These findings are in contrast with those of Gillespie & Sheng (1990) using the same tissue, who reported that 30 μM pyrogallol significantly reduced nitrgic relaxation at frequencies above 1 Hz and 100 μM significantly reduced those at frequencies above 0.5 Hz. We can offer no explanation for the discrepancy.

In the present experiments, inhibition of endogenous Cu/Zn SOD with DETCA did not significantly alter the effects of pyrogallol on NO or EFS-induced relaxations. In similar experiments with DETCA-treated rat anococcygeus muscles, Liu *et al.* (1997) reported that EFS-induced relaxations were $51.7 \pm 10.8\%$ ($n=10$) before pyrogallol and were reduced about 40% to $30.7 \pm 7.6\%$ ($n=7$) in the presence of pyrogallol. Although the difference they reported is not statistically significant by unpaired *t*-test; $P=0.17$, it may nevertheless have been a real effect since it was reversed by SOD. In contrast, we observed that pyrogallol produced a slight, but not significant increase in EFS-induced relaxations after DETCA treatment. The possible discrepancy between our findings and those of Liu *et al.* (1997) would require further experiments for its resolution.

Duroquinone weakly reduced responses to NO with an IC_{50} value of $240 \pm 56 \mu\text{M}$, but this effect was markedly enhanced after treatment with DETCA, the IC_{50} value then being $8.5 \pm 0.9 \mu\text{M}$. Treatment with DETCA likewise enhanced the reduction of responses to nitrgic nerve stimulation, the IC_{50}

value being $40 \pm 7 \mu\text{M}$. However, it should be noted that duroquinone still differentiated quantitatively between relax-

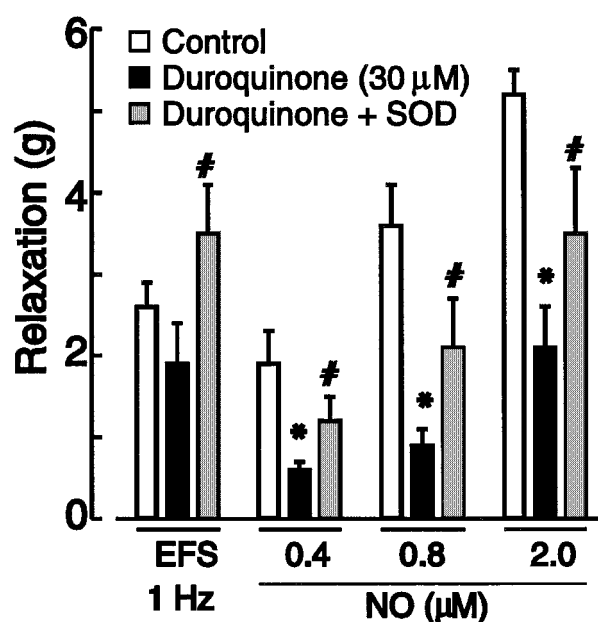


Figure 6 Effects of 30 μM duroquinone in DETCA-treated muscles in the absence and presence of 1000 u ml^{-1} SOD on relaxant responses to electrical field stimulation (EFS, 1 Hz; $n=3$) and NO ($n=4$). Column heights represent means and I-bars are s.e.means. *Indicates significant reduction from control. #Indicates significant increase produced by SOD.

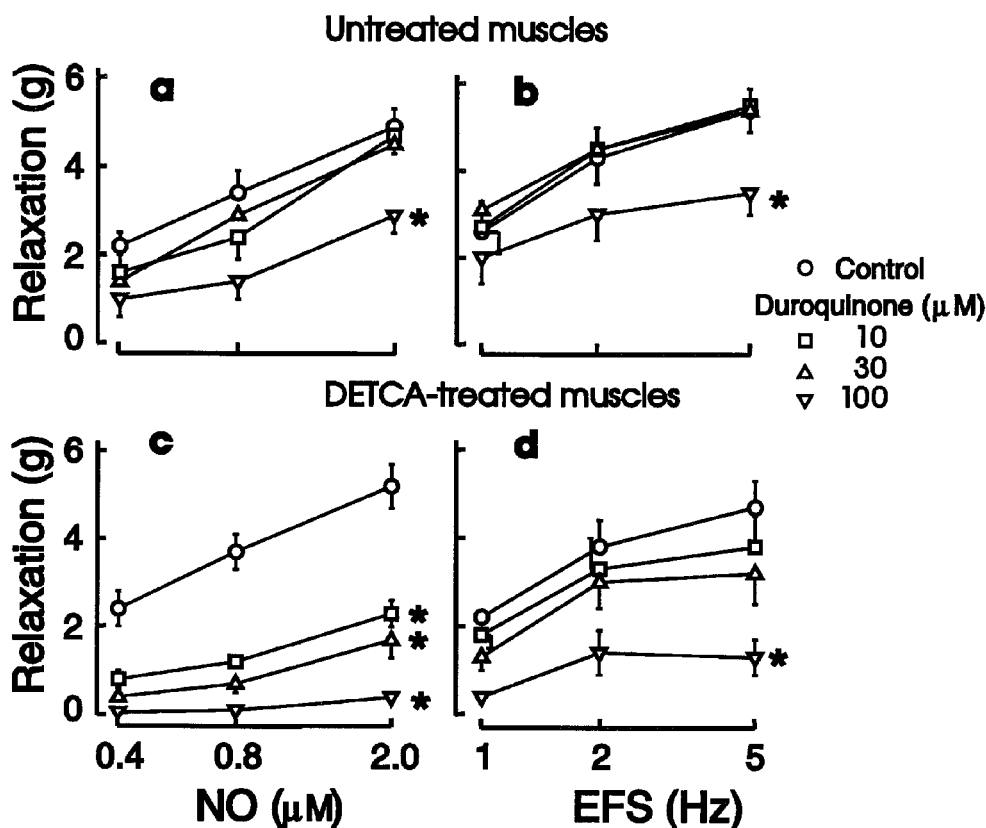


Figure 5 Effects of duroquinone on relaxant responses to NO (a and c, 0.4–2 μM) and electrical field stimulation (EFS: b and d, 1–5 Hz) in control (a and b) and DETCA-treated muscles (c and d). Symbol represents means and I-bars are s.e.means, $n=5$ for a and c, and $n=4$ for b and d. *Curves significantly different from control (ANOVA, $P<0.01$).

ant responses to EFS and NO after treatment with DETCA with a 4.7 fold difference in IC_{50} values. The findings provide some support for the hypothesis that the relative resistance of the nitrgic transmitter in the rat anococcygeus muscle to duroquinone could be due to elimination of the superoxide generated by it by Cu/Zn SOD, but this does not account fully for the differentiating action.

Recently, Lilley & Gibson (1997) reported that ascorbate is released by EFS, and it is possible that it acts as an antioxidant to protect NO from attack by scavenger molecules, and might modify the effects of superoxide generators on nitrgic transmission and NO-induced relaxations. Furthermore, the local concentration of neuronal ascorbate might preferentially protect the nitrgic transmitter. On the other hand, we have not observed any differences in responses to NO or EFS between experiments in which ascorbate (0.14 mM) was present or absent (unpublished data).

One explanation for the differential actions of the superoxide generator pyrogallol and the free radical scavenger hydroquinone is that the nitrgic transmitter is not NO but may be a NO-donating species. Another view is that it is NO but it is not appreciably affected by inactivating agents in the short time it would take for NO to diffuse from its site of generation to its site of action (Wood & Garthwaite, 1994). However, the rate of reaction of NO with superoxide to form peroxynitrite ($ONOO^-$) is $6.7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, and is described as near-diffusion limited (Huie & Padmaja, 1993). Further-

more, the interaction between NO and superoxide to produce peroxynitrite occurs 3.5 times faster than the dismutation of superoxide by SOD (Beckman & Koppenol, 1996). Thus, it is likely that any NO acting as a transmitter would be appreciably affected by superoxide, even in the presence of SOD, in its rapid diffusion across the neuroeffector junction from its site of formation to its site of action. This favours the view that the transmitter is not NO, but a contribution of the effect envisaged by Wood & Garthwaite (1994) can not be excluded.

It has recently been suggested that the product of NOS is the nitrosyl anion (NO^-) rather than free radical NO (Schmidt *et al.*, 1996). If the nitrgic transmitter were nitrosyl, it would not be expected to be scavenged by hydroquinone or to react rapidly with free radical superoxide generated by pyrogallol or duroquinone. It is of some interest that Schmidt *et al.* (1996) found that the Cu (II) of SOD reacts with the nitrosyl ion to form Cu (I) and NO free radical. Since the SOD inhibitor DETCA is a copper chelator, it may stabilize nitrosyl ions acting as the nitrgic transmitter.

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Growth-inhibitory effect of cyclic GMP- and cyclic AMP-dependent vasodilators on rat vascular smooth muscle cells: effect on cell cycle and cyclin expression

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1 The possibility that the antiproliferative effect of cyclic GMP- and cyclic AMP-dependent vasodilators involves an impaired progression of vascular smooth muscle cells (VSMC) through the cell cycle and expression of cyclins, which in association with the cyclin-dependent kinases control the transition between the distinct phases of the cell cycle, was examined.

2 FCS (10%) stimulated the transition of quiescent VSMC from the G₀/G₁ to the S phase (maximum within 18–24 h and then to the G₂/M phase (maximum within 22–28 h). Sodium nitroprusside and 8-Br-cyclic GMP, as well as forskolin and 8-Br-cyclic AMP markedly reduced the percentage of cells in the S phase after FCS stimulation.

3 FCS stimulated the low basal protein expression of cyclin D1 (maximum within 8–24 h) and E (maximum within 8–38 h) and of cyclin A (maximum within 14–30 h). The stimulatory effect of FCS on cyclin D1 and A expression was inhibited, but that of cyclin E was only minimally affected by the vasodilators.

4 FCS increased the low basal level of cyclin D1 mRNA after a lag phase of 2 h and that of cyclin A after 12 h. The vasodilators significantly reduced the FCS-stimulated expression of cyclin D1 and A mRNA.

5 These findings indicate that cyclic GMP- and cyclic AMP-dependent vasodilators inhibit the proliferation of VSMC by preventing the progression of the cell cycle from the G₀/G₁ into the S phase, an effect which can be attributed to the impaired expression of cyclin D1 and A.

Keywords: VSMC; proliferation; nitric oxide donor; sodium nitroprusside; forskolin; 8-Br-cyclic GMP; 8-Br-cyclic AMP; cell cycle; cyclins

Abbreviations: CAS 92-4678, 4-phenyl-furoxan-3-carboxylic acid [pyridyl-3-yl-methyl]-amide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-tetraacetic acid; FCS, foetal calf serum; HEPES, N-2-hydroxyethyl piperazine-N-2-ethanesulphonic acid; MOPS, 3-(N-morpholino)-propanesulphonic acid; NO, nitric oxide; NOS II, inducible nitric oxide synthase; NOS III, endothelial nitric oxide synthase; SDS, sodium dodecylsulphate; SNAP, S-nitroso-N-acetylpenicillamine; SNP, sodium nitroprusside; VSMC, vascular smooth muscle cells

Introduction

Endothelium-derived nitric oxide (NO) plays a pivotal role in the control of vascular homeostasis by regulating vascular tone (Furchgott & Vanhoutte, 1989) and the interaction of blood cells with the vascular wall (Radomski *et al.*, 1987). In addition, NO may also contribute to the maintenance of vascular smooth muscle cells (VSMC) in a non-proliferative state. Such a concept is supported by several lines of evidence. First NO donors, as well as NO generated in VSMC following gene transfer of endothelial NO synthase (NOS III), or the cytokine-induced expression of inducible NO synthase (NOS II), strongly inhibited the growth of cultured VSMC (Garg & Hassid, 1989; Kariya *et al.*, 1989; Scott-Burden *et al.*, 1992; Kullo *et al.*, 1997). Second, administration of NO donors reduced the development of intimal thickening in rat (Guo *et al.*, 1994) and porcine carotid (Groves *et al.*, 1995), and rabbit femoral arteries injured by a balloon catheter (Marks *et al.*, 1995), and also in humans

after percutaneous transluminal angioplasty (The ACCORD Study Investigators, 1997). Third, administration of L-arginine, the substrate of NOS, to rabbits attenuated intimal thickening of aortae in response to balloon injury (McNamara *et al.*, 1993) or to a high cholesterol diet (Cooke *et al.*, 1992), whereas NOS inhibitors exacerbated the accumulation of VSMC in the neointima (Cayette *et al.*, 1994). Furthermore, *in vivo* gene transfer of NOS III in rat carotid arteries prevented neointimal hyperplasia (Von der Leyen *et al.*, 1995).

The growth-inhibitory effect of NO donors seems to be mediated, at least in part, by a cyclic GMP-dependent effector pathway, since VSMC proliferation is also impaired by a lipophilic analogue of cyclic GMP (Garg & Hassid, 1989; Assender *et al.*, 1992), by an NO-independent activator of soluble guanylyl cyclase (Yu *et al.*, 1995) and by activators of the particulate guanylyl cyclase (Abell *et al.*, 1989). Although cyclic GMP may directly interfere with growth-related events, it is also conceivable that a cross-talk exists between cyclic GMP- and cyclic AMP-mediated signaling pathways since high concentrations of cyclic GMP have been reported to inhibit the degradation of cyclic AMP by phosphodiesterase III (Beavo & Reifsynder, 1990) and activate protein kinase A

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(Forte *et al.*, 1992; Cornwell *et al.*, 1994). Consistent with such a hypothesis it has been shown, that agents which elevate cyclic AMP, including prostaglandin E1 (Loesberg *et al.*, 1985), adenosine (Jonzon *et al.*, 1985; Loesberg *et al.*, 1985), forskolin (Assender *et al.*, 1992), or 8-Br-cyclic AMP (Jonzon *et al.*, 1985; Assender *et al.*, 1992) potently inhibited VSMC proliferation. Since the antiproliferative effect of NO donors or 8-Br-cyclic GMP is still observed when added up to 12 h after foetal calf serum (FCS) stimulation of growth-arrested VSMC (Kariya *et al.*, 1989; Sarkar *et al.*, 1997) it is conceivable that these vasodilators target growth-related events essential for the progression of the cell cycle from the G0/G1 into the S phase. NO may also inhibit growth-related events in the S phase and G2/M phase of the cell cycle since S-nitroso-N-acetylpenicillamine (SNAP) reduced DNA synthesis when added to VSMC synchronized in S phase (Sarkar *et al.*, 1997). Furthermore, NO produced following induction of NOS II blocked macrophage-like cells in the early G2/M phase of the cell cycle (Takagi *et al.*, 1994). These findings suggest that NO can interfere with the cell cycle progression, resulting in a growth arrest in defined phases. Since the sequential expression of cyclins, regulatory subunits of the cyclin-dependent kinases (Pines, 1995), is known to be determinant in the transition between the different phases of the cell cycle, it is conceivable that NO might exert its antiproliferative effect through a modulation of cyclin expression. Therefore the aim of the present study was to assess the effect of NO donors on the expression of cyclins and to better characterize the effect of cyclic GMP- and cyclic AMP-dependent vasodilators on the cell cycle of VSMC.

Methods

Materials

SNP was obtained from Merck (Darmstadt, Germany), forskolin and 8-Br-cyclic GMP from Fluka (Deisenhofen, Germany), and 8-Br-cyclic AMP from Biomol (Hamburg, Germany). CAS 92-4678 (4-phenyl-furoxan-3-carboxylic acid [pyridyl-3-yl-methyl]-amide) was kindly supplied by Hoechst (Frankfurt, Germany). Minimum essential medium containing Earle's salts, trypsin, penicillin and streptomycin were supplied by Gibco BRL (Eggenstein, Germany) and FCS by Biochrom (Berlin, Germany). All plastic-ware was purchased from Falcon (Becton Dickinson) and [α - ^{32} P]-dCTP (3000 Ci mmol $^{-1}$) from Hartmann Analytic (Braunschweig, Germany). All other chemicals were from Sigma (Deisenhofen, Germany). The *EcoRI* restriction fragment of human cyclin A was kindly provided by Dr T. Hunter (Salk Institute, La Jolla, U.S.A.) and the *EcoRI* restriction fragment of mouse cyclin D1 by Dr C.J. Sherr (St. Jude Children's Res. Hosp. Memphis, U.S.A.).

Cell culture

VSMC were isolated by elastase and collagenase digestion of thoracic aortae from male Wistar rats (250–300 g). Cells were cultured in minimum essential medium containing Earle's salts, 2 mM glutamine, 5 mM *N*-tris (hydroxymethyl)-methyl-2-aminoethanesulfonic acid NaOH, 5 mM *N*-2-hydroxyethyl-piperazine-*N*-2-ethanesulphonic acid (HEPES)-NaOH, (both at pH 7.3), 50 U ml $^{-1}$ penicillin, 50 μ g ml $^{-1}$ streptomycin, and FCS (10%, v/v). Confluent cultures of VSMC were serially passaged with 0.05% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA). All experiments were performed with confluent VSMC at passage 7–24. Cells were plated on either

24-well plates (growth studies) or on Petri dishes (35 mm diameter for flow cytometric analysis, 100 mm for Northern and Western blot analyses). Prior to the exposure of VSMC to the cyclic GMP- and cyclic AMP-dependent vasodilators, cells were incubated in fresh FCS-free medium containing 0.1% fatty acid-free bovine serum albumin every 24-h period for 2 days before treatment or as stated.

Cell growth studies

VSMC were seeded at approximately 70,000 cells/well in culture medium containing FCS (10%). After 24 h the culture medium was replaced with medium containing FCS (5%) prior to the addition of either SNP, the furoxan derivative CAS 92-4678 or forskolin. In the case of SNP, the medium containing the compound was replaced every 24 h. Cell number determinations were performed in triplicate, both 24 h after initial plating and 3 days after treatment, with a CASY-Coulter counter (Schaefer System, Reutlingen, Germany).

Flow cytometric analysis

After a 48-h incubation period in FCS-free medium, cells were left either untreated or were exposed first to either a cyclic GMP- or cyclic AMP-dependent vasodilator and 5 min later to FCS (10%) and then incubated for the indicated time periods. The 5-min treatment period with the vasodilators has been shown previously to be associated with maximal production of cyclic GMP (Gruetter *et al.*, 1981) and cyclic AMP (Leitman *et al.*, 1986), respectively. At the appropriate times VSMC were trypsinized, centrifuged at 200 $\times g$, washed with HEPES-buffered Tyrode's solution (pH 7.4), and then analysed for their DNA content by staining with propidium iodide according to the procedure of a commercial kit (Cycle TEST PLUS, Becton Dickinson, San Jose, CA, U.S.A.). Flow cytometric analysis was carried out using a FACScan (Becton Dickinson). DNA histogram analysis was performed using the ModFitLT software (Becton Dickinson).

Western blot analysis

VSMC were exposed to the cyclic GMP- and cyclic AMP-dependent vasodilators for 5 min prior to the addition of FCS (10%). Cells were then washed twice with cold HEPES-buffered Tyrode's solution (pH 7.4), harvested by scraping, and collected by centrifugation at 900 $\times g$ for 3 min at 4°C. The cell pellets were incubated in 300 μ l of buffer A (mM): HEPES pH 7.9, KCl 10, EDTA 0.1, ethyleneglycol-bis(β -aminoethyl ether)-tetraacetic acid (EGTA) 0.1, dithiothreitol 1, phenylmethylsulfonyl fluoride 0.5, and 2 μ g ml $^{-1}$ each of leupeptin, pepstatin A, chymostatin, antipain, aprotinin, and trypsin inhibitor for 15 min at 4°C. The crude nuclei were obtained following lysis with Nonidet P-40 (0.6%) and collected by centrifugation at 15,000 $\times g$ for 1 min at 4°C. The nuclear pellets were resuspended in 100 μ l of buffer B (HEPES pH 7.9 20 mM, NaCl 0.4 M, EDTA 1 mM, EGTA 1 mM, dithiothreitol 1 mM and 2 μ g ml $^{-1}$ each of the protease inhibitors). Nuclei were shaken for 15 min and clarified by centrifugation at 15,000 $\times g$ for 5 min at 4°C. The nuclear extracts (30–50 μ g) were subjected to sodium dodecylsulphate (SDS) polyacrylamide gel (12%) electrophoresis and the separated proteins were then transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). A Ponceau staining was performed to ensure equal amounts of protein in each lane. The immobilized cyclin D1, E and A proteins were detected by subsequent incubation with polyclonal rabbit antibodies directed against cyclin D1, E and A,

respectively, (dilution 1:1000, overnight at 4°C; Santa Cruz, Heidelberg, Germany) and then with a secondary polyclonal donkey anti-rabbit immunoglobulin antibody conjugated to horseradish peroxidase (dilution 1:10,000, 1 h at 22°C; Calbiochem). The immunocomplexes were visualized using an enhanced horseradish peroxidase/luminol chemoluminescence reaction. Prestained molecular-mass markers were used as standards for SDS-polyacrylamide gel immunoblot analysis. The autoradiographs were analysed by scanning densitometry (Pharmacia densitometer equipped with a Kappa CCD video camera and the ImageMaster software).

Northern blot analysis

Total cellular RNA was prepared by acid guanidinium thiocyanate extraction and quantified by absorbance at 260 nm. Total RNA (20–30 µg) was size-fractionated by electrophoresis on 1.2% agarose gels containing 2.2 M formaldehyde in a buffer of 20 mM 3-(N-morpholino)-propanesulphonic acid (MOPS), 5 mM sodium acetate, and 1 mM EDTA pH 7.0. RNA was transferred to nylon membranes (Hybond N⁺ Amersham Buchler, Braunschweig, Germany) and fixed by UV-crosslinking and baking at 80°C for 2 h. The membranes were prehybridized at 42°C for 4 h in a buffer containing 50% formaldehyde, 5 × Denhardt's solution, 5 × SSPE, 0.2% SDS, and 250 µg ml⁻¹ denatured salmon sperm DNA. *Eco*RI restriction fragments for mouse cyclin D1 (1.3 kb) and human cyclin A (2.2 kb) were labelled with 30 µCi of α -³²P-dCTP (3000 Ci mmol⁻¹) using a labelling kit (Pharmacia, Freiburg, Germany). The [³²P]-DNA was purified by gel filtration (Nick columns, Pharmacia). Northern blots were hybridized in the same buffer as used for prehybridization at 42°C for 16–20 h with the labelled DNA. Thereafter the blots were washed twice for 30 min at 22°C in 6 × SSPE and 0.1% SDS, then twice for 15 min at 42°C in 1 × SSPE and 0.1% SDS and finally for 30 min at 55°C in 1 × SSPE and 0.1% SDS. After hybridization with the cyclin probes, filters were hybridized with a ³²P-labeled probe for 18S ribosomal RNA. Autoradiography was performed with Fuji RX film with intensifying screens (DuPont de Nemours, Bad Homburg, Germany) at -70°C. The autoradiographs were analysed by scanning densitometry. Cyclic mRNA levels were normalized to their respective 18S ribosomal mRNA levels and expressed in per cent of the signal obtained with FCS-stimulated VSMC.

DNA fragmentation

A photometric enzyme immunoassay (Cell Death Detection ELISA, Boehringer Mannheim) was used for the detection of DNA fragmentation of the VSMC. Cells were incubated with the cyclic GMP- and cyclic AMP-dependent vasodilators for 5 min prior to the addition of FCS (10%). After an incubation period of 22 h, cells were harvested by scraping, centrifuged at 700 × g for 10 min, washed with phosphate-buffered saline and resuspended in incubation buffer. The nucleosomes contained in the sample bind *via* their histone components to a mouse anti-histone antibody and the DNA-parts of the nucleosomes are linked to a mouse anti-DNA-peroxidase antibody. The amount of peroxidase bound in the immunocomplex was determined photometrically at 405 nm with 2,2'-azino-di-(3-ethylbenzthiazoline sulphonate) as a substrate.

Statistical analysis

Results are shown as mean ± s.e.mean of *n* experiments with different batches of VSMC. Statistical analyses were performed with Student's *t*-test or when more than two treatments were compared with one-way analysis of variance (ANOVA) followed by a Bonferroni *t*-test. A value *P* < 0.05 was considered statistically significant.

Results

Effect of cyclic GMP- and cyclic AMP-dependent vasodilators on the FCS-induced proliferation of VSMC

Exposure of VSMC to FCS (5%) induced about a 4 to 5 fold increase in cell number after a 3-day incubation period (Figure 1). This FCS-stimulated cell growth was attenuated by the NO donors SNP (Figure 1a) and CAS 92 92-4678 (10 µM, reduction by 34%, *n* = 3), as well as by forskolin (Figure 1b).

To exclude the possibility that the antiproliferative effects of the agents tested were due to cytotoxicity, morphological appearance of the cells was assessed by light microscopy and DNA fragmentation by a photometric enzyme immunoassay. Exposure of VSMC to either a cyclic GMP- or a cyclic AMP-dependent vasodilator for 22 h did neither induce any major changes in cell shape nor provoke DNA fragmentation compared to control cells (Table 1). However, exposure of

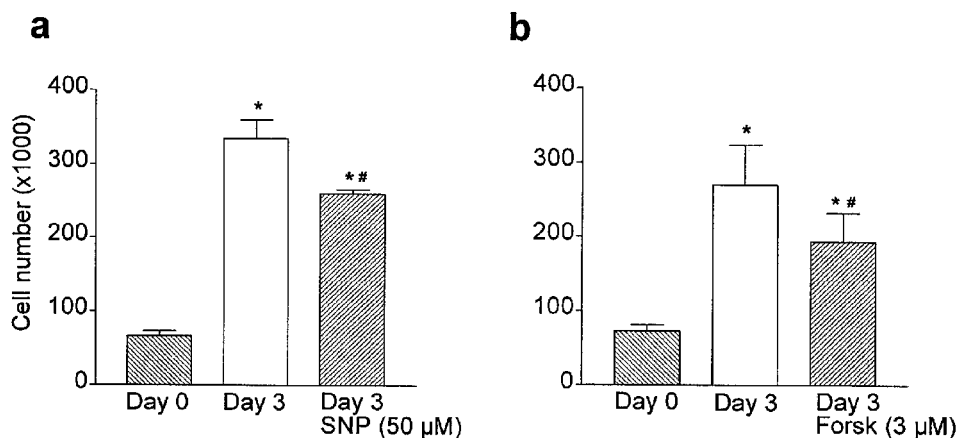


Figure 1 Effect of (a) SNP and (b) forskolin (Forsk) on the FCS (5%)-stimulated proliferation of cultured rat aortic smooth muscle cells. Cell numbers were determined 24 h after initial plating (Day 0) and 3 days after exposure of VSMC to FCS (Day 3) in the absence or presence of either SNP or forskolin. Data shown as mean ± s.e.mean of seven experiments for SNP and five experiments for forskolin. (**P* < 0.05 vs Day 0, #*P* < 0.05 vs Day 3).

VSMC to a 10 fold higher concentration of SNP (3 mM) induced an about 11 fold increase in DNA fragmentation in two independent experiments.

Effect of cyclic GMP- and cyclic AMP-dependent vasodilators on the cell cycle of VSMC

Flow cytometric analysis after 48 h of FCS starvation revealed that more than 90% of VSMC were arrested in the G0/G1 phase (Figure 2). About 14 h after exposure to FCS (10%), VSMC progressed from the G0/G1 phase of the cell cycle into the S phase, and then after about 18 h into the G2/M phase (Figure 2). The effect of cyclic GMP- and cyclic AMP-dependent vasodilators on the transition of VSMC from the G0/G1 phase into the S phase was assessed at various time points after FCS stimulation. Both SNP and forskolin significantly prevented the FCS-stimulated transition of VSMC from the G0/G1 phase into the S phase (Figure 2a and b), but affected minimally the percentage of VSMC in the G2/M phase (Figure 2c). The inhibitory effect of SNP was concentration-dependent with a maximal reduction of $43 \pm 5\%$

(300 μM , Figure 3a). In addition to SNP, the NO donor CAS 92-4678 blocked S phase entry of VSMC with increasing concentrations with a maximal effect of $37 \pm 7\%$ (30 μM , $n=4$). Moreover, 8-Br-cyclic GMP, the membrane-permeable, stable analogue of cyclic GMP, reduced the amount of VSMC in the S phase by $39 \pm 10\%$ (3 mM, Figure 3b). Similar to the cyclic GMP-dependent vasodilators forskolin and 8-Br-cyclic

Table 1 Effect of cyclic GMP- and cyclic AMP-dependent vasodilators on the DNA fragmentation in cultured rat aortic smooth muscle cells exposed to FCS

Treatment	DNA fragmentation (% of FCS value)
Untreated	146 $\pm$ 87
FCS (10%, 22 h)	100
FCS+SNP (300 μM)	102 $\pm$ 25
FCS+8-Br-cyclic GMP (3 mM)	90 $\pm$ 21
FCS+Forskolin (10 μM)	119 $\pm$ 17
FCS+8-Br-cyclic AMP (0.3 mM)	83 $\pm$ 6

Results shown as mean $\pm$ s.e.mean of three experiments.

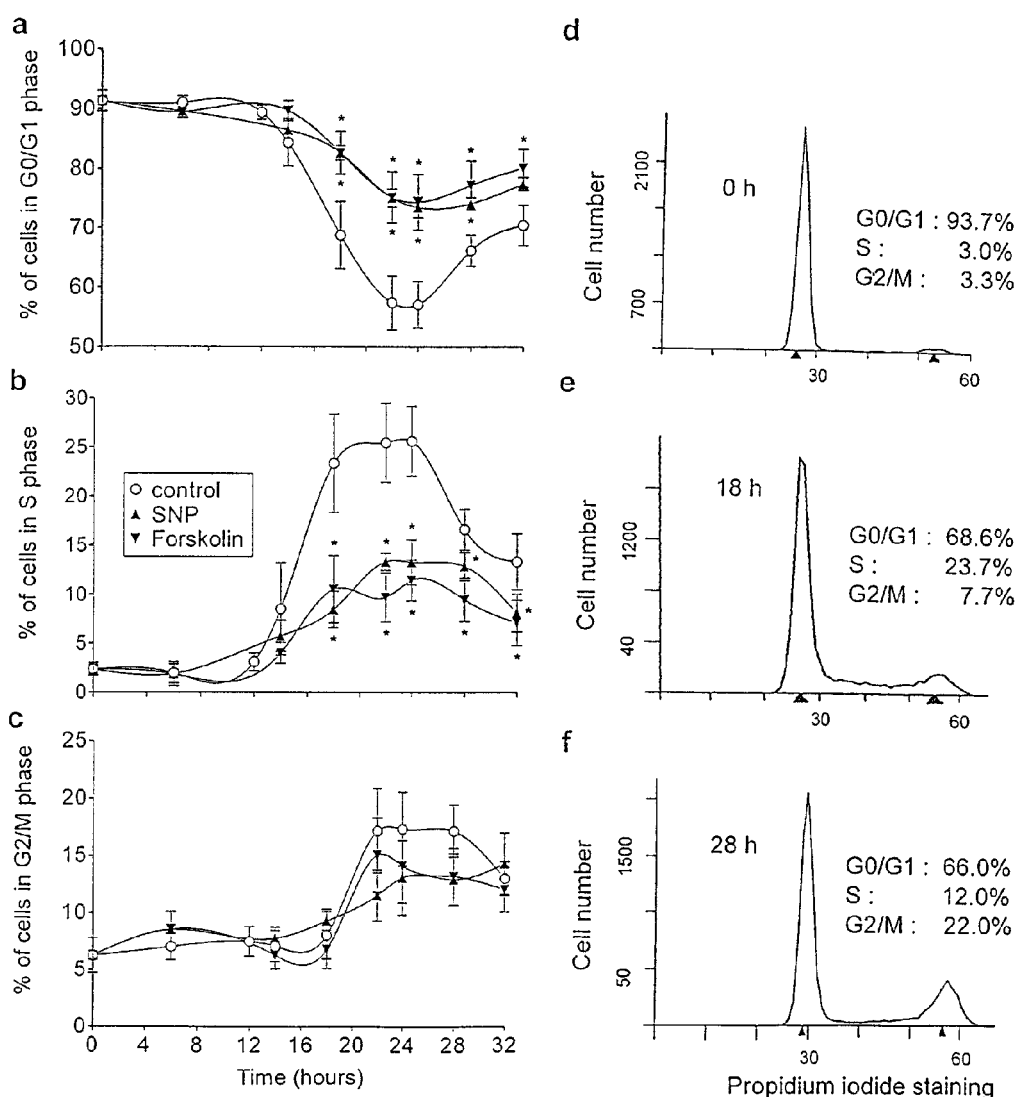


Figure 2 Flow cytometric analysis of cell cycle progression of cultured rat aortic smooth muscle cells. VSMC were arrested in the G0/G1 phase by FCS starvation for 48 h and then were stimulated to enter the cell cycle by the addition of FCS (10%). The effect of SNP (600 μM) and forskolin (3 μM) on the FCS-stimulated progression of VSMC through the cell cycle is also shown. In (a), (b) and (c) data shown as mean $\pm$ s.e.mean of five experiments for SNP and seven experiments for forskolin. In (d), (e) and (f) representative DNA histograms of VSMC at times 0, 18 and 28 h after FCS stimulation are depicted.

AMP markedly decreased the amount of VSMC in the S phase in a concentration-dependent manner with maximal reductions of $80 \pm 10\%$ ($10 \mu\text{M}$, Figure 4a) and $67 \pm 4\%$ (0.3 mM , Figure 4b), respectively.

Effect of cyclic GMP- and cyclic AMP-dependent vasodilators on the protein expression of cyclins

Low levels of the G1 cyclin D1 and cyclin E protein were found in quiescent VSMC, whereas S phase cyclin A protein levels were minimal or even below the detection limit (Figure 5). Exposure of VSMC to FCS (10%) caused a time-dependent increase first of cyclin D1 protein (maximum value within 8–24 h and cyclin E protein (maximum value within 8–38 h) and thereafter of cyclin A protein (maximum value within 14–30 h) (Figure 5). The effect of cyclic GMP- and cyclic AMP-dependent vasodilators on the FCS-induced expression of

cyclin D1, E and A protein was determined after 22 h of treatment, a time point at which a significant and consistent expression of the three cyclins was observed. Both SNP and 8-Br-cyclic GMP significantly reduced the FCS-stimulated expression of cyclin D1 protein by about 70 and 42%, and cyclic A protein by about 42 and 36%, respectively, whereas the expression of cyclin E protein was only slightly, though significantly attenuated by SNP, but not by 8-Br-cyclic GMP (Figure 6). The cyclic AMP-dependent vasodilators forskolin and 8-Br-cyclic AMP significantly decreased the FCS-induced expression of cyclin D1 protein by about 82 and 77%, and cyclin A protein by about 80 and 79%, respectively (Figure 6). In contrast, the cyclic AMP-dependent vasodilators exerted only minimal effects on the FCS-induced cyclin E expression (Figure 6). The FCS-stimulated cyclin A protein expression was inhibited to a significantly greater extent by the cyclic AMP-dependent vasodilators than by the cyclic GMP-dependent vasodilators.

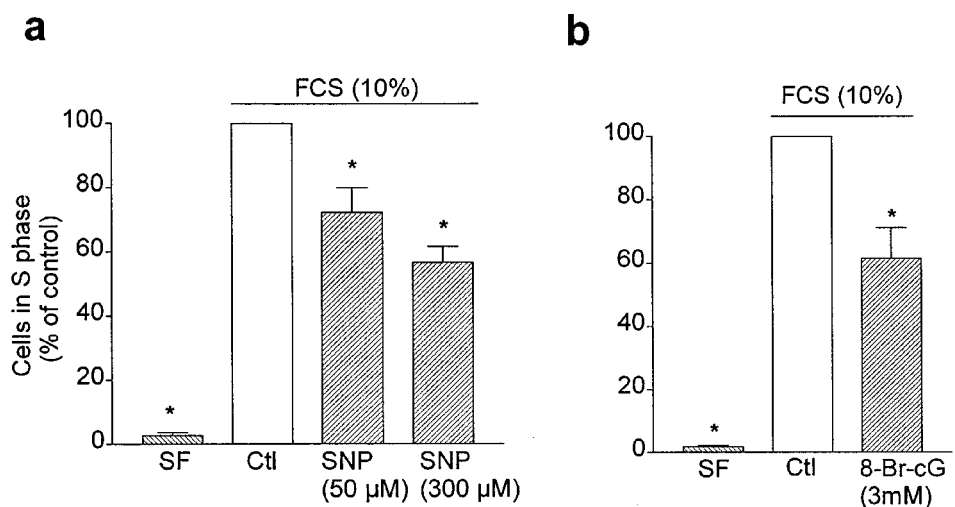


Figure 3 Effect of (a) SNP and (b) 8-Br-cyclic GMP (8-Br-cG) on the FCS-stimulated S phase entry of cultured rat aortic smooth muscle cells. Following growth arrest VSMC were stimulated to enter the cell cycle by the addition of FCS (10%) for 18 h. VSMC were exposed to a cyclic GMP-dependent vasodilator 5 min prior to FCS. Data shown as mean $\pm$ s.e. mean of six experiments for SNP and three experiments for 8-Br-cyclic GMP. (* $P < 0.05$ vs Ctl). Serum-free: SF; Control: Ctl.

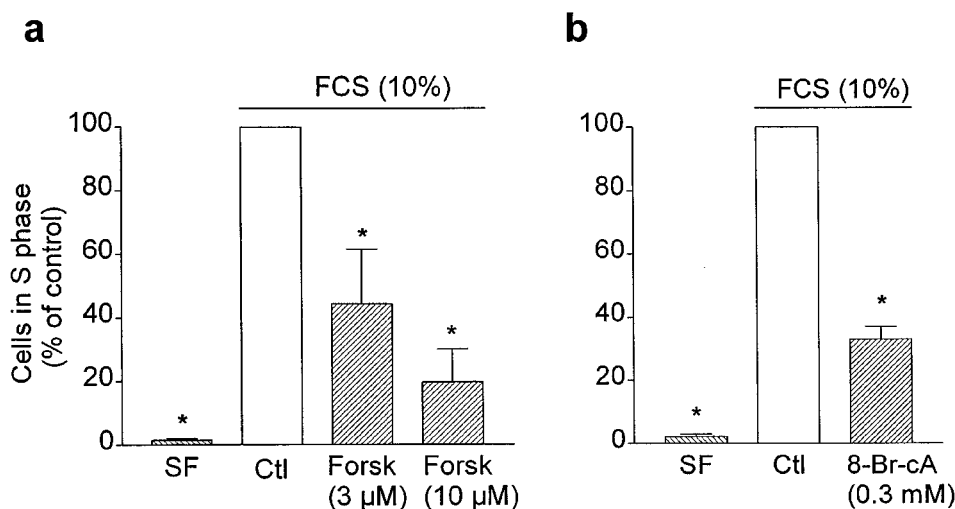


Figure 4 Effect of (a) forskolin (Forsk) and (b) 8-Br-cyclic AMP (8-Br-cA) on the FCS-stimulated S phase entry of cultured rat aortic smooth muscle cells. Following growth arrest VSMC were stimulated to enter the cell cycle by the addition of FCS (10%) for 18 h. VSMC were exposed to a cyclic AMP-dependent vasodilator 5 min prior to FCS. Data shown as mean $\pm$ s.e. mean of three experiments for forskolin and five experiments for 8-Br-cyclic AMP. (* $P < 0.05$ vs Ctl). Serum-free: SF; Control: Ctl.

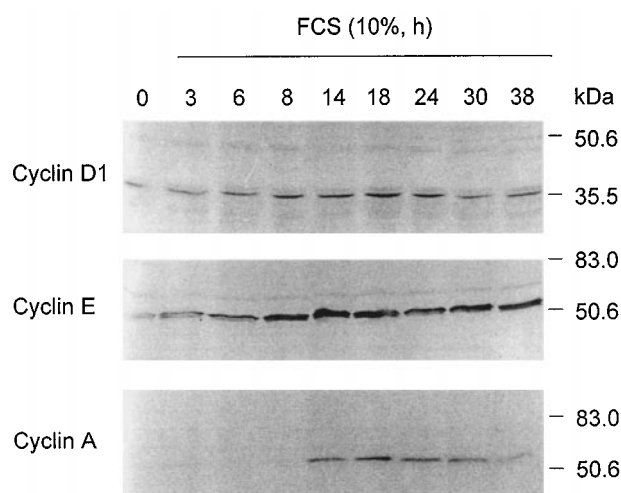


Figure 5 Representative Western blots showing the time course of cyclin D1, E, and A protein expression induced by FCS (10%) in cultured rat aortic smooth muscle cells. Equal amounts of protein from nuclear extracts were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with polyclonal antibodies against G1 phase cyclin D1 (top), and cyclin E (center) and S phase cyclin A (bottom). Similar results were obtained in two additional experiments.

Effect of cyclic GMP- and cyclic AMP-dependent vasodilators on the mRNA expression of cyclins

Low levels of cyclin D1 and cyclin A mRNA were detectable in FCS-deprived VSMC (Figure 7). Exposure of VSMC to FCS (10%) increased the expression of cyclin D1 mRNA after a lag-phase of about 2 h, and thereafter the cyclin D1 mRNA levels remained elevated for the next 28 h (Figure 7a). Cyclin A mRNA increased gradually after a delay of about 12 h to reach a maximum level at about 26 h (Figure 7b). Both SNP and 8-Br-cyclic GMP significantly reduced the FCS-stimulated expression of cyclin D1 mRNA by about 54 and 57% (Figure 8a) and cyclin A mRNA by about 44 and 74% (Figure 8b) as assessed after 16 h. Forskolin abolished the FCS-induced expression of cyclin D1 and A mRNA (Figure 8a and b) and 8-Br-cyclic AMP abolished the FCS-induced expression of cyclin D1 mRNA, whereas cyclin A mRNA was inhibited by about 75%, as assessed after 16 h.

Discussion

The present findings demonstrate that SNP and forskolin inhibit the proliferation of VSMC by preventing the progression of the cell cycle from the G0/G1 phase into the S phase. This cell cycle arrest seems to be due to an impaired expression of the G1 phase cyclin D1 and early S phase cyclin A via cyclic GMP- and cyclic AMP-dependent mechanisms.

Previous studies have shown that both NO-dependent (SNP) (Garg & Hassid, 1989; Kariya *et al.*, 1989; Sarkar *et al.*, 1997), and NO-independent (the indazole derivative YC-1) (Yu *et al.*, 1995) activators of soluble guanylyl cyclase, as well as activators of the particulate enzyme (atrial natriuretic peptide) (Abell *et al.*, 1989) inhibited the serum-stimulated [³H]-thymidine incorporation when added up to 12 h after serum stimulation of quiescent VSMC. Such an inhibitory effect of cyclic GMP-dependent vasodilators suggests that VSMC were growth-arrested in the G0/G1 phase of the cell cycle. In the present study, flow cytometric analysis provided

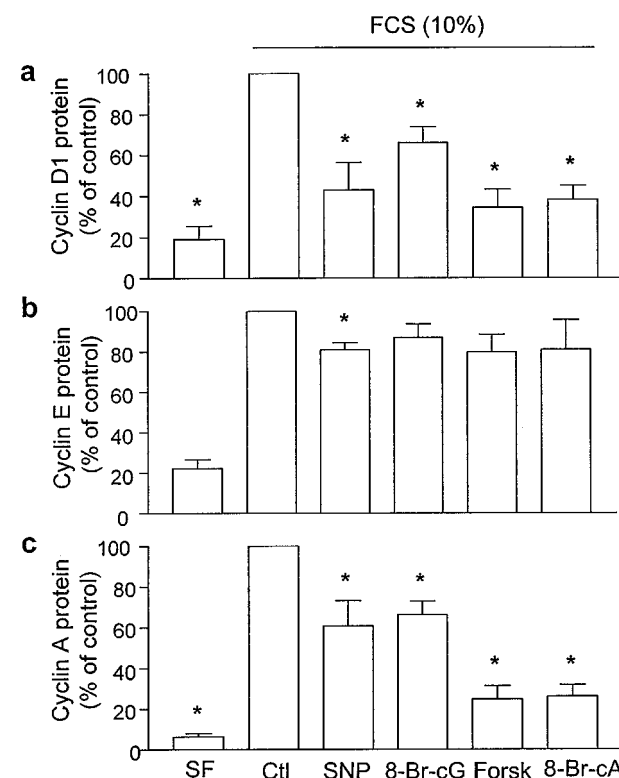


Figure 6 Cumulative data from Western blots showing the effect of cyclic GMP- and cyclic AMP-dependent vasodilators on the FCS-induced (a) cyclin D1, (b) cyclin E and (c) cyclin A protein expression in cultured rat aortic smooth muscle cells. VSMC were exposed to FCS (10%) for 22 h. The vasodilators were added 5 min prior to FCS. Results shown as mean $\pm$ s.e. mean of at least four experiments. (* $P < 0.05$ vs Ctl). Serum-free: SF; control: Ctl; SNP: 300 μ M; 8-Br-cyclic GMP: 8-Br-cG, 3 mM; forskolin: Forsk, 10 μ M; 8-Br-cyclic AMP: 8-Br-cA, 0.3 mM.

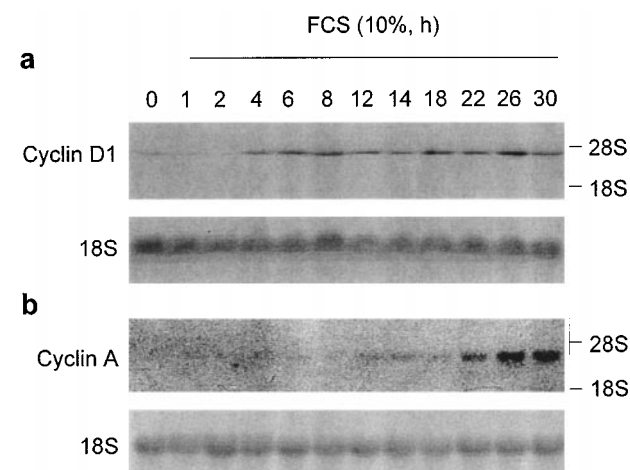


Figure 7 Representative Northern blots showing the time course of cyclin D1 and cyclin A mRNA expression induced by FCS (10%) in cultured rat aortic smooth muscle cells. (a) cyclin D1 cDNA probe (top), 18S ribosomal RNA (bottom), (b) cyclin A cDNA probe (top), 18S ribosomal RNA (bottom). Similar results were obtained in two additional experiments for cyclin D1 and in an additional experiment for cyclin A.

further evidence for this assumption. Since the inhibitory effect of the cyclic GMP-dependent vasodilators was mimicked by 8-Br-cyclic GMP, the membrane-permeable, stable analogue of cyclic GMP, it is likely, that the cyclic GMP effector pathway

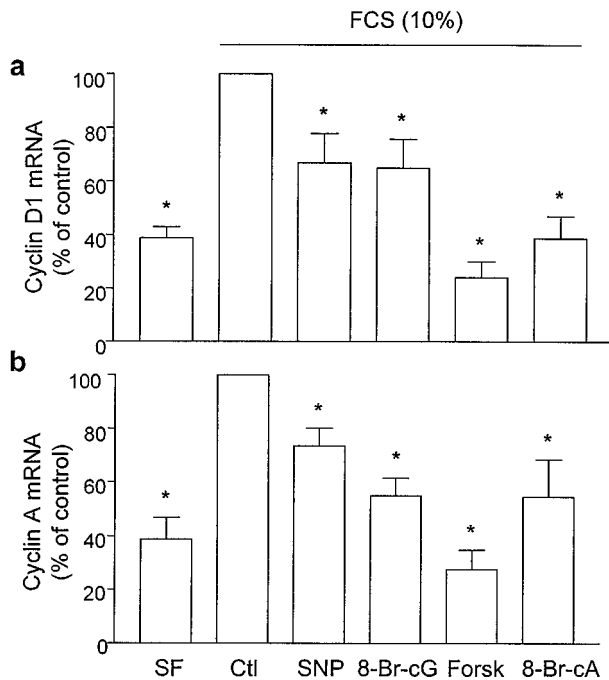


Figure 8 Cumulative data from Northern blots showing the effect of cyclic GMP- and cyclic AMP-dependent vasodilators on the FCS-induced (a) cyclin D1 and (b) cyclin A mRNA expression in cultured rat aortic smooth muscle cells. VSMC were exposed to FCS (10%) for 16 h. The vasodilators were added 5 min prior to FCS. Results shown as mean $\pm$ s.e. mean of at least four experiments. (* $P < 0.05$ vs Ctl). Serum-free: SF; control: Ctl; SNP: 300 μ M; 8-Br-cyclic GMP: 8-Br-cG, 3 mM; forskolin: Forsk, 10 μ M; 8-Br-cyclic AMP: 8-Br-cA, 0.3 mM.

is involved. NO donors have also been reported to inhibit DNA synthesis in VSMC synchronized in the S phase, an effect which was not mimicked by 8-Br-cyclic GMP (Sarkar *et al.*, 1997). Moreover, NO produced following induction of NOS II blocked macrophage-like cells in the early G2/M phase of the cell cycle (Takagi *et al.*, 1994). Since experiments have not been performed with VSMC synchronized in the S phase, an inhibitory effect of the cyclic GMP- and cyclic AMP-dependent vasodilators on the progression of VSMC through the S and G2/M phases cannot be ruled out in the present study.

Thus, NO donors may arrest the cell cycle *via* at least two different mechanisms, a cyclic GMP-dependent arrest of VSMC up to the late G1, and a cyclic GMP-independent blockage of cells in the S phase. Such a cyclic GMP-independent blockade in the S phase may be due to an inhibition of ribonucleotide reductase or thymidine kinase (Garg & Hassid, 1993; Kwon *et al.*, 1991).

In the present study, treatment of VSMC with SNP or 8-Br-cyclic GMP, in addition to preventing S phase entry, also impaired the expression of both G1 cyclin D1 and early S phase cyclin A mRNA and protein, whereas the expression of cyclin E was only minimally affected. Although a tight interregulation of the expression of cyclins has been demonstrated (Pines, 1995), the lack of effect on cyclin E expression might indicate that even low cyclin D1 levels may be sufficient to activate pathways leading to maximal cyclin E expression. The present findings are in agreement with those of a recent study reporting an impaired serum-stimulated expression of cyclin A but not of cyclin E by SNP and SNAP in rat aortic VSMC (Guo *et al.*, 1998). In addition, in human umbilical artery VSMC, SNAP reduced the serum-stimulated expression

of cyclin E and A mRNA, but not cyclin D1 mRNA, whereas no effect was found at the protein level (Ishida *et al.*, 1997). The heterogeneous effect of NO donors on the cyclin D1, E and A expression may be due to the use of different VSMC (i.e., species, arteries), but possibly also to the different ability of SNAP and SNP to release NO. Indeed, the antiproliferative effect of NO donors is critically dependent on the duration of NO release (Mooradian *et al.*, 1995). Furthermore, experimental conditions associated with the continuous release of small amounts of NO (i.e. gene transfer of NOS III, cytokine-induced expression of NOS II, cocultures of endothelial cell and VSMC) strongly inhibited proliferation (Scott-Burden *et al.*, 1992; Scott-Burden & Vanhoutte, 1993; Kullo *et al.*, 1997), whereas the release of NO by a large bolus of NO donors during a short time-period was much less effective (Mooradian *et al.*, 1995). In the present study a small but consistent inhibition of proliferation was observed by daily addition of SNP to VSMC, an effect that might have been further increased by a more frequent addition of the NO-donor. Beside the reduction of GP- and early S phase cyclin expression, the antiproliferative effect of NO donors may also involve an inhibition of cyclin-dependent kinase 2 or an enhanced expression of the cyclin-dependent kinase inhibitor p21 (Ishida *et al.*, 1997).

In the current study, forskolin and 8-Br-cyclic AMP, analogous to the cyclic GMP-dependent vasodilators, inhibited the progression of VSMC from the G0/G1 phase into the S phase of the cell cycle, and reduced the expression of both cyclin D1 and A mRNA and protein, whereas the expression of cyclin E was only slightly inhibited. Consistent with these findings, cyclin D1 and A expression has been reported to be under negative control of cyclic AMP in different cell types, including fibroblasts (Desdouets *et al.*, 1995; Barlat *et al.*, 1995; L'Allemain *et al.*, 1997), macrophages (Cocks *et al.*, 1992) and an astrocytic cell line (Gagelin *et al.*, 1994). The cyclic AMP-dependent vasodilators inhibited the FCS-stimulated expression of cyclin A to a greater extent than the cyclic GMP-dependent vasodilators. Thus, it is not unlikely that cyclic GMP acts *via* cross-activation of the cyclic AMP effector pathway. Such an assumption is supported by the findings that elevations of cyclic GMP levels in VSMC are associated with an activation of protein kinase A and that selective inhibitors of protein kinase A prevented the NO-mediated growth inhibition (Cornwell *et al.*, 1994). Moreover, cyclic GMP has been shown to inhibit the degradation of cyclic AMP by phosphodiesterase III (Beavo & Reifsnnyder, 1990).

Inhibitory effects of the cyclic GMP- and cyclic AMP-dependent vasodilators on the expression of cyclin D1 and A mRNA could reflect a repressed transcription of the cyclin D1 and A genes, respectively, and/or an increased degradation of the mRNA. Transcriptional regulation of the cyclin D1 and A genes could be mediated by the cyclic AMP responsive element, of which the consensus sequence has been identified in the promoters of these genes (Desdouets *et al.*, 1995; Barlat *et al.*, 1995; Herber *et al.*, 1994). In contrast to the cyclin D1 and A genes, no cyclic AMP responsive element has been found in the promoter region of the cyclin E gene (Koff *et al.*, 1991), a fact that could help explain the lack of effect of the cyclic GMP- and cyclic AMP-dependent vasodilators on cyclin E expression. Mutational analysis studies using promoter constructs of the cyclin A gene have shown that the cyclic AMP responsive element is required for the antiproliferative effect of cyclic AMP-elevating agents in fibroblasts (Barlat *et al.*, 1995).

An impaired bioavailability of endothelium-derived NO seems to be one of the early events during the development of

atherosclerosis (Busse & Fleming, 1996). The nature of this endothelial dysfunction is largely unknown, although possible mechanisms include decreased substrate availability, decreased expression of NOS III, imbalance between endothelium-derived relaxing and constricting factors, production of an endogenous NOS inhibitor and overproduction of oxygen free radicals. In human atherosclerotic plaques and also in restenotic lesions of rat aortae after balloon injury, a marked induction of cell cycle regulatory proteins, such as cyclin A, cyclin E, and their associated kinase, cyclin-dependent kinase 2, and also proliferating cell nuclear antigen was found, predominantly in areas rich in VSMC (Wei *et al.*, 1997). These previous findings in conjunction with the present data, support

the concept that removal of the inhibitory feedback of NO on the expression of G1 and early S phase cyclins might contribute to the excessive VSMC proliferation leading to intimal thickening at sites of vascular injury.

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Formal analysis of electrogenic sodium, potassium, chloride and bicarbonate transport in mouse colon epithelium

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1 The mammalian colonic epithelium carries out a number of different transporting activities simultaneously, of which more than one is increased following activation with a single agonist. These separate activities can be quantified by solving a set of equations describing these activities, provided some of the dependent variables can be eliminated. Using variations in the experimental conditions, blocking drugs and comparing wild type tissues with those from transgenic animals this has been achieved for electrogenic ion transporting activity of the mouse colon.

2 Basal activity and that following activation with forskolin was measured by short circuit current in isolated mouse colonic epithelia from normal and cystic fibrosis (CF) mice.

3 Using amiloride it is shown that CF colons show increased electrogenic sodium absorption compared to wild type tissues. CF mice had elevated plasma aldosterone, which may be responsible for part or all of the increased sodium absorption in CF colons.

4 The derived values for electrogenic chloride secretion and for electrogenic potassium secretion were increased by 13 and 3 fold respectively by forskolin, compared to basal state values for these processes.

5 The loop diuretic, frusemide, completely inhibited electrogenic potassium secretion, but apparently only partially inhibited electrogenic chloride secretion. However, use of bicarbonate-free solutions and acetazolamide reduced the frusemide-resistant current, suggesting that electrogenic bicarbonate secretion accounts for the frusemide-resistant current.

6 It is argued that the use of tissues from transgenic animals is an important adjunct to pharmacological analysis, especially where effects in tissues result in the activation of more than one sort of response.

Keywords: Electrogenic epithelial transport; potassium secretion; sodium absorption; chloride secretion; bicarbonate secretion; cystic fibrosis; CFTR; forskolin; loop diuretics

Abbreviations: CF SCC, short circuit current in CF; CF SCC_{amiloride}, SCC remaining after amiloride in CF; CF SCC_{forskolin}, SCC remaining after amiloride and forskolin in CF; CF SCC_{forskolin/frusemide}, SCC remaining after amiloride, forskolin then frusemide in CF; CF SCC_{frusemide}, SCC remaining after amiloride and frusemide in CF; KHS, Krebs Henseleit Solution; SCC, short circuit current; SCC_{amiloride}, SCC remaining after amiloride; SCC_{forskolin}, SCC remaining after amiloride and forskolin; SCC_{forskolin/frusemide}, SCC remaining after amiloride, forskolin then frusemide; SCC_{frusemide}, SCC remaining after amiloride and frusemide

Introduction

The epithelium lining the alimentary canal is responsible for a large number of processes designed to move ions, solutes and water into or out of the gut lumen. Of great significance in these transporting activities is the asymmetric nature of the epithelial cells, vectorial transport depending on the differential distribution of channels, pumps and carriers between the apical and basolateral membranes. In this study transporting activities in the mouse distal colon are investigated, but only those which are electrogenic. In particular, stimulation of electrogenic transport by cyclic AMP will be the focus of the study, transporting activity being measured by the short circuit current (SCC) technique. The cyclic nucleotide has a number of known targets in epithelia, as follows; apically located chloride channels (Li *et al.*, 1988), namely the cystic fibrosis transmembrane conductance regulator (CFTR), basolaterally located NaK2Cl cotransporter (Haas *et al.*, 1993) and basolaterally located cyclic AMP sensitive K⁺-channels, namely the KvLQT1 K⁺-channel sensitive to the chromanol, 293B (Lohrmann *et al.*, 1995; Loussouarn *et al.*, 1997). Thus the actions at the two membranes work in concert to promote

anion secretion, chloride concentration increases within the cell to a level above its electrochemical equilibrium and exits through CFTR channels in the apical membrane, anion secretion being maintained by apical membrane hyperpolarization caused by K⁺-channel activation. Ca²⁺-raising secretagogues, by contrast, produce only a transient anion secretory response dependent on activation of basolateral Ca²⁺-sensitive K⁺-channels without increasing the probability of CFTR channel opening (Dharmasathaphorn & Pandol, 1986; Cuthbert *et al.*, 1994). Cyclic AMP not only increases anion secretion but K⁺ ions entering *via* the cotransporter may leave *via* the apical membrane, as well as re-equilibrate across the basolateral membrane. Thus inhibition of the cotransporter with loop diuretics will block both chloride and potassium secretion. However, loop diuretics rarely cause complete inhibition of the responses to forskolin in chloride secreting epithelia, a point noted by many, but commented on by few (MacVinish *et al.*, 1998). Finally, in the colon, Na⁺ can enter through the apical membrane and exit the basolateral membrane using basolateral Na-K-ATPase. Here we have used pharmacological methods as the basis for producing a formal analysis of basal and cyclic AMP stimulated electrogenic transporting activity in the mouse distal colon.

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Methods

All experiments were performed on the distal colonic mucosa of mice. Animals were killed by CO₂ narcosis and the terminal colon removed into Krebs Henseleit Solution (KHS). The colon was opened immediately, cleaned and the muscle layers dissected away under microscopic control. The most terminal part of the colon was mounted in an Ussing chamber, window area 20 mm², and the tissue short circuited with series resistance compensation, exactly as described recently, elsewhere (Teather & Cuthbert, 1997). Only one preparation was taken from each mouse, so that any variation in the responses can not be due to variation in the part of the colon from which the preparations were derived. Two types of mice were used as follows; wild type mice which were littermates of cystic fibrosis (CF) mice, either *Cftr^{tm1Cam}* (Ratcliff *et al.*, 1993) or *Cftr^{tm2Cam}* (Colledge *et al.*, 1995). Previous studies have not revealed any differences between colonic epithelia of the CF null mice (*Cftr^{tm1Cam}*) and those with the $\Delta F508$ mutation (*Cftr^{tm2Cam}*) (Colledge *et al.*, 1995). Similarly, the wild type mice were a mixture of homo- and heterozygotes, and again no differences in the behaviour of the epithelium of the colon has been demonstrated (Ratcliff *et al.*, 1993; Cuthbert *et al.*, 1995). All litters were initially fed on a low fibre diet to avoid meconium ileus and the wild type transferred to normal chow once genotyping was completed. Animals were used as soon as possible after genotyping.

Three experimental paradigms were used as follows.

- Protocol 1.** After the SCC had stabilized, amiloride (100 μ M) was applied to the apical surface to block all electrogenic sodium absorption. Ten minutes later forskolin (10 μ M) was applied in both the apical and basolateral bathing solutions and finally, after a further 10 min, frusemide (1 mM) was added to the basolateral bathing fluid and the SCC recorded for a further period of 10 min. The following parameters were measured from all SCC records and tabulated before analysis; basal SCC, Δ SCC caused by amiloride, Δ SCC caused by forskolin, Δ SCC caused by frusemide and SCC after frusemide.
- Protocol 2.** The design was identical to Protocol 1 except that addition of forskolin was omitted. Protocols 1 and 2 were applied to both wild type and CF colonic epithelia.
- Protocol 3.** This experimental paradigm was applied only to wild type colonic epithelia, and was identical to Protocol 1 except the experiments were carried out in bicarbonate-free solution.

Krebs Henseleit Solution (KHS) was used in Protocols 1 and 2 and had the following composition (in mM); NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25 and glucose 11.1. This solution had a pH of 7.4 when bubbled with 95%O₂/5%CO₂ at 37°C. Bicarbonate-free solution had the same composition, except that NaCl was increased to 137 mM and NaHCO₃ was omitted. This solution was buffered with HEPES buffer, 10 mM, pH 7.4 and the solution bubbled with pure O₂. An unpaired Student's *t*-test was used to make comparisons between sets of data, a *P* value less than 0.05 was considered significant.

Plasma aldosterone concentration was measured for both CF and wild type mice by radioimmunoassay, after extraction from plasma with dichloromethane. Blood was collected from the thoracic cavity after severing major veins into heparinized syringes. The samples were immediately centrifuged to remove cells and the plasma stored at -20°C. Samples from eight wild type and eight CF animals were collected. On average 150 μ l of

plasma was obtained from each mouse, which was insufficient volume for the assay. Consequently the samples from wild type and CF mice were separately pooled. Sufficient plasma was obtained to allow three separate estimations in CF samples and five separate estimations in wild type samples of aldosterone, each measurement being made in triplicate.

Results

Typical SCC records from colonic epithelia subject to the regimens of Protocols 1 and 2 are illustrated in Figure 1. The differences between wild type and CF epithelia are particularly evident with the Protocol 1 procedure, where the SCC increases after forskolin in the former, yet falls after the same treatment in CF tissues. With Protocol 2 the SCC changes due to frusemide are rather small when applied without prior addition of forskolin. By pooling the data from all the Protocol 1 studies, a number of significant differences were revealed, as illustrated in Figure 2. Highly significant differences were seen between the responses of wild type and CF epithelia in response to both forskolin and frusemide. Also, the responses to amiloride were significantly greater in CF colons, even though the basal SCC was larger in wild type colons.

For the formal analysis of the responses to Protocols 1 and 2 it is more convenient to deal with the actual SCC values, rather than changes in SCC. The data of Figure 2 were replotted as SCC in Figure 3 where the responses to amiloride, forskolin and frusemide are shown as step functions. Peak responses were used to construct Figure 3 and no allowance was made for any decline of the response during the 10 min of exposure to the drugs. The final SCC value remaining after frusemide is therefore a consequence of additions and subtractions of peak responses taking the original basal current as the starting point. Therefore the actual SCC remaining after frusemide is not exactly that found by calculation. The predicted value for the residual SCC after exposure to forskolin and then frusemide in CF colons ($\text{CF SCC}_{\text{forskolin/frusemide}}$) was $17.5 \pm 4.0 \mu\text{A cm}^{-2}$ whereas the actual value was $18.8 \pm 2.3 \mu\text{A cm}^{-2}$. Similarly the predicted value in wild type colons ($\text{SCC}_{\text{forskolin/frusemide}}$) was $75.6 \pm 19.0 \mu\text{A cm}^{-2}$ while the actual value was $61.0 \pm 6.7 \mu\text{A cm}^{-2}$.

It is notable that frusemide failed to reverse completely the SCC increase caused by forskolin in wild type colons (Figure 3a) but completely reversed the forskolin effect in the CF colon (Figure 3b). Using Protocol 2, i.e. no addition of forskolin, frusemide had little effect on SCC in unstimulated colons (Figure 4a). Addition of acetazolamide after frusemide in wild type colons increased the inhibition of the forskolin response from 68 to 75% (Table 1). The data shows that the response to frusemide increases to $168.4 \pm 9.9 \mu\text{A cm}^{-2}$ from $108.1 \pm 14.1 \mu\text{A cm}^{-2}$ when bicarbonate-free solution is used. However when wild type colons were mounted in HCO₃⁻-free KHS the responses to forskolin were greater, but not significantly so, than those in KHS. Therefore, derived values were calculated to allow for the differences in the response to forskolin and analysis showed that inhibition by frusemide was significantly increased by bicarbonate removal. Inhibition of forskolin responses in bicarbonate-free solution were increased from 87% after frusemide to 91% by addition of acetazolamide (Figure 4b). Examples of responses in the two conditions are given in Figure 5.

Plasma aldosterone measurements gave values of $224 \pm 19 \text{ pmol l}^{-1}$ (five measurements) in wild type and $1086 \pm 23 \text{ pmol l}^{-1}$ in CF (three measurements) each made in triplicate.

Theory

Assuming that SCC represents the electrogenic transport of chloride, potassium, sodium and other unknown ions the following formalism can be developed; $SCC_{amiloride} = x + y + z$, where $SCC_{amiloride}$ is the SCC after amiloride, x is basal electrogenic chloride secretion, y is basal electrogenic potassium secretion and z is electrogenic transport of unknown species. In CF epithelia, chloride secretion can not occur because of the absence of CFTR and the relevant equation for

SCC after amiloride is $CF\ SCC_{amiloride} = y + z$. Forskolin stimulates chloride and potassium secretion, and assuming z is unaffected the relevant equations are $SCC_{forskolin} = x + \Delta x + y + \Delta y + z$ and $CF\ SCC_{forskolin} = y + \Delta y + z$. In Protocols 1 and 2, and with either wild type or CF epithelia the SCC remaining at the end of the experiments, after frusemide had been added, is z , whether or not frusemide was added in the absence of forskolin ($SCC_{frusemide}$ and $CF\ SCC_{frusemide}$) or after forskolin ($SCC_{forskolin/frusemide}$ and $CF\ SCC_{forskolin/frusemide}$). It follows therefore that:

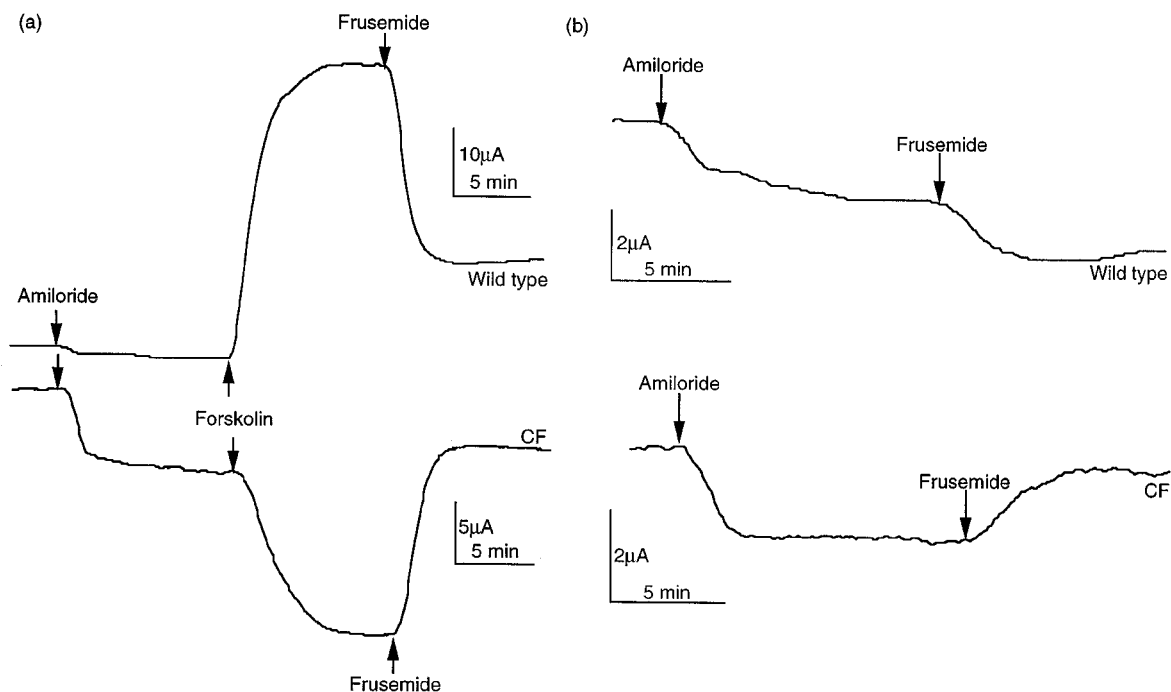


Figure 1 The effects of sequential administration of amiloride (100 μM , apical) and frusemide (1 mM, basolateral), with (a) or without (b) forskolin (10 μM , both sides) given between, in wild type and CF colonic epithelia. Specimen SCC traces for epithelia of 20 mm² are shown. Note when forskolin is not added (b) the changes caused by frusemide are small. In this situation frusemide increased or decreased the SCC in wild type resulting in only a small mean change, whereas in CF epithelia an overall significant increase in current was found (see Figure 4a).

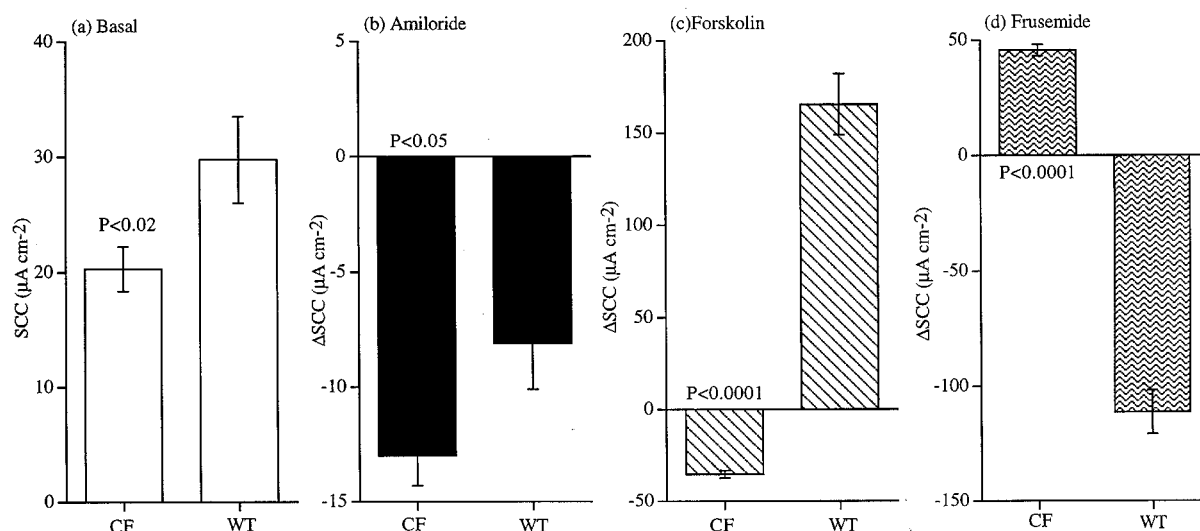


Figure 2 Cumulative data from 22 wild type colonic epithelia and 42 CF epithelia. Shown are the values (means $\pm$ s.e.mean) for the basal SCC (a) and changes in SCC caused by the sequential addition of amiloride (100 μM , apical) (b), forskolin (10 μM , both sides) (c) and frusemide (1 mM, basolateral) (d), all as $\mu A\ cm^{-2}$. P values are shown and were obtained with Student's t -test, comparing wild type with CF preparations.

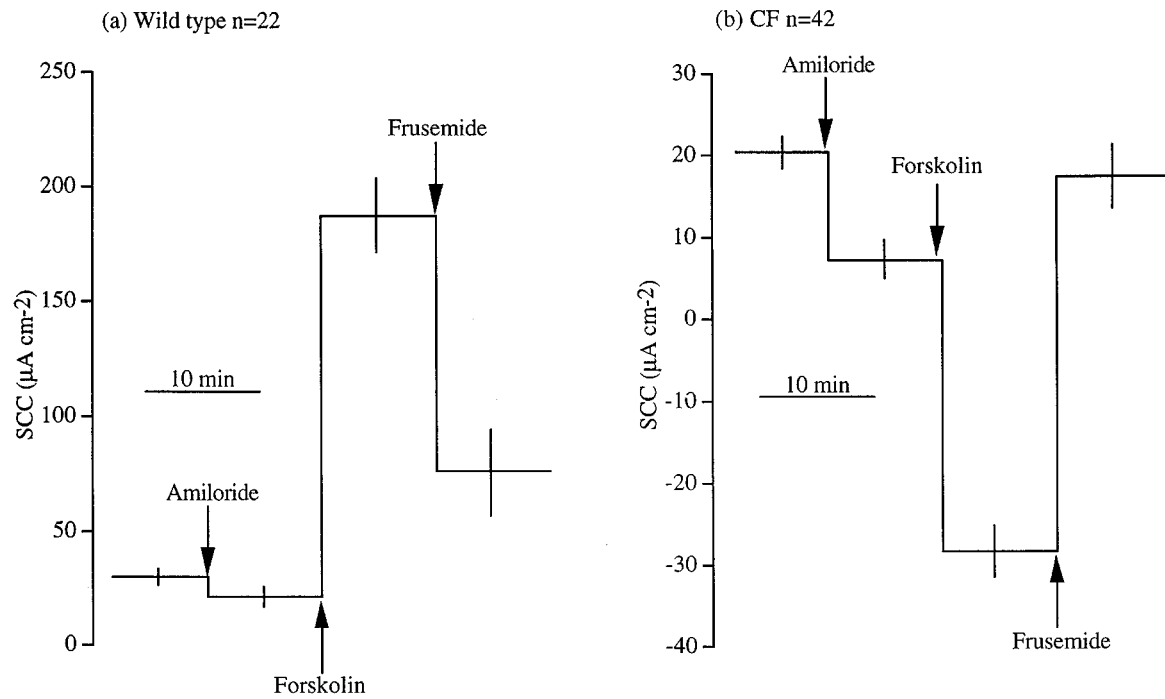


Figure 3 The total data from Figure 2 presented as actual SCC values. The mean values of the currents and their standard errors are given. Peak values have been used and no allowance made for any decline in the response during 10 min before the next drug was added.

Table 1 Effect of frusemide and acetazolamide on forskolin responses in normal and bicarbonate free conditions

All responses as $\mu\text{A cm}^{-2}$							
Conditions	$\text{SCC}_{\text{amiloride}}$	$\Delta\text{Forskolin}$	$\Delta\text{Frusemide}$	% residual SCC	$\Delta\text{Frusemide and acetazolamide}$	% residual SCC	n
KHS	23.3 ± 4.7	159.2 ± 27.8	108.1 ± 14.1 (131.9 ± 17.2 , $P < 0.04^*$)	32.1%	120.2 ± 15.5	24.5%	12
<i>P</i> values	N.S.	N.S.	< 0.002		< 0.007		
HCO_3^- -free	21.7 ± 5.5	194.4 ± 11.3	168.4 ± 9.9 (137.9 ± 8.1 , $P < 0.03$)	13.4%	176.3 ± 10.7	9.3%	12
KHS							

% residual is the percentage current remaining compared to $\Delta\text{Forskolin}$. n =number of experiments. *one tailed t -test. Values in parentheses are derived by multiplying the experimental values by a factor to allow for the difference in forskolin responses in the two groups. The value for frusemide response in the HCO_3^- -free solution ($168.4 \pm 9.9 \mu\text{A cm}^{-2}$) is significantly greater than the derived value in KHS. Similarly the response in KHS ($108.1 \pm 14.1 \mu\text{A cm}^{-2}$) is significantly smaller than the derived value in HCO_3^- -free solution. Thus it can be concluded that the effects of frusemide are greater in HCO_3^- -free solution.

$$\begin{aligned}
 (\text{SCC}_{\text{forskolin}} - \text{CF SCC}_{\text{forskolin}}) - \\
 (\text{SCC}_{\text{amiloride}} - \text{CF SCC}_{\text{amiloride}}) &= \Delta x \\
 (\text{CF SCC}_{\text{forskolin}} - \text{CF SCC}_{\text{amiloride}}) &= \Delta y \\
 (\text{SCC}_{\text{amiloride}} - \text{CF SCC}_{\text{amiloride}}) &= x \\
 (\text{CF SCC}_{\text{amiloride}} - \text{CF SCC}_{\text{frusemide}}) &= y
 \end{aligned}
 \tag{1}$$

$$\begin{aligned}
 \text{SCC}_{\text{frusemide}} &= \text{CF SCC}_{\text{frusemide}} = \\
 \text{SCC}_{\text{forskolin/frusemide}} &= \text{CF SCC}_{\text{forskolin/frusemide}} = z
 \end{aligned}
 \tag{5}$$

The values, taken from Figures 3 and 4a were:

$$\begin{aligned}
 \text{SCC}_{\text{amiloride}} &= 21.7 \pm 4.3 \mu\text{A cm}^{-2} \\
 \text{CF SCC}_{\text{amiloride}} &= 7.3 \pm 2.3 \mu\text{A cm}^{-2} \\
 \text{SCC}_{\text{forskolin}} &= 186.9 \pm 16.5 \mu\text{A cm}^{-2} \\
 \text{CF SCC}_{\text{forskolin}} &= -28.3 \pm 3.1 \mu\text{A cm}^{-2} \\
 \text{SCC}_{\text{frusemide}} &= 12.7 \pm 8.3 \mu\text{A cm}^{-2} \\
 \text{CF SCC}_{\text{frusemide}} &= 16.1 \pm 3.6 \mu\text{A cm}^{-2} \\
 \text{SCC}_{\text{forskolin/frusemide}} &= 75.6 \pm 19.0 \mu\text{A cm}^{-2} \\
 \text{CF SCC}_{\text{forskolin/frusemide}} &= 17.5 \pm 4.0 \mu\text{A cm}^{-2}
 \end{aligned}$$

This data gives values of the various transport parameters as follows:

$$\begin{aligned}
 \Delta x &= 200.9 \pm 17.5 \mu\text{A cm}^{-2}, \text{ from (1)} \\
 \Delta y &= -35.6 \pm 5.0 \mu\text{A cm}^{-2}, \text{ from (2)} \\
 x &= 14.4 \pm 4.9 \mu\text{A cm}^{-2}, \text{ from (3)} \\
 y &= -8.8 \pm 4.3 \mu\text{A cm}^{-2}, \text{ from (4)}
 \end{aligned}$$

Four estimates of z can be derived from (5), they are respectively 12.7 ± 8.3 , 16.1 ± 3.6 , 75.6 ± 10.0 and $17.5 \pm 4.0 \mu\text{A cm}^{-2}$. The first, second and fourth values are not significantly different from one another, while all three are significantly smaller than the third value ($P < 0.0001$). These derived values are close to the residual SCCs at the end of the experiments after frusemide had been applied, which were, respectively 11.4 ± 3.2 , 15.9 ± 3.1 , 61.0 ± 6.7 and $18.8 \pm 2.3 \mu\text{A cm}^{-2}$. Again the value for the residual current remaining after forskolin and frusemide in wild type colons is discrepant from the rest of the values for z . This point was commented upon in the Introduction, and is clarified by experiments using Protocol 3.

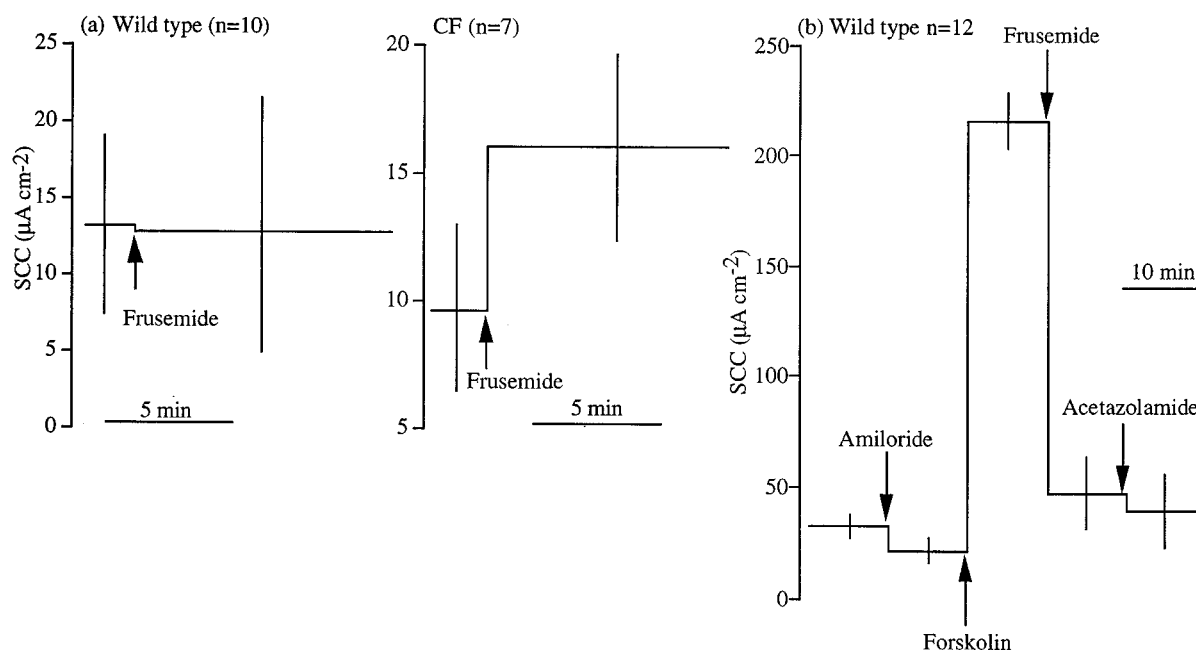


Figure 4 (a) Effects of frusemide (1 mM, basolateral) addition to wild type and CF colons, without addition of forskolin and in the presence of amiloride. (b) Effects on SCC by the sequential addition of amiloride (100 μM , apical), forskolin (10 μM , both sides) and frusemide (1 mM, basolateral) in 12 wild type colonic epithelia bathed in HCO_3^- -free medium. Means $\pm$ s.e.mean are given in both (a) and (b).

Discussion

The opportunity to collect a large body of data for CF colons arose from other studies on CF mice where the responses were used to check the genotyping from DNA tail clip analysis. Comparison with data for wild type colons showed that the amiloride sensitive SCC was significantly larger in the CF tissues. Aldosterone is known to increase electrogenic sodium transport in the mouse colon (Grubb & Boucher, 1997) and measurement of plasma aldosterone showed the level was greater in CF mice. Therefore the increase in sodium absorption in CF colons maybe due to the increase in hormone levels. In an earlier study (Cuthbert *et al.*, 1994) we failed to record any increase in amiloride sensitive sodium absorption in CF colons compared to wild type with numbers approaching those in this study. However in that instance two or three colonic preparations were taken from each mouse, whereas here only a single preparation was taken from each animal. As electrogenic sodium absorption occurs in only the most terminal part of the colon we have shown a difference in this study by using strictly comparable tissues. Amiloride sensitive sodium channels, (ENaC) are also upregulated by the lack of CFTR (Stutts *et al.*, 1997; Briel *et al.*, 1998). Whether part of the increase in CF colons is due to this effect can not be ascertained from this study, but it should be noted that the increase in sodium absorption in CF colons is modest compared to that found in airway epithelia (MacVinish *et al.*, 1997) which are apparently insensitive to aldosterone (Knowles *et al.*, 1985). In the airways sodium absorption and chloride secretory activities are expressed in most cells. As CFTR is produced only in the secretory crypts of the colon and sodium absorption occurs in the surface cells (Trezise *et al.*, 1997), it is probably only the cells near to the mouth of the crypts that can absorb sodium ions before all CFTR function is lost. Thus only a relatively small

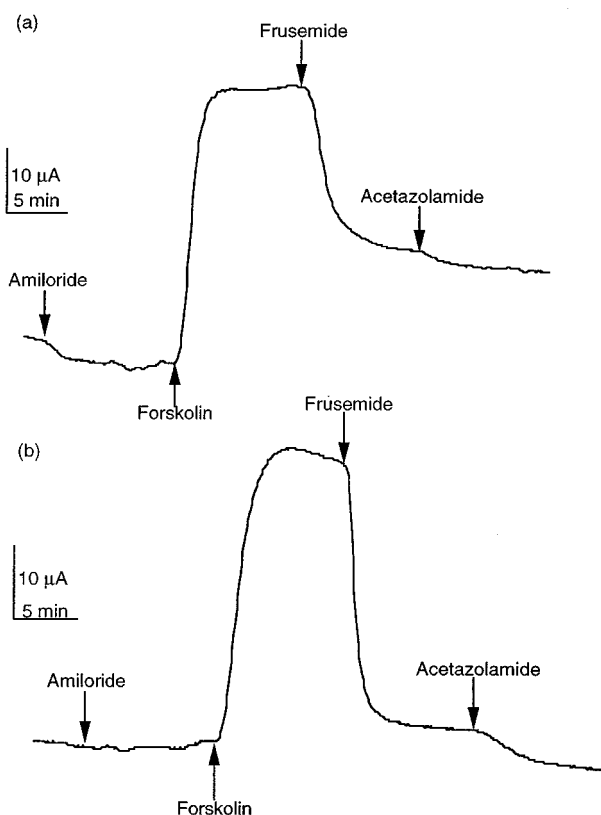


Figure 5 SCC records from two separate colonic epithelia, one bathed in KHS (a) and the other in HCO_3^- -free KHS (b). Each was subject to the sequential addition of amiloride (100 μM , apically), forskolin (10 μM , both sides), frusemide (1 mM, basolaterally) and acetazolamide (100 μM , both sides). Both tissues had an area of 20 mm^2 .

proportion of the total epithelium could be modified by loss of CFTR.

Throughout this investigation forskolin has been used to stimulate adenylate cyclase. The SCC increase in wild type colons caused by forskolin ($165.3 \pm 16.5 \mu\text{A cm}^{-2}$) underestimates the actual chloride secretory response by the SCC change caused by forskolin in CF colons ($35.6 \pm 2.1 \mu\text{A cm}^{-2}$). The values of basal chloride and potassium secretion are small, and of opposite polarity (14.4 ± 4.9 and $-8.8 \pm 4.3 \mu\text{A cm}^{-2}$ respectively), therefore frusemide added in the absence of prior forskolin addition is not expected to produce much effect. In wild type colons frusemide caused a SCC change of $-0.5 \pm 5.9 \mu\text{A cm}^{-2}$ (95% confidence limits of 12.8 to $-13.7 \mu\text{A cm}^{-2}$), while in CF colons, where chloride secretion does not occur, it is predictable that frusemide would cause a SCC increase. Experimentally the value was $6.5 \pm 1.7 \mu\text{A cm}^{-2}$ (95% confidence limits of 2.4–10.5 $\mu\text{A cm}^{-2}$). Forskolin increased the anion secretory current by 13 fold from the basal value, while potassium secretion was only increased 3 fold.

Three estimates of z gave values of 9–18 $\mu\text{A cm}^{-2}$, which were not significantly different from each other, while in wild type colons there was a large residual current after forskolin and frusemide. This large value of z is not predicted by the formal treatment given above and needs to be addressed. First, in wild type colons treated with both drugs the situation is competitive at the level of the cotransporter. Incomplete inhibition might explain the lack of complete reversal. However, a similar discrepancy would exist in CF colons but this was not apparent and the hypothesis is rejected. Secondly, other electrogenic transporting activities may be present in wild type colons, which are not inhibited by frusemide, but which are dependent on CFTR. It is known that the CFTR channel is permeable to Cl^- and HCO_3^- , but with a preference for Cl^- (Kunzelmann *et al.*, 1991; Poulsen *et al.*, 1994). However in the absence of Cl^- the colon can demonstrate electrogenic HCO_3^- secretion (Feldman *et al.*, 1988) and others have demonstrated bicarbonate secretion in the mouse intestine (Hogan *et al.*, 1997; Seidler *et al.*, 1997). Clearly then HCO_3^- secretion is not possible in CF colons because of the absence of CFTR, and neither do bicarbonate ions enter the cell on the cotransporter. However, when the cotransporter is inhibited with frusemide the ratio of intracellular $\text{Cl}^-/\text{HCO}_3^-$ will fall.

Throughout this study we have used supramaximally effective concentrations of drugs so that the maximal effect is achieved quickly and is easily discernible. For example, lower concentrations of frusemide produce the same maximal effect but take much longer to reach equilibrium. Acetazolamide only removed a small amount of the residual SCC after forskolin and frusemide in wild type colons, indicating that HCO_3^- transport is not dependent on the hydration of CO_2 , particularly as HCO_3^- can enter cells using a $\text{Cl}^-/\text{HCO}_3^-$ exchanger. Removal of HCO_3^- from the bathing fluid together with the addition of acetazolamide, after frusemide, was able to remove virtually all the current generated by forskolin (Table 1). The increase in the SCC response to forskolin in bicarbonate-free conditions, although this just failed to reach significance, may result from the removal of competition from

the less permeable HCO_3^- . This data can not be introduced into the formal analysis in a numerical way because it only refers to a sample of 12 experiments and the experimental conditions were different (HCO_3^- -free KHS), however it does seem as if a large part of the residual current in wild type colons after forskolin and frusemide is due to bicarbonate secretion. In unstimulated colons the residual current after frusemide is small compared to the residual value in stimulated colons from which it may be concluded that forskolin also stimulates bicarbonate transport. However this is not necessarily so. An alternative way to measure ion transport under SCC conditions is to measure the net flux of ions. When this is done the net flux of chloride is often larger than expected from integration of the SCC response (Cuthbert & Margolius, 1982; Cuthbert *et al.*, 1983). Here we have shown that the SCC response to forskolin underestimates the chloride secretory response due to the concomitant potassium secretory current. However, it would not be expected, if a large part of the anion secretory response was due to bicarbonate, that net flux would exceed the SCC. In this investigation it is shown that when bicarbonate is removed the chloride secretory response to forskolin is maintained, indicating that bicarbonate secretion is not an obligatory part of the response. It appears therefore that addition of frusemide to a forskolin stimulated colonic epithelium actually alters the experimental conditions to one which is essentially chloride free, as no chloride can enter the cell on the cotransporter, although entrance by other routes is possible. In this situation bicarbonate is free to leave the cell without competition from chloride *via* apical CFTR. The consequence is that it appears that frusemide is incapable of completely inhibiting chloride secretion when the bathing solution contains bicarbonate. Small residual currents, termed z , ($\sim 15 \mu\text{A cm}^{-2}$) are not accounted for in this study, but account for only 5–10% of the maximal current after forskolin stimulation. In summary, we have used a formal pharmacological analysis to quantify electrogenic transport of sodium, potassium, chloride and bicarbonate ions in the rat colon epithelium stimulated by forskolin.

In biological situations where a system shows more than one type of response to a stimulus then such systems can be analysed in a formal way if the equations describing complex actions can be solved by appealing to the data. To this end we have eliminated some part of the response using specific blocking protocols (amiloride, frusemide, bicarbonate-free), have compared the stimulated (forskolin) with the resting condition and have compared normal with transgenic tissues to analyse the transporting activity of the mouse colon. The increasing availability of transgenics in which some component of the response mechanism is 'knocked out' is likely to become an important method in understanding both function and drug action.

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Identification of a region of the C-terminal domain involved in short-term desensitization of the prostaglandin EP₄ receptor

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1 The prostaglandin EP₄ receptor, which couples to stimulation of adenylyl cyclase, undergoes rapid agonist-induced desensitization when expressed in CHO-K1 cells.

2 Truncation of the 488-amino acid receptor at residue 350 removes the carboxy-terminal domain and abolishes desensitization.

3 To further delineate residues involved in desensitization, the receptor was truncated at position 408, 383 or 369. Receptors truncated at position 408 or 383 underwent PGE₂-induced desensitization, whereas the receptor truncated at position 369 displayed sustained activity, indicating that the essential residues for desensitization lie between 370 and 383.

4 The six serines in the 14-amino acid segment between residues 370 and 383 were mutated to alanine, retaining the entire C-terminal domain. Desensitization was absent in cells expressing this mutant.

5 The results indicate involvement of serines located between 370 and 382 in rapid desensitization of the EP₄ receptor.

Keywords: Prostaglandin EP₄ receptor; cyclic AMP; agonist-induced desensitization; deletion mutagenesis; site-directed mutagenesis

Abbreviations: CHO-K1, Chinese hamster ovary K1; GRK, G protein coupled receptor kinase; IBMX, 3-isobutyl-1-methylxanthine; PGE₂, prostaglandin E₂; PKA, protein kinase A

Introduction

Prostaglandin E₂ (PGE₂) is an important modulator in many physiological and pathophysiological events (Campbell & Halushka, 1996), binding to four distinct G protein-coupled receptors (Negishi *et al.*, 1995). The EP₁ receptor couples to phospholipase C, the EP₂ and EP₄ receptors couple to stimulation of adenylyl cyclase, and the EP₃ receptor couples to inhibition of adenylyl cyclase. All the EP receptor subtypes have been cloned. The EP₃ receptor has six isoforms, generated from alternative splicing of a single gene, that differ in their C-terminal region (Regan *et al.*, 1994a; An *et al.*, 1994; Adam *et al.*, 1994; Schmid *et al.*, 1995; Kotani *et al.*, 1995). The EP₃ isoforms display differences in their degree of cyclase inhibition (Regan *et al.*, 1994a; An *et al.*, 1994) and susceptibility to desensitization (Negishi *et al.*, 1993).

PGE₂-induced increase in the cyclic AMP level is mediated by two receptors: EP₂ and EP₄. Both receptors display a broad tissue distribution with similar expression patterns (Katsuyama *et al.*, 1995), whereas each has distinctive structural and functional characteristics. EP₂ is a 358-amino acid protein with relatively short third intracellular and C-terminal domains (Regan *et al.*, 1994b). In contrast, EP₄, which has ~30% sequence identity to EP₂, consists of 488 amino acids, and has a long third intracellular loop and a long cytoplasmic tail that comprises almost one third of the receptor (An *et al.*, 1993; Bastien *et al.*, 1994). In addition, the two receptors display different sensitivities to metabolic inactivation of PGE₂, in that the first PGE₂ metabolite 15-keto-PGE₂ is more potent at the EP₂ receptor than EP₄ (Nishigaki *et al.*, 1996). Furthermore, the EP₂ receptor lacks agonist-induced short-term desensitization, whereas EP₄ desensitizes rapidly (Nishigaki *et al.*, 1996).

Agonist-induced desensitization is a common biological phenomenon that involves reduction of responsiveness despite continuous agonist challenge. Recent pharmacological evidence suggests that EP₄ may mediate several actions of PGE₂. Regulation by PGE₂ of matrix metalloproteinase activities in the RNK-16 cell line, a model for natural killer cells, is mediated by EP₄ (Zeng *et al.*, 1996) so that short-term desensitization may provide a tight regulatory control over the PGE₂-elicited chemokinesis. The EP₄ receptor has also been implicated in neonatal remodelling of the cardiovascular system triggered by a drop in PGE₂ level (Nguyen *et al.*, 1997), which is among actions of PGE₂ utilized in clinical therapy (Campbell & Halushka, 1996). Desensitization may play an important role in sensing the drop of PGE₂ level by the EP₄ receptor, which is critical for closure of the ductus arteriosus.

Structural determinants of desensitization have been studied extensively in the case of β -adrenergic receptors (see Bohm *et al.*, 1997 for a review). Short-term agonist treatment results in uncoupling of the receptor from its G protein, followed by receptor sequestration. The uncoupling event involves phosphorylation of the receptor by cyclic AMP-dependent protein kinase (PKA) and/or β -adrenergic receptor kinase at certain serine and threonine residues located in the third intracellular and/or C-terminal domains. Sequestration involves internalization of the receptor through endocytosis.

We have shown that the C-terminal domain is essential for agonist-induced desensitization of EP₄ (Bastepe & Ashby, 1997). We generated a mutant EP₄ severely truncated at its C-terminus, and demonstrated that the mutant, although intact in terms of ligand binding and G protein coupling, is resistant to short-term desensitization. Supporting our findings, the C-terminal domain of EP₄ has recently been shown to confer agonist-induced receptor desensitization in a chimeric receptor with the EP₃ β receptor

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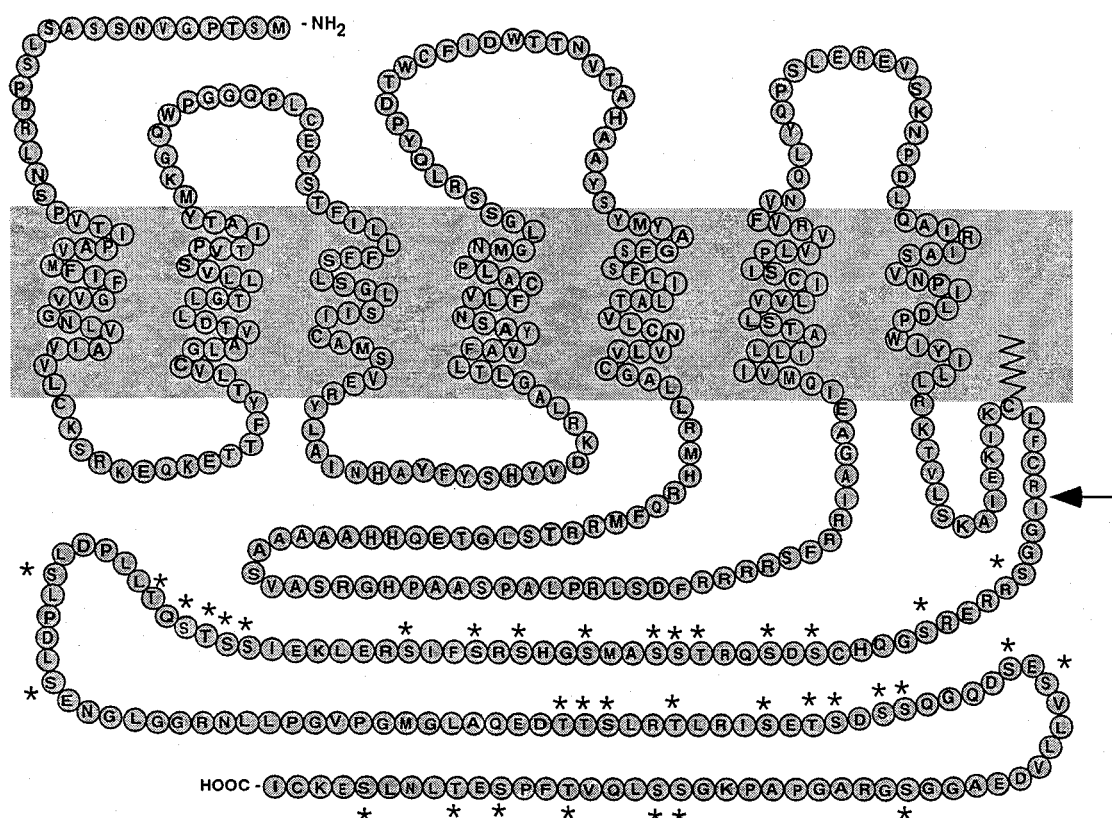


Figure 1 Membrane topography of the human EP₄ receptor. Amino acid sequences within the proposed transmembrane domains were determined by hydropathy analysis of the sequence. Asterisks indicate serine and threonine residues in the carboxy-terminal domain. The arrow indicates the position of the truncation at residue 350 that results in an active receptor that does not undergo short-term desensitization.

(Neuschafer et al., 1997). Figure 1 shows the membrane topography of the EP₄ receptor, indicating the 36 serine and threonine residues in the C-terminus and the position of the truncation at residue 350 that results in an active receptor that does not undergo agonist-induced short-term desensitization. In an effort to map the amino acid residues involved in agonist-induced desensitization of EP₄, we generated a series of mutants with successive deletions at their C-terminus, and one that carries serine-to-alanine point mutations at certain positions. Here we report that six serines within a 14-amino acid stretch in the proximal third of the cytoplasmic tail are essential residues involved in agonist-induced desensitization of EP₄.

Methods

Materials

Restriction enzymes were from New England Biolabs (Beverly, MA, U.S.A.). [³H]PGE₂, [2,8-³H]adenine and [¹⁴C]cyclic AMP were from DuPont NEN (Boston, MA, U.S.A.). Other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CHO-K1 cells were from the American Type Culture Collection (Rockville, MD, U.S.A.). The plasmid pRc/CMV was from Invitrogen (San Diego, CA, U.S.A.). DNA sequencing was carried out by automated sequencing at the University of Pennsylvania DNA Sequencing Facility (Philadelphia, PA, U.S.A.). Custom-order oligonucleotides were from Life Technologies Inc. (Gaithersburg, MD, U.S.A.).

Deletion mutagenesis

Construction of the plasmid pRc/CMV-hEP₄-wt was described previously (Bastepe & Ashby, 1997). A fragment of pRc/CMV-hEP₄-wt, extending from the unique *Sac*II site at nucleotide 694 to the desired truncation point (nucleotides 1224, 1149 and 1107 for hEP₄-t408, hEP₄-t383 and hEP₄-t369, respectively), was amplified by PCR. Forward primer was 5'-CCA CCG CGG CCG CCT CGG TTG CCT CC-3' (underlined bases indicate the *Sac*II site). Three different stop codons located in three different reading frames and an *Apa*I site were introduced in the 3' end of the PCR product via reverse primer, which was 5'-GGA GGG CCC TAT TTA TTC AGC CAT TTT CAC TGA GGT CTG-3', 5'-GGA GGG CCC TAT TTA TTC ACC GGG AGA TGA AGG AGC GAG-3' or 5'-GAG GGG CCC TAT TTA TTC ATG TCC TTT GAC TGT CTG AG-3' (underlined bases indicate the *Apa*I site; bases in bold-type indicate stop codons) for hEP₄-t408, hEP₄-t383 or hEP₄-t369, respectively. Following purification and restriction enzyme digestion with *Sac*II and *Apa*I, each PCR product was ligated with the 6773-bp linearized piece of the *Sac*II/*Apa*I digestion products of pRc/CMV-hEP₄-wt. The plasmids, designated pRc/CMV-hEP₄-t408, pRc/CMV-hEP₄-t383 and pRc/CMV-hEP₄-t369, were sequenced in order to check for undesired mutations and sequence integrity. To maintain consistency in plasmid structures, the 3'-untranslated region of the wild-type EP₄ cDNA was deleted by a similar strategy using the reverse primer: P4 = 5'-GGA GGG CCC TAT TTA TTC ATA TAC ATT TTT CTG ATA AGT TCA G-3' (underlined bases indicate the *Apa*I site; bases in bold-type indicate stop codons).

Site-directed mutagenesis

Serine-to-alanine mutations were carried out by oligonucleotide-directed mutagenesis according to Higuchi *et al.* (1988). Initially, Ser370Ala and Ser371Ala mutations were introduced. Two overlapping fragments of pRc/CMV-hEP₄-wt were amplified by PCR using the primers: P1 = 5'-CCA CCG CGG CCG CCT CGG TTG CCT CC-3' and P2 = 5'-GGC AGC AGC TGT CCT TTG ACT G-3', or P3 = 5'-CAG TCA AAG GAC AGC TGC TGC C-3' and P4. One of the PCR products extended from the unique *Sac*II site to nucleotide 1116 and the other from nucleotide 1095 to the *Apa*I site adjacent to the termination codon. Both PCR products carried the same T to G mutations at nucleotides 1108 and 1111 which were introduced *via* mismatch in primers P2 and P3. Small aliquots of the overlapping PCR products were combined, denatured, reannealed and subjected to additional 30 cycles of PCR using primers P1 and P4. Following purification and restriction enzyme digestion with *Sac*II and *Apa*I, the resultant PCR product was substituted with the corresponding region of pRc/CMV-hEP₄-wt. The construct was sequenced, and used as template in the PCR for generation of the cDNA encoding the mutant hEP₄ with serine-to-alanine mutations at positions 370, 371, 374, 377, 379 and 382. The first overlapping PCR product, generated using primers P1 and P5 (5'-GGC GAT GAA GGC GCG AGC GTG GCC TGC CAT GGC AGA AG-3'), extended from the unique *Sac*II site to nucleotide 1146 and the second, generated using primers P6 (5'-TGG CAG GCC ACG CTC GCG CCT TCA TCG CCC GGG AG-3') and P4, extended from nucleotide 1118 to the *Apa*I site adjacent to the termination codon. Both PCR products carried the same T to G mutations at nucleotides 1120, 1129, 1135, and 1144, introduced *via* mismatches in primers P5 and P6. The final plasmid construct, designated pRc/CMV-hEP₄-S370-382A, was generated as described above and the mutations confirmed by sequencing.

Expression in CHO-K1 cells

Stable transfection of CHO-K1 cells with each cDNA plasmid and isolation of clonal cells were performed as previously described (Bastepe & Ashby, 1997). Positive clones were identified by measuring the PGE₂-induced cyclic AMP formation and selected clonal cells maintained in medium containing 10% foetal bovine serum, 350 µg ml⁻¹ G-418 sulphate and 1 mM acetyl salicylic acid (used to inhibit endogenous PGE₂ formation). For transient expression, CHO-K1 cells were seeded at a density of 5 × 10⁶ cells per 100-mm culture plate. Twenty-four hours later, transfection was carried out with 8 µg of plasmid DNA and 12.5 µg ml⁻¹ Lipofectamine reagent (Life Technologies Inc.) according to the manufacturer's instructions. Cells were assayed 48 h after the start of transfection.

Determination of adenylyl cyclase activity

Cells grown in 6-well 35-mm culture plates were labelled overnight with 2 µCi ml⁻¹ of [³H]adenine (25 Ci mmol⁻¹). Labelling medium was removed and cells incubated for 10 min with fresh medium containing 2 mM 3-isobutyl-1-methyl-xanthine (IBMX). Cells were challenged with forskolin (30 µM) or PGE₂ (10 µM) for various times. Reactions were stopped by the replacement of the medium with a solution containing HCl (0.2 M), 0.2% sodium dodecyl sulphate and 2000 c.p.m. of [¹⁴C]cyclic AMP as recovery standard. [³H]cyclic AMP was determined according to Salomon (1979) as percentage of the

total labelled adenine nucleotides. Kinetic data obtained from PGE₂ challenge of each clonal cell line were normalized to initial rate of cyclic AMP formation determined over the first 2 min and expressed as percentage of cyclic AMP expected to accumulate within 30 min at the initial rate.

Membrane preparation and binding experiments

Cells grown in 100-mm culture plates were washed and scraped in hypotonic lysis buffer containing (in mM) Tris-HCl 20 (pH 7.5), MgCl₂ 10, EDTA 1, EGTA 1, Leupeptin (10 µM) and soybean trypsin inhibitor (10 µg ml⁻¹). Following incubation on ice for 30 min, the lysate was sonicated and centrifuged at 80,000 × g for 30 min at 4°C. The pellet was washed once with (in mM) Tris-HCl 50 (pH 7.5), MgCl₂ 10, EDTA 1, and 10 µM Leupeptin (Buffer A), re-centrifuged and resuspended in Buffer A. Protein concentrations were determined by the Coomassie® Plus protein assay reagent (Pierce, Rockford, IL, U.S.A.). Membranes were incubated with 1 nM [³H]PGE₂ (200 Ci mmol⁻¹) in the presence of varying concentrations of non-labelled PGE₂ in Buffer A for 60 min at room temperature. Binding was determined as described previously (Kunapuli *et al.*, 1994a) except the filters were presoaked in Tris-HCl 50 mM, (pH 7.5) containing 0.1 mg ml⁻¹ bovine serum albumin and 0.2% polyethylenimine and washed with ice-cold Tris-HCl 50 mM, (pH 7.5) buffer. Data were analysed using EBDA and LIGAND softwares (Biosoft, Cambridge, U.K.) (Mcpherson, 1983; Munson & Rodbard, 1980).

Results

Truncation mutants of the EP₄ receptor

Figure 2A shows a schematic representation of the deletion mutants in comparison with wild-type EP₄. The cytoplasmic C-terminal domain of the wild-type EP₄ receptor consists of 156 amino acids. In the mutants, designated hEP₄-t408, hEP₄-t383 and hEP₄-t369, the number of amino acid residues deleted are 80, 105 and 119, respectively. Eighteen, 25 and 31 of the 38 serine and threonine residues fall within the deleted fragment in hEP₄-t408, hEP₄-t383 and hEP₄-t369, respectively.

We expressed the truncated receptors transiently in CHO-K1 cells to characterize their agonist binding properties. Specific binding of [³H]PGE₂ to membranes of transfected cells was saturable for each receptor and K_d values were similar to the wild-type EP₄ receptor (Table 1).

Functional features of the truncated receptors were studied in clonal CHO-K1 cells stably expressing wild-type or each of the mutant receptors. The time-course of cyclic AMP formation was examined in selected clonal cells challenged with PGE₂ in the presence of the phosphodiesterase inhibitor IBMX, which inhibits break-down of cyclic AMP, so that a decrease in the rate of cyclic AMP synthesis represents a decline in adenylyl cyclase activity. As a means of assessing desensitization, this method has been validated by detailed studies of prostaglandin receptor desensitization in intact cells (Ashby, 1989; 1990). As shown in Figure 3A, cells stably expressing wild-type EP₄, hEP₄-t408 and hEP₄-t383 displayed similar kinetic patterns of 10 µM PGE₂-induced cyclic AMP formation. The time-course, which appeared linear over the first 2–3 min of agonist stimulation, curved off with time and reached a plateau within 15 min, indicating a decline in rate of cyclic AMP synthesis. In contrast, the kinetic pattern obtained in the clonal cells expressing hEP₄-t369 was significantly

To confirm our findings, we repeated the experiments with two other clonal cell lines for wild-type EP₄, hEP₄-t383 and hEP₄-t369. Figure 4 shows the average 2- or 20-min level of PGE₂-induced cyclic AMP in different clonal cell lines expressing the same type of receptor. Expressed as percentage of the hypothetical 30-min level reached at the initial rate of synthesis, the levels attained after 2 min were similar in all the selected clonal cells. However, those reached after 20 min displayed significant differences. Although the average level of cyclic AMP accumulated in the clonal cell lines expressing wild-type EP₄ and the hEP₄-t383 mutant were still similar to each other, the amount accumulated in cells expressing hEP₄-t369 was significantly higher than the others (Figure 4). The observation that the mutant terminating at Thr369 (hEP₄-t369) does not desensitize in response to short-term agonist challenge, unlike the one terminating at Arg383 (hEP₄-t383), indicates that the residues of the EP₄ receptor involved in agonist-induced desensitization are embedded in a 14 amino-

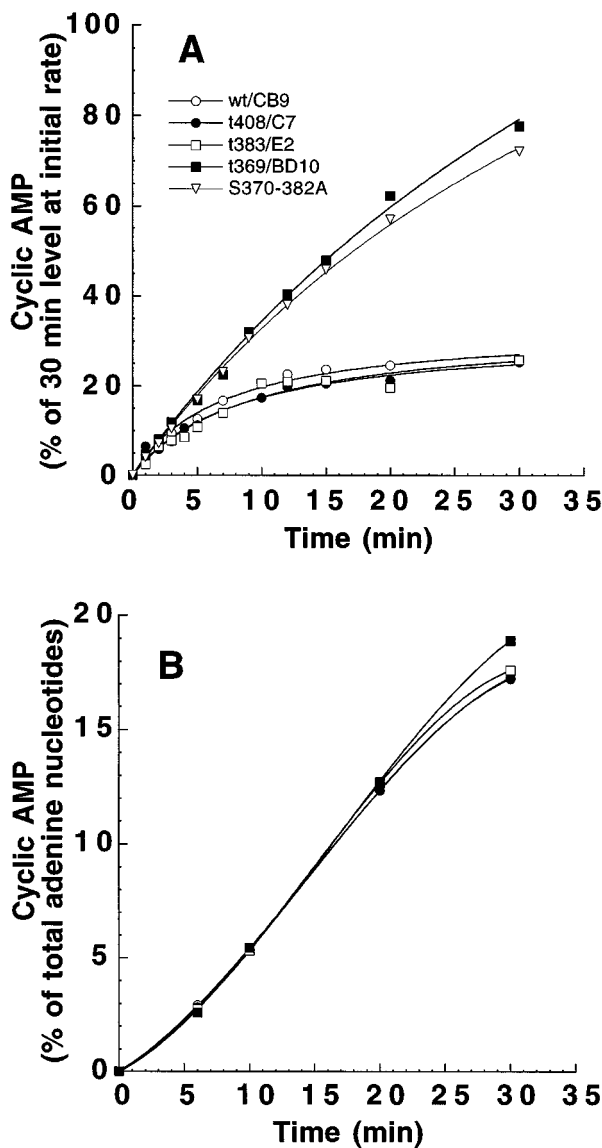


Figure 3 Time-course of PGE₂ (A) or forskolin (B)-induced cyclic AMP formation in CHO-K1 cells stably expressing the wild-type EP₄ receptor or each of the deletion mutants. Clonal hEP₄-wt/CB9, hEP₄-t408/C7, hEP₄-t383/E2, hEP₄-t369/BD10 or hEP₄-S370-382A/AC8 cells were stimulated with PGE₂ (10 μ M) or forskolin (30 μ M) for indicated times in the presence of IBMX (2 mM). Reactions were stopped and cyclic AMP measured as described in Methods. Values are representative of at least three separate experiments with similar results. [³H] cyclic AMP was determined as percentage of total labelled adenine nucleotides. The level obtained with PGE₂ stimulation was normalized for each clonal cell to the initial rate of cyclic AMP formation determined over the first 2 min. After subtraction of the base-line, each value was expressed as a percentage of the amount of cyclic AMP expected to accumulate within 30 min at the initial rate of synthesis, giving an indication of the degree of desensitization. Note that all of the constructs are represented in Figure 3B but the data are hidden by other symbols since they are so similar.

acid stretch located between positions 370 and 383 within the proximal one third of the C-terminal domain.

We also determined specific binding of PGE₂ to the wild-type and truncated receptors expressed in the clonal cells to assure that receptor expression levels were comparable. As presented in Table 1, the amount of [³H]PGE₂ specifically bound to membranes prepared from individual clonal cells displayed little variation. The average amount of specifically-bound [³H]PGE₂ in each group of clonal cells expressing the

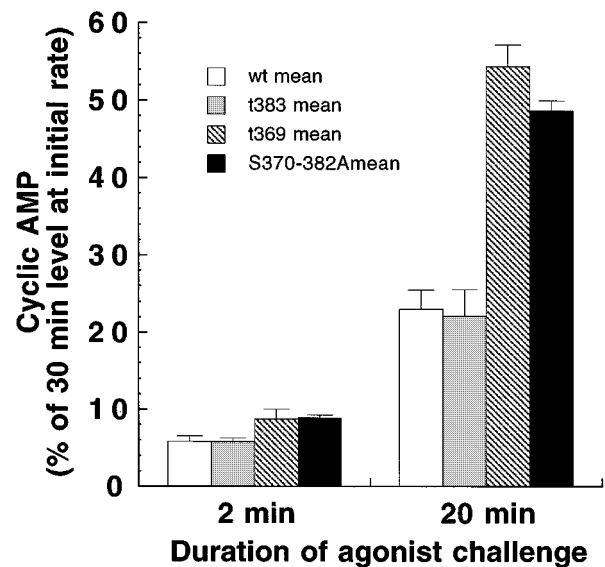


Figure 4 PGE₂-induced cyclic AMP formation in clonal CHO-K1 cells expressing hEP₄-wt, hEP₄-t383, hEP₄-t369, or hEP₄-S370-382A. Clonal cells were stimulated with PGE₂ (10 μ M) for indicated time periods in the presence of IBMX (2 mM). Reactions were stopped and cyclic AMP measured as described in Methods. Values represent means $\pm$ s.e. mean of 4–6 separate experiments with a total of three clonal cell lines for each receptor. [³H] cyclic AMP was determined as a percentage of total adenine nucleotides, and normalized, for each clonal cell, to the initial rate of cyclic AMP formation determined over the first 2 min as described in Methods. * P < 0.001 compared with wild-type or hEP₄-t383 at the same time point according to Student's t -test.

same type of receptor was comparable to one another, with no indication of correlation with susceptibility to receptor desensitization.

Site-directed mutagenesis of the EP₄ receptor

There are six serines within the identified region of the C-terminal domain of the EP₄ receptor which are potential targets for phosphorylation. To ascertain the importance of these serines in desensitization of EP₄, we generated another mutant, designated hEP₄-S370-382A, that retained the entire C-terminal domain with serine-to-alanine substitutions at positions 370, 371, 374, 377, 379 and 382 (Figure 2B).

The agonist affinity of hEP₄-S370-382A expressed transiently in CHO-K1 cells was determined by measuring binding of [³H]PGE₂ to membranes and the K_d value was similar to that of wild-type receptor (Table 1). We examined the time-course of PGE₂-induced cyclic AMP response in clonal cells stably expressing hEP₄-S370-382A (Figure 3A). The kinetic pattern indicated a sustained increase in the level of cyclic AMP, which was similar to that observed in clonal cells expressing hEP₄-t369 but significantly different from that observed in clonal cells expressing wild-type EP₄. This finding clearly shows that desensitization is substantially impaired by serine-to-alanine substitutions at positions 370, 371, 374, 377, 379 and 382.

We examined the time-course of PGE₂-induced cyclic AMP formation in two additional clonal cell lines expressing hEP₄-S370-382A. A sustained elevation of cyclic AMP formation was observed in both clonal cells. As shown in Figure 4, whereas the average cyclic AMP levels were similar after 2 min of agonist stimulation in multiple clonal cells expressing wild-type EP₄, hEP₄-t369 or hEP₄-S370-382A, the level attained after 20 min was significantly higher in cells expressing hEP₄-

S370-382A than those expressing wild-type EP₄. We also examined the forskolin-stimulated adenylyl cyclase activity in each of the clonal cells, and showed that it was similar to those measured in cells expressing wild-type or truncated EP₄ receptors (data not shown). [³H]PGE₂ binding in the clonal cell lines was comparable with each other and with wild-type or the truncated receptors (Table 1).

Discussion

The EP₄ subtype of the PGE₂ receptor is a member of the G protein-coupled receptor superfamily. Agonist stimulation of EP₄ leads to activation of adenylyl cyclase and the receptor is susceptible to agonist-induced short-term desensitization. In this study, we measured the time-course of cyclic AMP formation in CHO-K1 cells expressing wild-type or mutant EP₄ receptors in order to determine the structural features involved in receptor desensitization.

The desensitization process examined here is essentially complete by 10 min which predominantly reflects a short-term desensitization process rather than down-regulation as is evident from the work of Nishigaki *et al.* (1996). These authors compared desensitization of EP₂ and EP₄. They showed rapid desensitization of EP₄ (complete after 10 min) but no rapid desensitization of EP₂, even after 30 min. By contrast, both EP₂ and EP₄ showed loss of receptor binding with half times on the order of 1 h or longer. While it is clear that some down-regulation takes place during the time period that we observe, the data of Nishigaki *et al.* (1996) show that it is possible to differentiate between short- and long-term desensitization by conducting experiments of 30 min or shorter duration.

Mechanisms of short-term receptor desensitization have been extensively studied in the β_2 -adrenergic receptor. The putative third intracellular and C-terminal domains have been highlighted as the most important structural features involved in agonist-induced short-term desensitization (Bohm *et al.*, 1997). Earlier, we reported that the C-terminal domain of EP₄ is required for desensitization by showing that deletion of 138 amino acids at the C-terminus results in a mutant EP₄ receptor that is unable to undergo desensitization (Bastepe & Ashby, 1997).

In the present study, our findings from mutant EP₄ receptors carrying successive deletions in their cytoplasmic tail indicate that, despite the lengthiness of the C-terminal domain, residues of importance with respect to rapid desensitization reside within a short stretch of amino acids. Even though deletion of the last 105 amino acids in the C-terminus (hEP₄-t383) does not alter the susceptibility of EP₄ to agonist-induced desensitization, removal of an additional 14 residues (hEP₄-t369) results in the loss of receptor desensitization upon short-term agonist stimulation.

Desensitization of G protein-coupled receptors in response to short-term agonist exposure entails receptor/G protein uncoupling mediated by phosphorylation of the receptor at certain serine or threonine residues located in the third intracellular loop and/or C-terminal domain (Bohm *et al.*, 1997). The kinases involved include cyclic AMP-dependent protein kinase, which leads to heterologous desensitization, and specific GRKs, which lead to homologous desensitization. Potential targets for phosphorylation in the identified 14-amino acid region of the EP₄ receptor are six serine residues at positions 370, 371, 374, 377, 379 and 382. We eliminated these residues in another mutant by substituting each with an alanine. Our results, from experiments involving functional

analysis of the latter mutant, strongly suggest that at least one of the six mutated serines is indeed involved in agonist-induced short-term desensitization of the EP₄ receptor. The mutant receptor, which differs from wild-type by only 6 amino acids, displays a significantly impaired agonist-induced desensitization, characterized by a sustained increase in the PGE₂-induced cyclic AMP formation.

The kinases involved in desensitization of G protein coupled receptors include cyclic AMP-dependent protein kinase, which leads to heterologous desensitization, and specific G protein-coupled receptor kinases (GRKs), which lead to homologous desensitization. Among the six serines identified, Ser370 and/or Ser371 seem likely candidates for phosphorylation by PKA. Although the PKA consensus sequence, Arg-Arg-Xaa-Ser, does not exactly appear in this region, a similar sequence, Gln-Arg-Thr-Ser370-Ser371, is present in the human receptor (An *et al.*, 1993; Bastien *et al.*, 1994). Moreover, the corresponding regions of the mouse and rat EP₄ receptors bear the exact consensus sequence, carrying an arginine (Arg395 and Arg370, respectively) instead of the glutamine in human (Honda *et al.*, 1993; Sando *et al.*, 1994). Nevertheless, a recent study has shown that heparin, a compound with known non-specific GRK inhibitory activity, but not the PKA inhibitor peptide (PKI), prevents agonist-induced short-term desensitization of EP₄ (Nishigaki *et al.*, 1996), suggesting involvement of GRKs, but not PKA.

A consensus sequence for GRK-mediated phosphorylation has yet to be unequivocally identified. It appears from studies using synthetic peptides that, whereas some GRKs, including rhodopsin kinase and β -adrenergic receptor kinase, may actively phosphorylate serines flanked N- or C-terminally by acidic residues (Onorato *et al.*, 1991) others may display higher activity for serines C-terminal to basic residues (Kunapuli *et al.*, 1994b; Loudon & Benovic, 1994). In the C-terminal domain of the EP₄ receptor, there are multiple serine residues, most of which are closely flanked by acidic or basic residues. Those within the identified region, however, appear to be predominantly flanked by basic residues, which may be helpful in future studies to identify the GRKs involved in desensitization of EP₄. Demonstration of agonist-induced phosphorylation by use of receptor-specific antibodies and over-expression of individual GRKs should provide more detail of EP₄ desensitization.

The primary difference between the two PGE₂ receptors EP₂ and EP₄ is their susceptibility to agonist-induced short-term desensitization (Nishigaki *et al.*, 1996). This distinction suggests that, whereas EP₂ may be involved in mediating more sustained actions of PGE₂, EP₄ may mediate rapidly waning events. Although boundaries between the physiological roles of the EP₂ and EP₄ receptors remain to be clarified, agonist-induced short-term desensitization is clearly a considerable regulatory element in EP₄-mediated signalling, but not in EP₂-mediated signalling. Identification of structural features of EP₄ involved in this response should prove useful for future investigations involving the physiology and pharmacology of these receptors.

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Acute troglitazone action in isolated perfused rat liver

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1 The thiazolidinedione compound, troglitazone, enhances insulin action and reduces plasma glucose concentrations when administered chronically to type 2 diabetic patients.

2 To analyse to what extent thiazolidinediones interfere with liver function, we examined the acute actions of troglitazone (0.61 and 3.15 μM) on hepatic glucose and lactate fluxes, bile secretion, and portal pressure under basal, insulin- and/or glucagon-stimulated conditions in isolated perfused rat livers.

3 During BSA-free perfusion, high dose troglitazone increased basal ($P < 0.01$), but inhibited glucagon-stimulated incremental glucose production by $\sim 75\%$ (10.0 ± 2.5 vs control: 40.0 ± 7.2 $\mu\text{mol g liver}^{-1}$, $P < 0.01$). In parallel, incremental lactate release rose ~ 6 fold (13.1 ± 5.9 vs control: 2.2 ± 0.8 mmol g liver^{-1} , $P < 0.05$), while bile secretion declined by $\sim 67\%$ [0.23 ± 0.02 vs control: 0.70 ± 0.05 $\text{mg g liver}^{-1} \text{ min}^{-1}$], $P < 0.001$]. Low dose troglitazone infusion did not enhance the inhibitory effect of insulin on glucagon-stimulated glucose production, but rapidly increased lactate release ($P < 0.0005$) and portal venous pressure ($+0.17 \pm 0.07$ vs $+0.54 \pm 0.07$ cm buffer height, $P < 0.0001$).

4 These results indicate that troglitazone exerts both insulin-like and non-insulin-like hepatic effects, which are blunted by addition of albumin, possibly due to troglitazone binding.

Keywords: Troglitazone; insulin; glucagon; glucose production; glycogenolysis; lactate; portal pressure; bile secretion; liver; rat

Abbreviations: ADP, adenosine-diphosphate; AMP, adenosine-monophosphate; BSA, bovine serum albumin; DMSO, dimethyl sulphoxide; PPAR γ , peroxisome proliferator activated receptor γ

Introduction

Thiazolidinedione derivatives represent a new class of insulin-sensitizers that improve insulin resistance, glycemia, and dyslipidemia in animal models of obesity and diabetes (Saltiel & Olefsky, 1996). One of these compounds, troglitazone (CS-045) also decreases hyperglycemia and ameliorates insulin sensitivity in human type 2 diabetes mellitus (Iwamoto *et al.*, 1991; Schwartz *et al.*, 1998; Inzucchi *et al.*, 1998). The improvement of hyperglycemia requires the presence of insulin and usually develops after several days to weeks of oral treatment. This chronic effect of troglitazone could be linked to activation of the nuclear peroxisome proliferator activated receptor γ (PPAR γ), which induces adipocyte differentiation in cell cultures (Lehmann *et al.*, 1995; Teboul *et al.*, 1995). However, PPAR γ is only rarely detectable in skeletal muscle or liver and no association between PPAR γ gene expression and insulin-resistant states like obesity and type 2 diabetes mellitus could be found (Aubouef *et al.*, 1997).

Thiazolidinediones also exert short-term effects on glucose metabolism, which cannot be explained by regulation of gene expression and/or transcription (Fujiwara *et al.*, 1988; Kreutter *et al.*, 1990; Lee & Olefsky, 1995; Fürnsinn *et al.*, 1997). In nondiabetic rats, troglitazone increases peripheral glucose disposal and decreases hepatic glucose production within 1 h (Lee & Olefsky, 1995). The acute stimulatory effect on muscle glucose metabolism is not insulin-dependent and

exhibits hypoxia-like features (Fürnsinn *et al.*, 1997). The reduction of hepatic glucose production could be due to an insulin-like action as suggested by inhibition of gluconeogenesis in isolated rat hepatocytes (Fulgencio *et al.*, 1996; Raman *et al.*, 1998). Alternatively, these effects could also result from unspecific troglitazone action. In this context it is of note that liver dysfunction or hepatocellular injury has occurred during troglitazone treatment in man (Ault, 1997; Imura, 1998; Watkins & Whitcomb, 1998).

To understand better detrimental hepatic troglitazone action this study analyses: (a) basal and stimulated net glucose production, (b) transhepatic lactate fluxes, simultaneously with the time-course of (c) bile secretion and (d) portal pressure in isolated perfused rat liver.

Methods

Animals

Male Sprague-Dawley rats (Him: OFA/SPF, Tierforschungsinstitut, Himberg, Austria) weighing from 220–300 g were kept at a constant 12 h day-night cycle with free access to standard laboratory rat chow and water. Experiments were started before 10.00 a.m. by intraperitoneal thiopental anaesthesia (80 mg g body weight $^{-1}$ intraperitoneally; Sanabo, Vienna, Austria) of the non-fasted rat to provide full hepatic glycogen stores (Burton & Ishida, 1965). All protocols were performed according to local legislation and to the principles of laboratory animal care.

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Isolated liver perfusion

The preparation and perfusion was performed as previously reported (Roden *et al.*, 1992, 1995; Graf & Peterlik, 1976) with the described modifications. Briefly, the portal vein and the vena cava were cannulated and the isolated liver was perfused in anterograde direction in a non-recirculating system (flow rate: $\sim 3.5 \text{ ml g liver}^{-1} \text{ min}^{-1}$). The perfusion was performed at 37°C with oxygenated (95% O_2 /5% CO_2) perfusion medium [Krebs-Henseleit buffer + 5 mM D(+)-glucose, pH 7.4] with or without added 0.2% (w/v) bovine serum albumin (BSA, fraction V; Boehringer Mannheim, Mannheim, Germany). A small flexible cannula was inserted into the bile duct in order to allow continuous monitoring of bile flow by using a drop counter connected to an IBM-compatible personal computer. Portal hydrostatic pressure was measured by using a Y-tubing located 15 cm before the liver and liver viability was checked during the equilibration period ($t = -40$ – 0 min) by uniform colour of the blood-free liver, constant lactate release, stable portal hydrostatic pressure ($< +5 \text{ cm}$ buffer height), and bile flow ($> 0.5 \text{ mg g liver}^{-1} \text{ min}^{-1}$) (Gores *et al.*, 1986; Roden *et al.*, 1995).

Drugs and hormones

Troglitazone was generously donated by Sankyo (Tokyo, Japan). The drug was dissolved and diluted in dimethyl sulphoxide (DMSO) and continuously infused into the portal tubing at final concentrations of 0.61 or $3.15 \mu\text{M}$. The DMSO concentration did not exceed 0.2% (v/v) and was identical in the control experiments in which DMSO was infused without added troglitazone. Human insulin (calculated final concentrations: 5 nM; Actrapid, Novo-Nordisk 40 U, Copenhagen, Denmark) and glucagon (final concentration: 0.1 or 1 nM; Lilly, Indianapolis, IN, U.S.A.) were diluted in Krebs-Henseleit buffer containing 2% BSA.

Experimental protocols

After the equilibration period, insulin and/or troglitazone or DMSO alone (control) were continuously admixed to the influent perfusate (0.5 ml min^{-1} ; Perfusor V, Braun Melsungen, Germany). During the second phase of the experiments, glucagon was infused to stimulate glycogen breakdown and to test the liver's hormonal responsiveness (Roden *et al.*, 1992). Perfusate samples were taken from the portal tubing (pre-hepatic: $t = 0, 30$, and 80 min) and from the vena cava (post-hepatic: every 5 min).

Three study protocols were performed to examine (I) acute troglitazone action, (II) effects of BSA on acute troglitazone action, and (III) effects of insulin on acute troglitazone action. During protocol (I) 0.6 or $3.15 \mu\text{M}$ troglitazone or DMSO alone were infused for 90 min with 1 nM glucagon being infused during the last 30 min. For protocol (II), $3.15 \mu\text{M}$ troglitazone or DMSO were infused for 90 min in the presence of 0.2% BSA with added glucagon (1 nM) during the last 30 min. During protocol (III), 5 nM insulin alone or with $0.61 \mu\text{M}$ troglitazone or DMSO only were infused for 60 min and an infusion of 0.1 nM glucagon was commenced for the last 60 min.

Analytical procedures

Glucose and lactate concentrations were determined enzymatically by the hexokinase (Glucose liquiUV; Human, Taunusstein, Germany) and the lactate dehydrogenase method (ACA,

Du Pont Company, Wilmington, DE, U.S.A.), respectively. Rates of glucose/lactate production were calculated by multiplying the transhepatic concentration difference (post-hepatic minus pre-hepatic concentration) times flow rate divided by liver wet weight and are expressed as $\mu\text{mol g liver}^{-1} \text{ min}^{-1}$. Incremental glucose or lactate production in $\mu\text{mol g liver}$ was assessed by the trapezoidal rule from the area under the time curve corrected for glucose or lactate production rates at zero time for the first period and at $t = 60 \text{ min}$ for the second (glucagon) period, respectively. Bile flow was calculated by multiplying single drop weight (8 mg per drop) times drop frequency per liver wet weight and is expressed in $\text{mg bile g liver}^{-1} \text{ min}^{-1}$. Portal pressure is given as the buffer height in cm with the liver support plate as the reference level.

Data analysis and statistical evaluation

Data are presented as means $\pm$ s.e.mean. Differences between groups were analysed with the unpaired, two-tail *t*-test. *P* values less than 0.05 were considered to indicate significant differences.

Results

Acute troglitazone action

Hepatic glucose production rates decreased slightly during infusion of low dose ($0.61 \mu\text{M}$) troglitazone or DMSO alone, but remained unchanged during the 60 min infusion of high dose ($3.15 \mu\text{M}$) troglitazone (Figure 1). Stimulation of

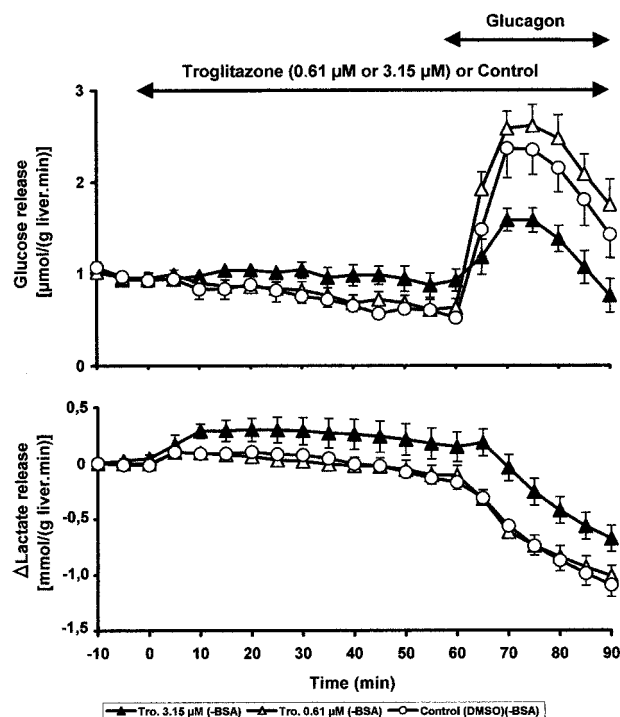


Figure 1 Acute troglitazone action. Time-course of glucose production rates and Δ lactate release during the basal period and during glucagon stimulation (1.0 nM) in isolated perfused rat liver. Effect of troglitazone infusion ($0.61 \mu\text{M}$: $n = 7$, $3.15 \mu\text{M}$: $n = 8$) compared with control (Krebs-Henseleit buffer + 0.2% DMSO: $n = 7$) under BSA-free conditions. Data are given as means $\pm$ s.e.-mean.

glycogenolysis by glucagon resulted within 10 min in a 4–5 fold rise of glucose production rates in both the low dose troglitazone and control groups. However, in the presence of high dose troglitazone, glucagon-stimulated glucose production rates increased by only ~1.7 fold and were ~36% lower than those elicited in the low dose troglitazone ($P<0.005$) or control ($P<0.05$) groups. Thus, glucagon-stimulated incremental glucose release was not affected by low dose troglitazone, but suppressed by high dose troglitazone to ~25% of control values (Table 1).

Lactate production rates (in $\text{mmol g liver}^{-1} \text{ min}^{-1}$) did not differ at zero time in the three treatment groups (high dose troglitazone: 1.25 ± 0.09 , low dose troglitazone: 1.48 ± 0.12 , control: 1.34 ± 0.03). Rates of hepatic lactate release corrected for zero-time lactate production (Δ lactate release) were similar during the basal period, while the glucagon-induced fall in lactate production rates was less pronounced in the presence of high dose troglitazone ($P<0.005$ vs low dose troglitazone and control; Figure 1).

Hepatic bile secretion remained constant during infusion of low dose troglitazone or vehicle; in both groups glucagon infusion induced a transient decrease followed by a small increment in bile secretion which then returned to control values (Figure 2). In the presence of high dose troglitazone, bile secretion rates rapidly declined in a nearly linear manner until the start of the glucagon infusion. At this time ($t=60$ min) bile secretion rates were decreased by ~70% compared with the low dose troglitazone and control groups ($P<0.00001$). Portal venous pressure was similar in all treatment groups prior to the glucagon infusion (Figure 2). Infusion of glucagon produced a gradual increase in mean portal pressure in livers exposed to high dose troglitazone did not differ significantly from those of the low dose troglitazone and control groups.

Effect of BSA on acute troglitazone action

In order to test whether the observed effects of high dose ($3.15 \mu\text{M}$) troglitazone could be prevented by albumin, the above described experiments were repeated in the presence of 0.2% BSA. Addition of BSA reduced hepatic glucose production rates in livers exposed to high dose troglitazone ($P<0.05$) to a level similar to that observed in control livers exposed to BSA (Figure 3). The rise in glucose production

induced by subsequent stimulation by glucagon was similar in livers treated with high dose troglitazone + BSA exposure or BSA alone but ~35% higher than in livers treated with high dose troglitazone alone ($P<0.05$). In the presence of BSA incremental glucose release was similar between the high dose troglitazone and control groups before and during glucagon stimulation (Table 1).

Lactate production rates (in $\text{mmol g liver}^{-1} \text{ min}^{-1}$) were again not different at zero time (high dose troglitazone without BSA: 1.25 ± 0.09 , high dose troglitazone + BSA: 1.10 ± 0.03 , control + BSA: 1.21 ± 0.08). Lactate production rates were

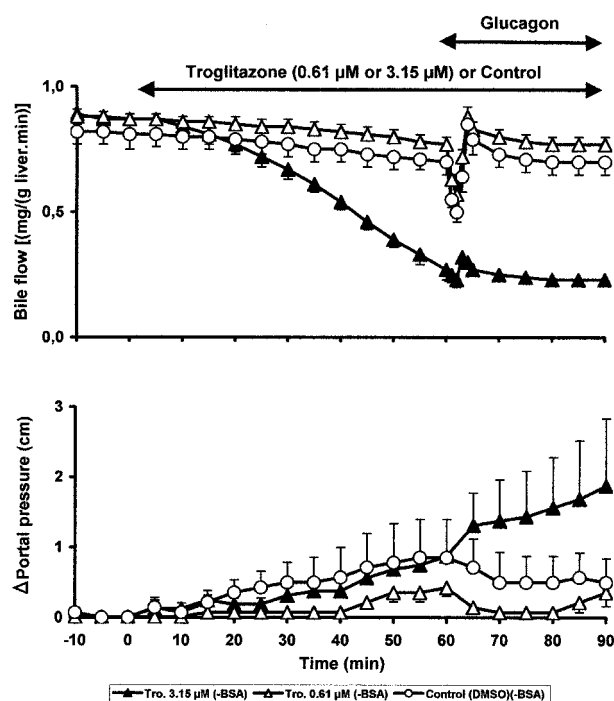


Figure 2 Acute troglitazone action. Time-course of bile flow and Δ portal pressure during the basal period and during glucagon stimulation (1.0 nM) in the isolated perfused rat liver. Effect of troglitazone infusion ($0.61 \mu\text{M}$: $n=6$, $3.15 \mu\text{M}$: $n=6$) compared with control (Krebs-Henseleit buffer + 0.2% DMSO; $n=6$) under BSA-free conditions. Data are given as means $\pm$ s.e.mean.

Table 1 Incremental basal and stimulated hepatic glucose release (means $\pm$ s.e.mean) during infusion of DMSO, troglitazone $\pm$ BSA and/or insulin

Troglitazone (μM)	BSA	Insulin	n	Incremental glucose production ($\mu\text{mol g liver}^{-1}$)	
				Basal	Glucagon-stimulated
<i>Acute troglitazone action</i>					
0	—	—	7	-15.4 ± 2.7^a	40.0 ± 7.2^b
0.61	—	—	7	-10.9 ± 3.4	45.4 ± 3.8^c
3.15	—	—	8	$-0.2 \pm 3.7^{a,d,e}$	$10.0 \pm 2.5^{b,c,f,g}$
<i>Effect of BSA on acute troglitazone action</i>					
0	+	—	6	-30.1 ± 9.9^e	37.3 ± 6.4^f
3.15	+	—	6	-25.8 ± 4.8^d	38.6 ± 4.0^g
<i>Effect of BSA on acute troglitazone action</i>					
0	—	—	6	-33.6 ± 4.4^h	60.8 ± 10.3^i
0	—	+	6	$-55.1 \pm 5.5^{h,i}$	29.2 ± 6.9^j
0.61	—	+	6	-39.5 ± 3.2^i	48.4 ± 5.7

Superscripts indicate significant differences between the groups compared. $^aP<0.01$, $^bP<0.01$, $^cP<0.001$, $^dP<0.001$, $^eP<0.01$, $^fP<0.05$, $^gP<0.01$, $^hP<0.05$, $^iP<0.05$, $^jP<0.05$.

lower in livers treated with high dose troglitazone + BSA than in those treated with troglitazone alone at 10–70 min ($P < 0.05$) but did not differ from the controls (Figure 3). In parallel, incremental lactate release (0–60 min: $13.1 \pm 0.6 \mu\text{mol g liver}^{-1}$) was markedly higher than in the absence of BSA ($-7.8 \pm 3.3 \text{ mmol g liver}^{-1}$, $P < 0.05$) and in the controls ($2.2 \pm 0.8 \text{ mg g liver}^{-1}$, $P < 0.05$).

Hepatic bile secretion also decreased within 25 min of high dose troglitazone + BSA exposure ($P < 0.01$), but the decline was less pronounced than that observed in BSA-free conditions (Figure 4). After 90 min, rates of bile secretion were about twice those found with high dose troglitazone without BSA ($P < 0.00001$), but still $\sim 37\%$ lower than in BSA-free control conditions ($P < 0.00001$). Portal pressure was similar in all three experimental groups (Figure 4).

Effect of insulin on acute troglitazone action

In order to compare the acute troglitazone effect on insulin action under both basal and glucagon-stimulated conditions, insulin (5 nM) $\pm$ troglitazone ($0.61 \mu\text{M}$) was infused during the basal period from zero time to 60 min and glucagon was added from $t = 60$ –120 min. Glucagon was used at a 10 fold lower concentration than in the previous studies in order to examine effects of insulin and troglitazone on stimulated glucose production. Insulin alone and with low troglitazone decreased ($P < 0.05$) hepatic glucose production rates during the basal period and also during glucagon stimulation (Figure 5). Similarly, incremental glucose release was lower with insulin alone than either control or insulin + low dose troglitazone during the basal period (Table 1). Under glucagon-stimulated

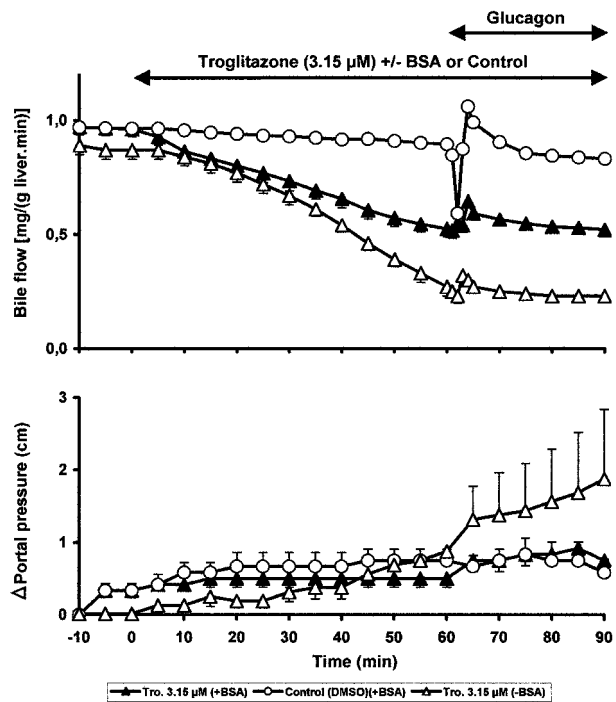


Figure 4 Effect of BSA on acute troglitazone action. Time-course of bile flow and Δ portal pressure during the basal period and during glucagon stimulation (1.0 nM) in the isolated perfused rat liver. Effect of troglitazone infusion ($3.15 \mu\text{M}$) with ($n = 6$) or without ($n = 6$, see also Figure 1) addition of 0.2% BSA compared with control (Krebs-Henseleit buffer + 0.2% DMSO + 0.2% BSA; $n = 6$). Data are given as means $\pm$ s.e.mean.

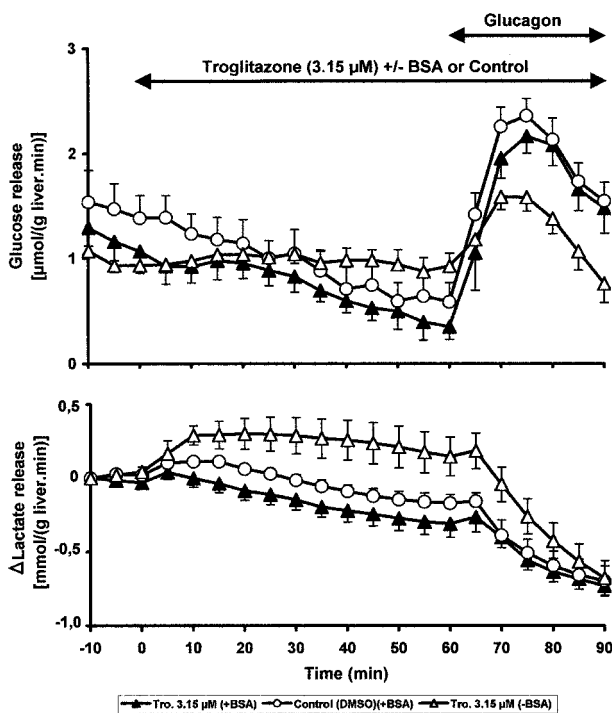


Figure 3 Effect of BSA on acute troglitazone action. Time-course of glucose production rates and Δ lactate release during the basal period and during glucagon stimulation (1.0 nM) in the isolated perfused rat liver. Effect of troglitazone infusion ($3.15 \mu\text{M}$) with ($n = 6$) or without ($n = 6$, see also Figure 1) addition of 0.2% BSA compared with control (Krebs-Henseleit buffer + 0.2% DMSO + 0.2% BSA; $n = 6$). Data are given as means $\pm$ s.e.mean.

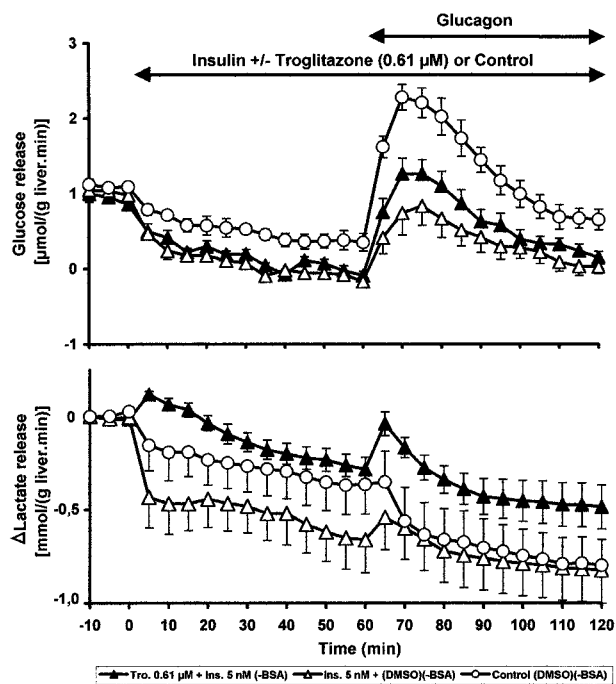


Figure 5 Effect of insulin on acute troglitazone action. Time-course of glucose production rates and Δ lactate release during the basal period and during glucagon stimulation (0.1 nM) in the isolated perfused rat liver. Effect of infusion of insulin with or without troglitazone ($0.61 \mu\text{M}$) compared with control (Krebs-Henseleit buffer + 0.2% DMSO) under BSA-free conditions. Data are given as means $\pm$ s.e.mean of six experiments in each group.

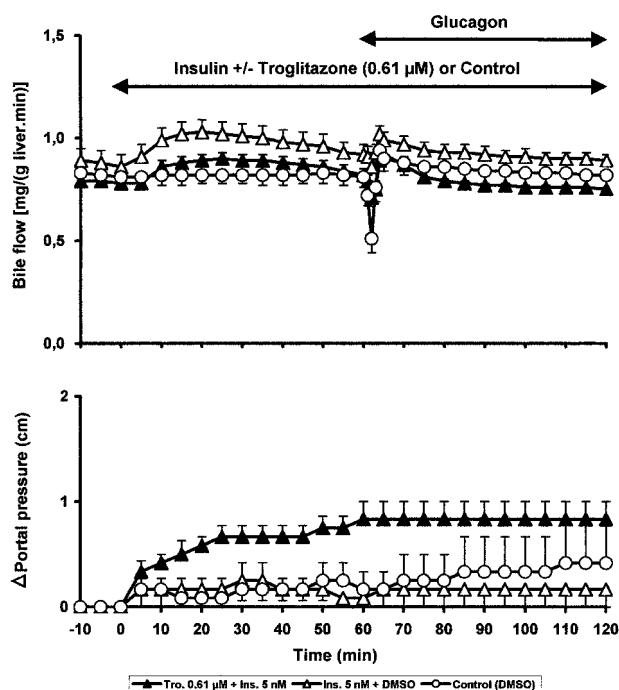


Figure 6 Effect of insulin on acute troglitazone action. Time-course of bile flow and Δ portal pressure during the basal period and during glucagon stimulation (0.1 nM) in the isolated perfused rat liver. Effect of infusion of insulin with or without troglitazone (0.61 μ M) compared with control (Krebs-Henseleit buffer + 0.2% DMSO) under BSA-free conditions. Data are given as means $\pm$ s.e. mean of six experiments in each group.

conditions, only insulin alone resulted in a decrease of glucose release; the gradual fall in glucose release observed with low dose troglitazone did not reach significance. Insulin induced a small decrease ($P < 0.01$) in lactate production rates with rapid onset; this was not seen when insulin and low dose troglitazone were administered together (Figure 5).

Low dose troglitazone with or without insulin did not affect hepatic bile secretion (Figure 6). Portal pressure rapidly (in cm buffer height) increased within 15 min during infusion of low dose troglitazone + insulin (0.54 ± 0.07) and was higher than observed during infusion of insulin (0.17 ± 0.07 , $P < 0.005$) or vehicle alone (0.08 ± 0.06 , $P < 0.0001$; Figure 6).

Discussion

Acute troglitazone exposure did not decrease basal hepatic glucose production, but inhibited glucagon-stimulated glucose release. The effect was observed only at perfusate troglitazone concentrations of 3.15 μ M, which is ~ 6 fold higher than plasma concentrations measured in effectively treated rodents (Lee *et al.*, 1994; Khourshed *et al.*, 1995). It is of note that 3.15 μ M troglitazone was ineffective in the presence of albumin as reported for isolated rat muscle (Fürnsinn *et al.*, 1997). This is most likely due to $\sim 99.9\%$ binding of troglitazone to albumin solutions and plasma (Shibukawa *et al.*, 1995) making it difficult to compare *in vitro* concentrations with those measured in plasma during treatment.

The present study employed an experimental model of hepatic glycogenolysis, since the livers from fed rats are full of glycogen and perfusion with 5 mM glucose favours hepatic glucose release (Brunengraber *et al.*, 1973). The observed

partial inhibition of glucagon-stimulated glucose production is therefore primarily due to decreased glycogenolysis. The reduction by troglitazone of glucagon-stimulated glycogenolysis can be interpreted rather as an unspecific toxic effect than an acute insulin-like action as shown for other compounds (Roden *et al.*, 1995). Previous reports examined effects of thiazolidinediones on lactate-dependent gluconeogenesis in isolated rat hepatocytes. At high concentrations of 100–1000 μ M, pioglitazone and more effectively troglitazone inhibited free fatty acid oxidation, triglyceride synthesis, and glucose production from lactate and pyruvate (Fulgencio *et al.*, 1996). The decrease of free fatty acid oxidation could likely account for the observed reduction of gluconeogenesis. But although the authors argued that a generalized toxic effect is unlikely, since other metabolic actions like phospholipid synthesis or carnitine palmitoyl transferase-1 activity were not affected, an unspecific troglitazone effect cannot be excluded from those data. By using lower troglitazone concentrations (30 μ M) gluconeogenesis decreased by $\sim 23\%$ in hepatocytes obtained from fasted rats, but not in those from fed rats (Raman *et al.*, 1998). It has been suggested (Raman *et al.*, 1998; Nishimura *et al.*, 1997) that thiazolidinediones decrease gluconeogenesis by elevation of hepatocyte fructose-2,6-bisphosphate which inhibits the key enzyme of gluconeogenesis, fructose-1,6-bisphosphatase (Van Schaftingen & Hers, 1981), while it activates the glycolytic key enzyme 6-phosphofructo-1-kinase (Pilkis *et al.*, 1990). However, considering the high concentrations employed in these studies, the decrease of lactate-dependent gluconeogenesis may have also resulted from an unspecific thiazolidinedione effect which would favour the glycolytic flux and thereby reduce gluconeogenesis. Recently it was reported that troglitazone at a comparable concentration of 3.25 μ M acutely induces an intracellular shift of glucosyl units into glycolysis and rather mimicks hypoxic than insulin-like action in isolated rat muscle (Fürnsinn *et al.*, 1997).

This study also found that 3.15 μ M troglitazone transiently increased hepatic lactate production. During simultaneous insulin exposure, lactate production rates were elevated even in the presence of 0.61 μ M troglitazone as observed in isolated rat muscle tissue (Fürnsinn *et al.*, 1997). In liver, insulin does not directly affect glucose transport but directs the flux of intracellular glucose to glycogen synthesis so that lactate production will decrease. Thus, the observed lactate release by troglitazone cannot be attributed to an insulin-like effect but rather indicates augmentation of anaerobic glycolysis in the liver cell, which is in keeping with a hypoxia-like mechanism for acute troglitazone action. It is of note that troglitazone must have led only to moderate hypoxia in the present study, since severe hypoxia or anoxia would have rapidly increased intracellular adenosine-monophosphate (AMP) or adenosine-diphosphate (ADP), which activate phosphorylase and thereby increase glycogenolysis (Hems & Whitton, 1980; Cheng *et al.*, 1985). During the not-stimulated perfusion period, no such increase in glycogenolysis was induced by troglitazone. Interestingly, higher troglitazone concentrations increase the ADP/ATP ratio, but not glucose production in hepatocytes obtained from fed rats (Fulgencio *et al.*, 1996).

Independently of the presence of BSA, 3.15 μ M troglitazone rapidly decreased bile flow during the basal period. Such acute reduction of bile flow has been described for classical hepatotoxic agents like carbon tetrachloride (Fracasso *et al.*, 1980) or phenothiazine (Eckhart *et al.*, 1963). The explanation for this action may be direct effects on the hepatocyte resulting in decreased bile formation and/or indirect effects resulting from redistribution of hepatic microcirculation (Tavoloni *et al.*

al., 1979; Krell & Dietze, 1989). The latter might in turn also increase portal pressure, which we noted to some extent in the present study. However, the effect of troglitazone on portal pressure was not as consistent as that on bile flow. In contrast, the concept of direct thiazolidinedione action on hepatocyte function is supported by pharmacokinetic studies demonstrating troglitazone uptake and metabolism by livers of rats, mice, and dogs and high biliary excretion rates of the resulting metabolites (Kawai *et al.*, 1997). This might explain the mild impairment of liver function, which was observed in ~2% of patients enrolled in clinical troglitazone trials, as well as the rare cases of severe liver failure requiring liver transplantation (Neuschwander-Tetri *et al.*, 1998).

Taken together, troglitazone given at concentrations generally used to study its metabolic actions decreased

glucagon-stimulated glucose production, but also increased lactate production, reduced bile flow, and slightly increased portal pressure. These data therefore provide evidence for an acute, non-insulin-like toxic effect of troglitazone in perfused liver.

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