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SPECIAL REPORT

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Inhibition by L-glutamine of the release of endothelium-derived relaxing factor from cultured endothelial cells

[1]Markus Hecker, Jane A. Mitchell, Tomasz A. Swierkosz, William C. Sessa & John R. Vane

The William Harvey Research Institute, St. Bartholomew's Hospital Medical College, London EC1 6BQ

L-Glutamine (0.02–2 mM) but not D-glutamine (0.2 mM and 2 mM) inhibited the release of endothelium-derived relaxing factor (EDRF) from bovine aortic endothelial cells cultured in the presence or absence of L-arginine. Inhibition was maximal at a concentration of 200 μM, and was reversed by L-arginine (50 μM) but not D-arginine (100 μM). L-Glutamic acid (2 mM) or ammonium chloride (1 mM), putative products of the metabolism of L-glutamine in endothelial cells, had no effect on EDRF release. L-Glutamine (0.2 mM and 2 mM) but not D-glutamine (2 mM), L-glutamic acid (2 mM) or ammonium chloride (1 mM) also inhibited the generation of L-arginine in endothelial cells. Thus, L-glutamine inhibits EDRF release by preventing the generation of L-arginine.

Introduction Endothelium-derived relaxing factor (EDRF) has been identified as nitric oxide or a closely related molecule derived from the guanidino group of L-arginine (L-Arg) (Palmer *et al.*, 1988a). The release of EDRF from cultured endothelial cells is inhibited by L-Arg analogues, such as N^G-monomethyl-L-arginine, an effect that is reversed by L-Arg but not D-Arg (Palmer *et al.*, 1988b). However, L-Arg elicits little or no relaxation of freshly isolated vascular preparations (Thomas *et al.*, 1989), probably due to their high L-Arg content (Hecker *et al.*, 1990). These findings indicate that L-Arg is the endogenous substrate of EDRF biosynthesis and that its availability is rate-limiting for EDRF release.

EDRF release from cultured endothelial cells appears to depend on the generation of L-Arg (Mitchell *et al.*, 1990a). L-Arg is generated when the cells are transferred from culture medium to Krebs solution, suggesting an inhibitory factor to be present in the medium. Interestingly, this transfer coincided with a rapid fall of L-glutamine (L-Gln) (Mitchell *et al.*, 1990b). As L-Gln was also a major constituent of the culture medium, we investigated whether L-Gln inhibits the release of EDRF from cultured endothelial cells.

Methods Endothelial cells cultured from bovine aorta were grown on Cytodex-3 microcarrier beads as described (de Nucci *et al.*, 1988). After reaching confluence, they were cultured for another 24 h in either Dulbecco's modified Eagle's medium (DMEM) containing 0.6 mM L-Arg and 4 mM L-Gln or Eagle's minimum essential medium (MEM) containing 2 mM L-Gln but not L-Arg. The cells were packed into a jacketed chromatography column (approx. 6×10^7 cells/column) and perfused at 5 ml min^{-1} with warmed (37°C), oxygenated (95% O_2/5% CO_2) Krebs solutions containing indomethacin (5.6 μM) and superoxide dismutase (10 u ml^{-1}). The effluent from the column superfused a cascade (Vane, 1964) of 4 spirally cut rabbit aortic strips (RbA) denuded of endothelium and preconstricted with 30 nM 9,11-dideoxy-9_α,11_α-methanoepoxy-prostaglandin $F_{2\alpha}$ (U46619). The length of the RbAs was monitored with auxotonic levers attached to a Watanabe WR3101 recorder. Glyceryl trinitrate (GTN) was used to calibrate the response of the detector tissues which were electronically adjusted to give similar relaxations to a standard dose. EDRF release from endothelial cells is characterized by its progressive disappearance down the cascade. The intracellular concentration of L-Arg, L-Gln and other amino acids was determined by reversed phase high performance liquid chromatography/fluorescence detection analysis as previously described (Hecker *et al.*, 1990). Adenosine diphosphate di(monocyclohexylammonium) salt (ADP), the amino acids and indomethacin were obtained from Sigma Chemical Co. (Poole, U.K.). Superoxide dismutase from bovine erythrocytes was a gift from Grünenthal (Stolberg, West Germany). GTN (Nitronal) was supplied by Lipha Pharmaceuticals Ltd (West Drayton, U.K.).

Results The effect of L-Gln on the release of EDRF from endothelial cells cultured in the presence of L-Arg is shown in Figure 1. L-Gln (0.2 mM and 2 mM) had no effect on the detector tissues but significantly reduced the release of EDRF induced by ADP (46.5 ± 5.5% inhibition ($n = 8$) and 48.1 ± 7.2% inhibition ($n = 3$); mean ± s.e.mean; $P < 0.05$) when infused through the column of endothelial cells. This inhibition was overcome by co-infusions of 100 μM L-Arg (83.6 ± 5.7% of control ($n = 6$) and 106.0 ± 2.7% of control ($n = 3$); $P < 0.05$) but not D-Arg (53.5 ± 9.9% of control in the presence of 200 μM L-Gln), ($n = 3$).

Infusions of L-Gln (2 mM) similarly inhibited the release of EDRF from Arg-depleted endothelial cells (Figure 2). As described previously (Mitchell *et al.*, 1990a), there was a time-dependent increase in stimulated EDRF release over 60 min which was prevented when L-Gln (0.2 mM or 2 mM) was present from the beginning of the experiment ($n = 3$). The release of EDRF induced by ADP (6 nmol) was attenuated by L-Gln in concentrations as low as 20 μM (31.1 ± 2.3% inhibition ($n = 3$); $P < 0.05$) and maximally inhibited at 200 μM (52.5 ± 4.5% inhibition ($n = 3$) as compared to 53.2 ± 4.3% inhibition ($n = 4$) at 2 mM). L-Arg (50 μM) but not D-Arg reversed the inhibitory effect of 2 mM L-Gln (94.6 ± 10.2% of control), ($n = 4$). D-Gln (0.2 mM and 2 mM), L-glutamic acid (0.2 mM and 2 mM) or ammonium chloride (1 mM) had no effect on EDRF release ($n = 4$).

When transferred to Krebs solution for 60 min at 37°C, endothelial cells cultured in the absence or presence of L-Arg increased their L-Arg levels by 9.5 ± 2.0 fold from 34 ± 4 μM to 210 ± 24 μM ($n = 26$) and 4.5 ± 1.1 fold from 160 ± 21 μM to 641 ± 125 μM ($n = 6$), respectively. L-Gln (0.2 mM and 2 mM) significantly inhibited the rise in intracellular L-Arg in both Arg-depleted (81.5 ± 6.3% inhibition ($n = 5$) and 79.7 ± 10.0% inhibition ($n = 6$); $P < 0.05$) and non-depleted cells (95.9 ± 2.1% inhibition at 0.2 mM ($n = 4$); $P < 0.05$). D-Gln (2 mM) potentiated (2.4 ± 0.7 fold), ($n = 3$) rather than inhibited the generation of L-Arg, whereas L-glutamic acid (2 mM) and ammonium chloride (1 mM) were not active (16.5 ± 7.2% inhibition and 14.1 ± 7.9% inhibition), ($n = 4$).

[1] Author for correspondence.

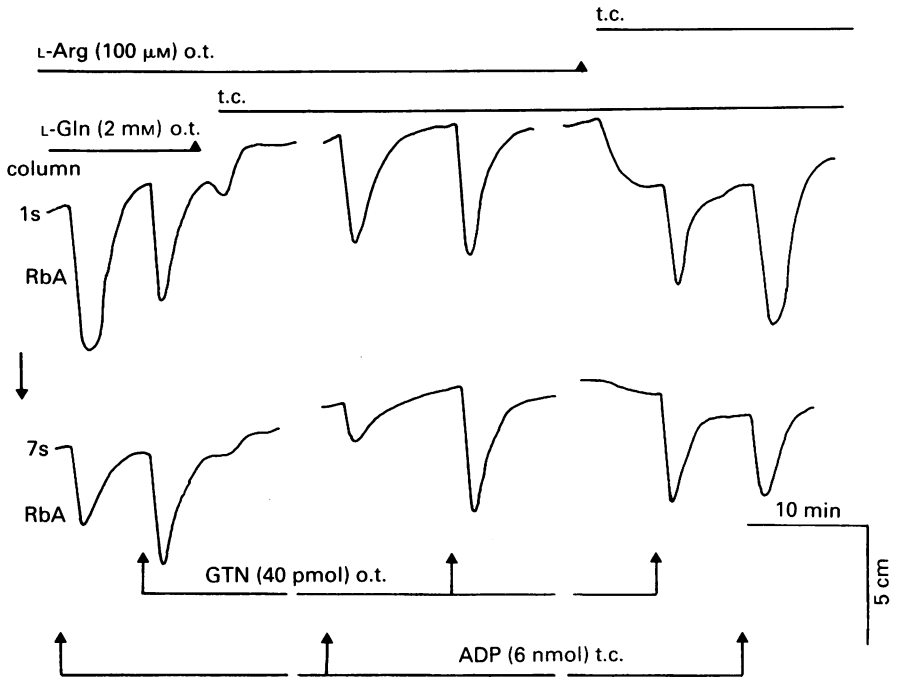

Figure 1 The effect of L-glutamine (L-Gln) on the release of endothelium-derived relaxing factor (EDRF) from endothelial cells cultured in the presence of L-arginine (L-Arg). Of four rabbit aortic strips (RbA) two are shown preconstricted with 30 mM U46619 and separated by 1 s and 7 s from the column of endothelial cells. Adenosine diphosphate (ADP, 6 nmol) injected through the column of endothelial cells (t.c.) elicited a release of EDRF which caused a greater relaxation of the top RbA than glyceryl trinitrate (GTN, 40 pmol) given over the tissues (o.t.) L-Gln (2 mM) infused t.c. caused an increase in the tone of the RbAs and a reduction in stimulated EDRF release (the relaxation of the top RbA was smaller as compared to GTN). These effects were reversed by a co-infusion of L-Arg (100 μM). Relaxations to GTN (40 pmol) given o.t. were not affected.

Discussion Endothelial cells cultured in the absence of L-Arg ($[Arg]_i < 10\,\mu M$) generate L-Arg ($[Arg]_i > 100\,\mu M$) when transferred to Krebs solution and the availability of L-Arg appears to be rate-limiting for EDRF release (Mitchell *et al.*, 1990a). As the generation of L-Arg in these cells coincided with a rapid fall in L-Gln (Mitchell *et al.*, 1990b), probably due to their high glutaminase activity (Leighton *et al.*, 1987), and since L-Gln was a major constituent of the culture medium (2 mM), it was tempting to speculate that L-Gln may block the generation of L-Arg and, hence, EDRF biosynthesis. Indeed, L-Gln but not D-Gln inhibited the release of EDRF from both Arg-depleted and non-depleted endothelial cells. L-Gln also

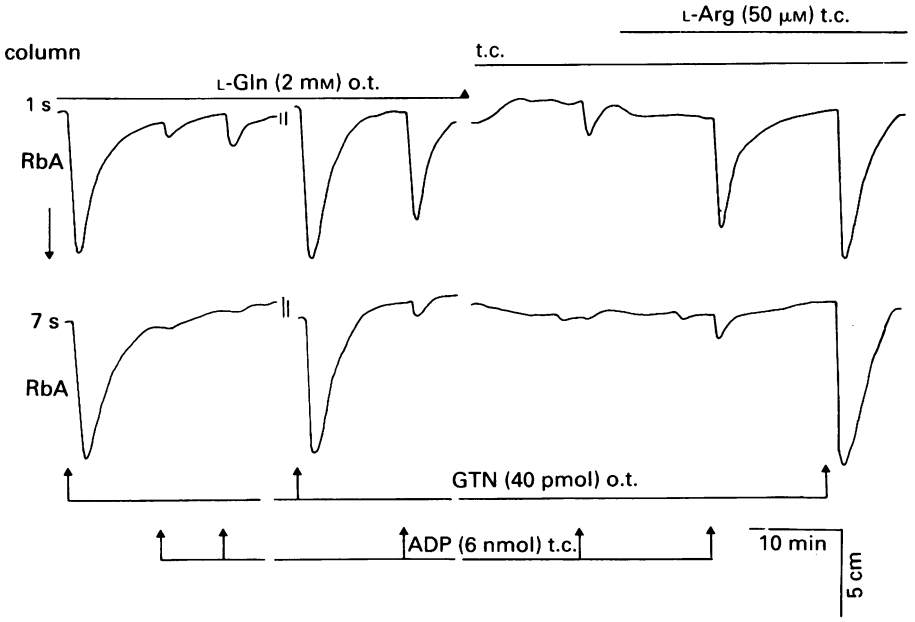

Figure 2 The effect of L-glutamine (L-Gln) on the release of endothelium-derived relaxing factor (EDRF) from endothelial cells cultured in the absence of L-arginine (L-Arg) for 24 h. Two of four rabbit aortic strips (RbA) are shown separated as in Figure 1. Repetitive injections of adenosine diphosphate (ADP, 6 nmol) through the column of endothelial cells (t.c.) elicited a release of EDRF which increased over 60 min. L-Gln (2 mM) infused t.c. reduced the release of EDRF induced by ADP. This effect was reversed by a subsequent co-infusion of L-Arg (50 μM). Relaxations induced by glyceryl trinitrate (GTN) given over the tissues (o.t.) were not affected.

prevented the time-dependent increase in stimulated EDRF release from Arg-depleted endothelial cells. This process is probably linked to the generation of L-Arg (Mitchell *et al.*, 1990a), and, consequently, the effect of L-Gln on the generation of L-Arg was investigated. L-Gln but not D-Gln, L-glutamic acid or ammonium chloride inhibited the generation of L-Arg by both Arg-depleted and non-depleted endothelial cells in the same concentration range as in the bioassay experiments, strongly suggesting that L-Gln, indeed, inhibits EDRF biosynthesis by blocking the generation of L-Arg.

L-Gln also stereospecifically inhibited the release of EDRF from non-depleted endothelial cells, supporting the notion that these cells maintain their L-Arg level and, hence, sustain their ability to release EDRF by generating L-Arg possibly from L-citrulline, the by-product of EDRF biosynthesis (Mitchell *et al.*, 1990b). The glutaminase metabolites, L-glutamic acid and ammonia, had no effect on either EDRF release or L-Arg generation. Although the precise mechanism by which L-Gln interferes with the generation of L-Arg remains to be elucidated, this finding together with the lack of inhibitory effect of D-Gln indicates a direct interaction of L-Gln with the enzyme(s) involved in L-Arg generation.

L-Gln is the most abundant amino acid in the body (Bulus *et al.*, 1989), its concentrations is 0.5–0.9 mM in plasma and about 2 mM in tissues or cells, including endothelial cells (Mitchell *et al.*, 1990b). The inhibition of EDRF release by L-Gln *in vitro* was maximal at 200 μM, a concentration well within the physiological range. Thus, L-Gln may be an endogenous inhibitor of EDRF biosynthesis *in vivo* when the plasma level of L-Arg is low or when the uptake of L-Arg by the endothelium is limited.

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References

BULUS, N., CERSOSIMO, E., GHISHAN, F. & ABUMRAD, N.N. (1989). Physiologic importance of glutamine. *Metab. Clin. Exp.*, **38** (Suppl. 1), 1–5.

DE NUCCI, G., GRYGLEWSKI, R.J., WARNER, T.D. & VANE, J.R. (1988). Receptor mediated release of endothelium-derived relaxing factor and prostacyclin from bovine aortic endothelial cells is coupled. *Proc. Natl. Acad. Sci., U.S.A.*, **85**, 2334–2338.

HECKER, M., MITCHELL, J.A., HARRIS, H.J., KATSURA, M., THIEMERMANN, C. & VANE, J.R. (1990). Endothelial cells metabolize N^G-monomethyl-L-arginine to L-citrulline and subsequently to L-arginine. *Biochem. Biophys. Res. Commun.*, **176**, 1037–1043.

LEIGHTON, B., CURI, R., HUSSEIN, A. & NEWSHOLME, E.A. (1987). Maximum activitities of some key enzymes of glycolysis, glutaminolysis, Krebs cycle and fatty acid utilization in bovine pulmonary endothelial cells. *FEBS Lett.*, **225**, 93–96.

MITCHELL, J.A., HECKER, M. & VANE, J.R. (1990a). The generation of L-arginine in endothelial cells is linked to the release of endothelium-derived relaxing factor. *Eur. J. Pharmacol.*, **176**, 253–254.

MITCHELL, J.A., HECKER, M., KATSURA, M., ÄNGGÅRD, E.E. & VANE, J.R. (1990b). Relationship between L-arginine concentration and EDRF release in bovine aortic endothelial cells. *Br. J. Pharmacol.*, **99**, 101P.

PALMER, R.M.J., ASHTON, D.S. & MONCADA, S. (1988a). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664–666.

PALMER, R.M.J., REES, D.D., ASHTON, D.S. & MONCADA, S. (1988b). L-Arginine is the physiological precursor for the formation of nitric oxide and endothelium-dependent relaxation. *Biochem. Biophys. Res. Commun.*, **153**, 1251–1256.

THOMAS, G., HECKER, M. & RAMWELL, P.W. (1989). Vascular activity of polycations and basic amino acids: L-arginine does not specifically elicit endothelium-dependent relaxation. *Biochem. Biophys. Res. Commun.*, **158**, 177–180.

VANE, J.R. (1964). The use of isolated organs for detecting active substances in the circulating blood. *Br. J. Pharmacol. Chemother.*, **23**, 360–373.

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Electrical effects of okadaic acid extracted from black sponge on rabbit sinus node

[1]Noriaki Kondo, Itsuo Kodama, Hiroshi Kotake & Shoji Shibata

Department of Pharmacology, University of Hawaii, Honolulu, Hawaii, U.S.A.; Mitsubishi-Kasei Institute of Life Sciences, Machida, Tokyo, Japan; Department of Circulation and Respiration, The Research Institute of Environmental Medicine, Nagoya University, Nagoya, Japan and Department of Medicine, Tottori University, Tottori, Japan

1 Effects of okadaic acid on electrical responses and spontaneous activity in the dominant pacemaker cells of rabbit sinus node were investigated by use of microelectrode techniques.

2 Okadaic acid (10^{-5} M to 4×10^{-5} M) caused a shortening of cycle length of spontaneous firing (SPCL) accompanied by increases in both maximum upstroke velocity at phase 0 ($\dot{V}_{max}$) and amplitude of action potential.

3 All of the effects of okadaic acid were relatively well preserved in a low-Ca^{2+} medium (0.12 mM). Okadaic acid restored the spontaneous activity of sinus node pacemaker cells even in a Ca^{2+}-deficient medium.

4 The effects of okadaic acid were markedly inhibited or abolished in a low Na^+ medium (24 mM or 70 mM) and in the presence of a slow channel blocking agent, verapamil (10^{-6} M).

5 In voltage-clamp experiments using a two-microelectrode technique, okadaic acid (10^{-5} M) caused an increase in the slow inward current without affecting the outward current. At a higher concentration (4×10^{-5} M), the drug increased the outward current.

6 These results indicate that okadaic acid causes an increase in spontaneous activity of sinus node pacemaker cells mediated by an enhancement of slow inward current (I_{si}) through verapamil-sensitive Ca^{2+} channels.

Introduction

Okadaic acid is a monocarboxylic acid ($C_{44}H_{66}O_{13}$) isolated from a common black sponge, *Halichondria* (Tachibana *et al.*, 1981). The molecular structure of this substance is reminiscent of the Ca^{2+} ionophores A-23187 and X-537A but its pharmacological properties are markedly different (Shibata *et al.*, 1982). Okadaic acid causes tonic and long-lasting contraction of smooth muscle even in the absence of Ca^{2+} (Shibata *et al.*, 1982; Shibata, 1987). In saponin-treated 'skinned' smooth muscle in the absence of Ca^{2+} (Ozaki *et al.*, 1987b), this agent causes a contraction. Recently, such a contraction has been attributed to myosin light-chain phosphorylation (Ozaki *et al.*, 1987; Bialojan *et al.*, 1988; Erdodi *et al.*, 1988) mediated through inhibition of myosin phosphatase activity by okadaic acid (Bialojan *et al.*, 1988; Erdodi *et al.*, 1988). In cardiac muscle, okadaic acid also causes an increase in the contractile force induced by electrical stimuli (Kodama *et al.*, 1986). This positive inotropic effect, unlike the case of vascular smooth muscle, was blocked by Ca^{2+} channel blockers and reducing $[Ca]_o$. Our previous study on guinea-pig isolated ventricular muscle (Kodama *et al.*, 1986) further suggested that this effect may be mediated by an enhancement of slow Ca^{2+} inward current (I_{si}) through the cell membrane. A similar effect of I_{si} was reported in isolated myocytes from guinea-pig cardiac ventricle (Hescheler *et al.*, 1988). This study suggested that the effect of okadaic acid on I_{si} is related to suppressed dephosphorylation through inhibition of protein phosphatase activity by this agent. This may result in facilitation of Ca^{2+} channel activation through an increase in phosphorylated Ca^{2+} channels. Thus, okadaic acid is a unique substance that exerts a cardiotonic effect probably through the inhibition of the protein phosphatases. In the present study, in order to evaluate the effectiveness of okadaic acid on spontaneous activity of the sinus node cell, we examined its action on electrical responses of sinus node pacemaker cells where I_{si} plays an important role in the genesis of spontaneous activity and the action potential.

[1] Author for correspondence at: Department of Pharmacology, Mitsubishi-Kasei Institute of Life Sciences, Machida, Tokyo 194, Japan

Methods

Thirty five rabbits of either sex, weighing 1.5 to 2.0 kg, were anaesthetized by sodium pentobarbitone (30 mg kg^{-1}, i.v.). The hearts were excised rapidly and placed in oxygenated Krebs-Ringer solution to obtain small specimens of sinus node pacemaker tissue. Since the procedure has been given in detail in a previous paper (Kodama & Boyett, 1985), it is described here only briefly. The right atrium was separated from the remaining part of the heart and was opened by a longitudinal incision in the free wall to expose the endocardial surface. After the sinus node had been identified in the centre of the intercaval region, several strands of tissue (0.2–0.4 mm in width, 2 mm in length) were cut from the sinus node in a direction perpendicular to the crista-terminalis. They were trimmed with a razor blade leaving the endocardial surface intact, and tied by a fine silk thread to obtain small ball-like specimens composed of dominant pacemaker cells. The final size of these specimens was about 0.3 mm in diameter. The distance from the right branch of the sinoatrial ring bundle to the centre of the specimen was 1.0 to 1.5 mm. These specimens usually resumed their spontaneous activity 10–30 min after the ligatures were tied. Once the dissection procedure was complete, the specimens were transferred to another tissue bath, which was also superfused with Krebs-Ringer solution gassed with a mixture of 95% O_2 and 5% CO_2 at 30°C. The rate of solution flow was 5 ml min^{-1}. The composition of the solution was as follows (mM): NaCl 120.3, KCl 4.8, $CaCl_2$ 1.2, $MgSO_4 \cdot 7H_2O$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 24.2 and glucose 5.5 (pH 7.4). In the low-Ca^{2+} solution, the $CaCl_2$ concentration was reduced to 0.12 mM, and in the Ca^{2+}-deficient solution the $CaCl_2$ was omitted. In the low-Na^+ solution, the Na^+ concentration was reduced to 70 mM or 24 mM by replacing NaCl with choline chloride or with lithium chloride, respectively.

When choline chloride was used as a substitute, atropine sulphate (10^{-6} M) was added to the solution to eliminate cholinergic side-effects.

Transmembrane potentials were recorded with two glass microelectrodes filled with 3 M KCl, one intracellularly and the other extracellularly placed closely. Each electrode was connected by an Ag-AgCl wire to a high-input impedance buffer amplifier connected to a differential amplifier. The action potential was differentiated electronically in order to measure the maximum upstroke velocity ($\dot{V}_{max}$). Cycle length of spontaneous firing (SPCL) was determined by measuring the interval between two successive spikes of the first derivative of action potentials.

The preparations were allowed to equilibrate in the tissue bath for about 1 h before any tests were performed. Sixteen out of 43 ball-like specimens (37%) showed unstable or no spontaneous activity during this equilibration period and they were omitted. The remaining 27 specimens with regular and stable spontaneous activity were used for the present experiments.

In the voltage clamp experiments, a two-microelectrode voltage clamp technique was employed. Two conventional glass microelectrodes filled with 3M KCl (10–30 MΩ) were used, one to record the membrane potential and the other to apply the current intracellularly. The membrane potential and current were displayed on an oscilloscope (Kikusui Denshi, 5516 ST), and recorded on cassette tape recorder (TEAC, MR-10) and with a penrecticorder (Nihon Kohden, RJG-4100). The principle of the feedback circuit was the same as that reported by Noma et al. (1980) and a virtual ground amplifier, essentially the same as that described by New & Trautwein (1972), was used. When both microelectrodes recorded identical action potentials, the preparation was clamped to −40 mV (holding potential) where the holding current was nearly zero (Noma et al., 1979).

The following drugs were used: okadaic acid (Fujisawa, Tokyo, Japan); verapamil (Knoll, Whippany, West Germany); tetrodotoxin (TTX, Sankyo Tokyo, Japan). The preparations were superfused for 20–40 min with Krebs-Ringer solution containing these drugs. The okadaic acid was dissolved in absolute ethanol, and then diluted with deionized water. The maximum concentration of ethanol in the superfusate was 0.4%.

Values were expressed as the mean ± s.e. unless otherwise stated. Statistical significance was evaluated by a paired t test, and P values of less than 0.05 were considered to indicate a significant difference. More details of each procedure are given in the Results.

Results

Transmembrane action potential under control conditions

The observed transmembrane potentials always showed a smooth transition from phase 4 to phase 0 (Figure 1). Furthermore, there were no changes in the shape of the action potential that were suggestive of a shift of a pacemaker site within the small specimens. These observations suggest that there was a nearly uniform distribution of transmembrane potential within the specimens. This is to be expected because the diameter (about 0.3 mm) of the small specimens was less than the space constant (0.5–0.8 mm) of this tissue (Bonke, 1973; Seyama, 1976). The amplitude of the action potential (APA), maximum diastolic potential (MDP), $\dot{V}_{max}$ and SPCL under control conditions are presented in Tables 1 to 3. These values are similar to those recorded from the leading pacemaker site of the intact rabbit sinus node (Bleeker et al., 1980; Kodama & Boyett, 1985).

Treatment of the preparations with TTX (10^{-6} M) for 30 min did not affect the shape of action potential (not shown in Figures or Tables). This may mean that the fast sodium inward current (I_{Na}) does not contribute to the action potentials of these preparations.

Effects of okadaic acid

Effects of okadaic acid on action potentials were examined in five preparations bathed in the normal medium. Okadaic acid at 10^{-5} M caused a shortening of SPCL (increase in firing rate) and an increase in $\dot{V}_{max}$ (Figure 1, Table 1), but had no effect on either the amplitude of the action potential (APA) or the maximum diastolic potential (MDP). Okadaic acid at a higher

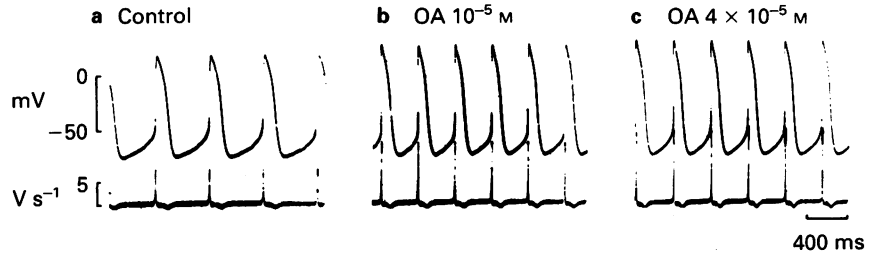

Figure 1 Effects of okadaic acid (OA) on the action potential of sinus node pacemaker cells. (a) Control; (b) 10^{-5} M okadaic acid; (c) 4×10^{-5} M okadaic acid. Panels (b) and (c) were recorded 20 min after application of okadaic acid. The upper trace represents the action potential and the lower trace shows the first derivative of the action potential.

Table 1 Effects of okadaic acid on the membrane action potentials of sinus node cells

	APA (mV)	*MDP* (mV)	$\dot{V}_{max}$ (Vs^{-1})	*SPCL* (ms)
Control	83.6 ± 1.8	69.1 ± 1.0	8.1 ± 0.6	462 ± 18
Okadaic acid (10^{-5} M)	87.1 ± 2.7	69.3 ± 0.8	11.2 ± 1.0*	378 ± 19*
Okadaic acid (4×10^{-5} M)	93.5 ± 3.0*	70.6 ± 0.6	13.9 ± 1.9*	324 ± 5*

Values are means ± s.e. ($n = 5$) before and 20 min after application of okadaic acid; APA: action potential amplitude; MDP: maximum diastolic potential; $\dot{V}_{max}$: maximum upstroke velocity at phase 0; SPCL: spontaneous firing cycle length. *Significantly different from the values of control at $P < 0.05$.

concentration (4×10^{-5} M) caused a significant increase in APA, but had no effect on MDP.

The onset of action of okadaic acid was approximately 6 min after application of the substance and the maximum effect was observed with 20 min. After washing out the drug, all the parameters of the action potential gradually returned to the control levels within 30 min.

Effects of the vehicle (ethanol) were examined in three preparations. The highest concentration of ethanol used in the present study (0.4%) had no effect on either the action potential or spontaneous activity.

Low-calcium medium

In eight preparations, the effects of okadaic acid were examined in either a low Ca^{2+} or a Ca^{2+}-deficient medium. When the preprations were superfused with Krebs-Ringer solution containing 0.12 mM Ca^{2+} for 20 min, APA and $\dot{V}_{max}$ were progressively decreased, resulting in the complete cessation of spontaneous activity at the resting membrane potential (-44.4 ± 4.0 mV, $n = 4$). In such preparations, the treatment with okadaic acid (4×10^{-5} M) restored regular spontaneous action potentials. APA, MDP and $\dot{V}_{max}$ of these action potentials were all considerably less than those observed under the control conditions, but SPCL was comparable to the control value (Figure 2, Table 2). Similar restoration of spontaneous activity by okadaic acid, but with much smaller APA and $\dot{V}_{max}$, was observed in the Ca^{2+}-deficient medium (Figure 2, Table 2).

Low-sodium medium

In the other eight preparations, the effects of okadaic acid were tested in the presence of low concentrations of Na^+. When the preparations were superfused with Krebs-Ringer solution containing 70 mM Na^+ for 20 min, APA, MDP and $\dot{V}_{max}$ were decreased and SPCL was prolonged significantly. In such preparations okadaic acid (4×10^{-5} M) caused a shortening of SPCL, but the other parameters of the action potential (MDP, APA, and $\dot{V}_{max}$) were not significantly affected (Figure 3, Table 3).

When the preparations were exposed to Krebs-Ringer solution containing 24 mM Na^+, APA and $\dot{V}_{max}$ were progressively decreased, resulting in complete cessation of spontaneous activity at the resting potential (-35.6 ± 3.1 mV, $n = 4$). In such preparations, okadaic acid (4×10^{-5} M) failed to restore the spontaneous activity (Figure 3, Table 3). The resting potential was also unaffected by this substance.

Influence of verapamil

Effects of okadaic acid were examined in four preparations pretreated with a cardiac slow-channel blocker, verapamil.

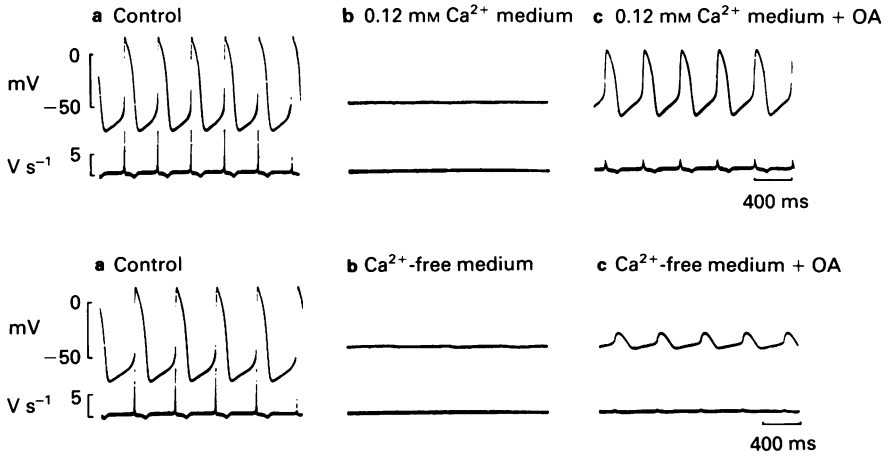

Figure 2 Effects of okadaic acid (OA) on the membrane action potential of sinus node pacemaker cells in low-Ca^{2+} and Ca^{2+}-free medium. Upper and lower panels show experiments in a low-Ca^{2+} (0.1 mM) and Ca^{2+}-free medium respectively. (a) Control; (b) 20 min after exposure to the low-Ca^{2+} or the Ca^{2+}-free medium; (c) 20 min after application of okadaic acid (4×10^{-5} M) in either low-Ca^{2+} or Ca^{2+}-free medium.

Table 2 Effects of okadaic acid (4×10^{-5} M) on the sinus node cell in low calcium and calcium-free medium

	APA (mV)	$\dot{V}_{max}$ (Vs^{-1})	*SPCL* (ms)
Control	85.5 ± 6.0	10.5 ± 1.9	384 ± 31
Low Ca^{2+} medium (0.12 mM)	0	0	—
Low Ca^{2+} medium + okadaic acid	60.8 ± 4.3*	2.2 ± 0.1*	433 ± 51*
Control	84.0 ± 5.6	10.3 ± 1.5	376 ± 18
Ca^{2+}-free medium	0	0	—
Ca^{2+}-free medium + okadaic acid	11.1 ± 2.2*	0.3 ± 0.1*	411 ± 33*

Values are means ± s.e. ($n = 4$). The preparations were exposed to low calcium and calcium-free medium for 20 min and okadaic acid (4×10^{-5} M) was further applied for 20 min.

* Significantly different from the values before application of okadaic acid at $P < 0.05$.

Abbreviations are the same as in Table 1.

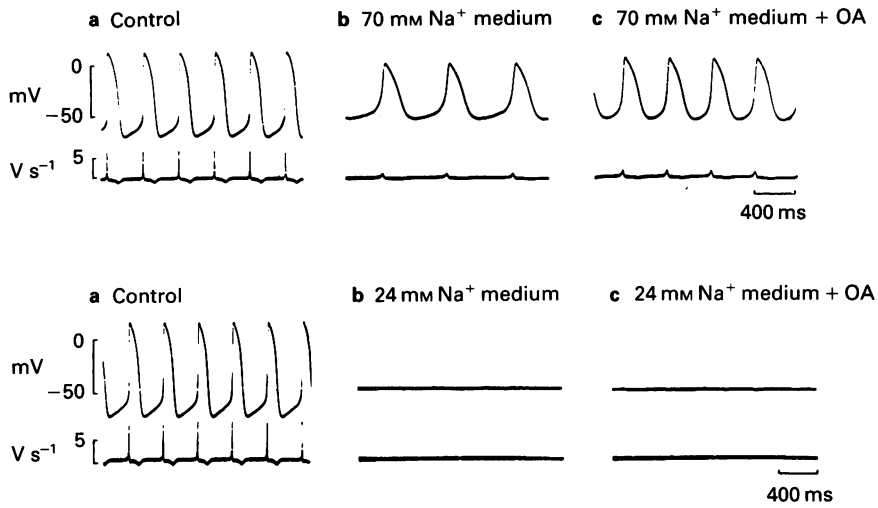

Figure 3 Effects of okadaic acid (OA) on the action potential of sinus node pacemaker cells in a low Na^+ medium (70 and 24 mM). Upper and lower panels show experiments in medium containing 70 and 24 mM Na^+ respectively. (a) Control; (b) 20 min after exposure to a low Na^+ medium; (c) 20 min after application of okadaic acid (4×10^{-5} M) in a low Na^+ medium.

Representative experiments are shown in Figure 4. The 30 min pretreatment with verapamil (10^{-6} M) resulted in a cessation of spontaneous activity at the resting potential (-37.8 ± 1.1 mV, $n = 4$). In these preparations, okadaic acid (4×10^{-5} M) failed to restore spontaneous activity.

Influence of propranolol and atropine

Effects of okadaic acid were also examined in preparations pretreated with propranolol (10^{-6} M), a β-adrenoceptor antagonist, and atropine (10^{-6} M), a acetylcholine receptor antagonist. Neither atropine nor propranolol had any effect on electrical responses to okadaic acid (data not shown).

Membrane currents

Effects of okadaic acid on the slow inward current and on the outward currents were investigated in four preparations by a two-microelectrode voltage clamp technique. Representative experiments are shown in Figure 5. A depolarization step

Table 3 Effects of okadaic acid (4×10^{-5} M) on the sinus node cells in low sodium medium

	APA (mV)	$\dot{V}_{max}$ (Vs^{-1})	*SPCL* (ms)
Control	81.6 ± 5.8	7.7 ± 2.2	411 ± 43
Low (70 mM) Na^+ medium	48.4 ± 6.4	1.2 ± 0.3	800 ± 69
Low (70 mM) Na^+ medium + okadaic acid	50.4 ± 7.6	1.4 ± 0.3	504 ± 27*
Control	83.3 ± 2.1	7.2 ± 0.6	489 ± 44
Low (24 mM) Na^+ medium	0	0	—
Low (24 mM) Na^+ medium + okadaic acid	0	0	—

Values are means ± s.e. ($n = 4$). The preparations were exposed to low sodium (70 and 24 mM) medium for 20 min and okadaic acid (4×10^{-5} M) was further applied for 20 min.
* Significantly different from the values before application of okadaic acid at $P < 0.05$.
Abbreviations are the same as in Table 1.

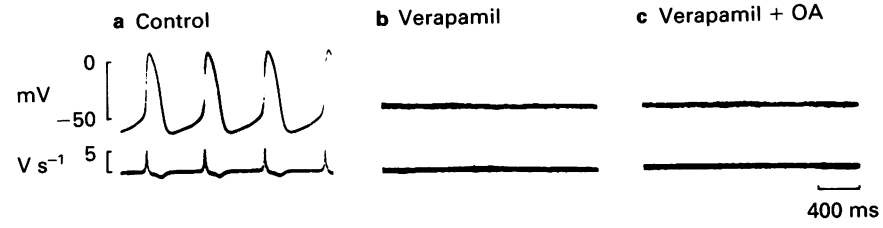

Figure 4 Effects of okadaic acid (OA) on the action potential of sinus node pacemaker cells pretreated with verapamil. (a) Control; (b) 30 min after application of verapamil (10^{-6} M); (c) 20 min after application of okadaic acid (4×10^{-5} M).

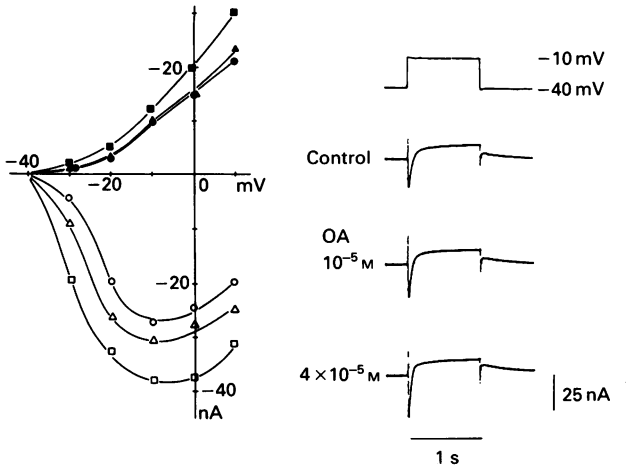

Figure 5 Effects of okadaic acid (OA) on membrane currents of sinus node pacemaker cells. Right panel: voltage clamp traces during depolarization (from −40 mV to −10 mV). Left panel: current-voltage relation for I_{si} (open symbols) and I_{out} at the end of depolarization step (closed symbols) in the absence (circles) and the presence of okadaic acid at 10^{-5} M (triangles) and 4×10^{-5} M (squares).

from the holding potential of −40 mV to −10 mV elicited a slow inward current (I_{si}) followed by a gradually increasing outward current (I_{out}). When the membrane potential was returned to −40 mV, an outward tail current (I_{tail}) was recorded. The amplitude of I_{si} was estimated by taking the difference between the inward peak of I_{si} and the current level at 200 ms after the onset of the depolarization step. The amplitude of I_{out} was estimated by the shift by the steady-state current level at the holding potential. After treatment with okadaic acid (10^{-5} M) the amplitude of I_{si} was significantly increased (117 ± 3.0% of control, $n = 4$), while both I_{out} at the end of the depolarization step and I_{tail} were unaffected. At a higher concentration of the drug (4×10^{-5} M), I_{si} was further increased (141 ± 5.6% of control, $n = 4$) and both I_{out} and I_{tail} were enhanced.

Discussion

In the present study small ball-like specimens of sinus node tissue were used to investigate the effects of okadaic acid. These preparations are considered to be composed of dominant pacemaker cells of the sinus node because the specimens were obtained from a restricted area of sinus node: the centre of the intercaval region, 1.0 to 1.5 mm away from the right branch of the sinoatrial ring bundle on the medial border of the crista terminalis. Previous reports have shown that the leading (or dominant) pacemaker sites of rabbit sinus node are located in this area (Bleeker *et al.*, 1980; Kodama & Boyett, 1985). It has also been previously found that most of the electrophysiological properties of the pacemaker cells of the intact sinus node are well preserved in such very small specimens (Kodama & Boyett, 1985). TTX (10^{-6} M) had no effect on either the transmembrane action potential or the spontaneous firing rate of the preparations, both of which indicate little or no contribution of the fast sodium inward current (I_{Na}) to the electrical activity of the preparations. TTX has been shown to have little effect on dominant pacemaker cells (Strauss *et al.*, 1977; Irisawa, 1978).

Okadaic acid at concentrations above 10^{-5} M caused a significant shortening of cycle length of spontaneous firing (SPCL) and an increase in both $\dot{V}_{max}$ and amplitude of action potentials (APA). It is well known that the upstroke phase of the action potentials of the dominant pacemaker cells of mammalian sinus nodes is generated by an activation of slow inward current (I_{si}). I_{si} has also been shown to play an important role in the development of pacemaker depolarization (diastolic depolarization) especially in its later phase (Noma *et al.*, 1980; Yanagihara *et al.*, 1980; Noble, 1984). In the present experiments, the spontaneous activity of the preparations was completely suppressed by a slow-channel blocking agent, verapamil (10^{-6} M), and okadaic acid failed to overcome this inhibitory effect of verapamil. Voltage clamp data also revealed that okadaic acid markedly augmented I_{si}. These facts suggest that the effect of okadaic acid on sinus node pacemaker cells is mediated by the enhancement of I_{si} by the agent.

The effects of okadaic acid on the sinus node action potential were markedly attenuated or nearly abolished in a low-Na^+ medium, whereas they are relatively well preserved in a low-Ca^{2+} medium. Okadaic acid restored spontaneous activity of sinus node cells even in a Ca^{2+}-deficient medium. These results can be explained by the mechanism through Na^+-Ca^{2+} exchange. In a low Na^+ medium, the intracellular Ca^{2+} concentration may have been increased to the inhibition of Na^+-Ca^{2+} exchange mechanism (Mullins, 1981). Intracellular Ca^{2+} accumulation would lower the Ca^{2+} concentration gradient across the cell membrane, and secondarily induce various changes in membrane properties, such as an increase in K^+ conductance (Isenberg, 1977), or Ca^{2+}-induced inactivation of the slow channel (Tsien, 1983). These secondary changes may inhibit or counteract I_{si} carried by Ca^{2+}. Thus, lowering $[Na]_o$ may inhibit slow inward current across cell membrane. The involvement of the ion selectivity of the slow channel may also be considered. Noma & Irisawa (1976) and Noma *et al.* (1977) suggested that the slow inward current of the sinus node cell is carried by Na^+ as well as by Ca^{2+}. Okadaic acid may increase I_{si} carried not only by Ca^{2+} but also by Na^+. In either the low-Ca^{2+} or the Ca^{2+}-deficient medium, therefore, okadaic acid can increase I_{si} carried by Na^+, because the normal Na^+ concentration gradient across the cell membrane may have been maintained under these conditions. In a low Na^+ medium, the decreased gradient of Na^+ concentration may reduce the contribution of Na^+ to I_{si}. However, since a recent study in bull-frog atrial cells (Campbell *et al.*, 1988) has suggested that I_{Ca} is carried almost exclusively by Ca^{2+} ions, it seems more likely that the present effect of low $[Na^+]_o$ is mediated through a Na^+-Ca^{2+} exchange mechanism. In our previous experiments with guinea-pig papillary muscles (Kodama *et al.*, 1986) the prolongation of action potential duration by okadaic acid was enhanced in a low-Ca^{2+} medium, while it was nearly abolished in a low-Na^+ medium. The present results seem analogous to those earlier findings.

Since a deactivating K^+ outward current is thought to be one component of the development of pacemaker depolarization, a decrease in this current may also cause a positive chronotropic effect on sinus node pacemaker cells. However, in the present voltage clamp experiments, K^+ outward current was increased. Therefore, K^+ outward current seems unlikely as a possible mechanism for the action of okadaic acid.

The possibility that okadaic acid causes release of neurotransmitters from nerve terminals in the sinus node which, then, modulate the spontaneous activity may be excluded, since neither a β-adrenoceptor nor a ACh receptor antagonist had any effect on the response of the tissue to okadaic acid.

In conclusion, the present study has revealed that okadaic acid has an intrinsic effect on sinus node dominant pacemaker cells, resulting in an increase in spontaneous activity. It was also suggested that this positive chronotropic effect is mediated by the enhancement of slow inward current through verapamil-sensitive Ca^{2+} channels. Recently, Hescheler *et al.* (1988) demonstrated that okadaic acid potently inhibited the protein phosphatases. They also observed an increasing effect of okadaic acid on cardiac I_{Ca} in the presence of isoprenaline which stimulates I_{Ca} through cyclic AMP-dependent phosphorylation of Ca channels (Reuter, 1983; Brum *et al.*, 1984). From these results, they have assumed that the effect of okadaic acid on I_{Ca} is related to suppression of phosphatase

activity resulting in inhibition of dephosphorylation of phosphorylated Ca channel proteins which enhances I_{Ca}. The present results would also be consistent with such an assumption. Okadaic acid is a useful tool for studying the involvement of protein phosphatase activity in the regulation of cardiac function, such as spontaneous activity and contractility.

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References

BIALOJAN, C., RUEGG, J.C. & TAKAI, A. (1988). Effects of okadaic acid on isometric tension and myosin phosphorylation of chemically skinned guinea-pig taenia coli. *J. Physiol.*, **398**, 81–95.

BLEEKER, W.K., MACKAAY, A.J., MASSON-PEVET, M., BOUMAN, L.N. & BECKER, A.E. (1980). Functional and morphological organization of the rabbit sinus node. *Circ. Res.*, **46**, 11–22.

BONKE, F.I.M. (1973). Electrotonic spread in the sinoatrial node of the rabbit heart. *Pflugers Arch.*, **339**, 17–23.

BRUM, G., OSTERRIEDER, W. & TRAUTWEIN, W. (1984). β-Adrenergic increase in the calcium conductance of cardiac myocytes studied with the patch clamp. *Pflugers Arch.*, **401**, 111–118.

CAMPBELL, D.L., GILES, W.R., HUME, J.R., NOBLE, D. & SHIBATA, E.F. (1988). Reversal potential of the calcium current in bullfrog atrial myocytes. *J. Physiol.*, **403**, 267–286.

ERDODI, F., ROKOLYA, A., SALVO, J.D., BARANY, M. & BARANY, K. (1988). Effect of okadaic acid on phosphorylation-dephosphorylation of myosin light chain in aortic smooth muscle homogenate. *Biochem. Biophys. Res. Commun.*, **153**, 156–161.

HESCHELER, J., MIESKER, G., RUEGG, J.C., TAKAI, A. & TRAUTWEIN, W. (1988). Effects of a protein phosphatase inhibitor, okadaic acid, on membrane currents of isolated guinea-pig cardiac myocytes. *Pflugers Arch.*, **412**, 248–252.

IRISAWA, H. (1978). Comparative physiology of the cardiac pacemaker mechanism. *Physiol. Rev.*, **58**, 461–498.

ISENBERG, G. (1977). Cardiac Purkinje fibers. $[Ca^{2+}]_i$ controls steady state potassium conductance. *Pflugers Arch.*, **371**, 71–76.

KODAMA, I. & BOYETT, M.R. (1985). Regional differences in the electrical activity of the rabbit sinus node. *Pflugers Arch.*, **404**, 214–226.

KODAMA, I., KONDO, N. & SHIBATA, S. (1986). Electromechanical effects of okadaic acid isolated from black sponge in guinea-pig ventricular muscles. *J. Physiol.*, **378**, 359–373.

MULLINS, L.J. (1981). *Ion Transport in Heart*. New York: Raven Press.

NEW, W. & TRAUTWEIN, W. (1972). Inward membrane current in mammalian myocardium. *Pflugers Arch.*, **334**, 1–23.

NOBLE, D. (1984). The surprising heart: A review of recent progress in cardiac electrophysiology. *J. Physiol.*, **353**, 1–50.

NOMA, A. & IRISAWA, H. (1976). Effects of calcium ion on the rising phase of the action potential in rabbit sinoatrial node cells. *Jpn. J. Physiol.*, **26**, 93–99.

NOMA, A., KOTAKE, H. & IRISAWA, H. (1980). Slow inward current and its role mediating the chronotropic effect of epinephrine in rabbit sinoatrial node. *Pflugers Arch.*, **388**, 1–9.

NOMA, A., PEPER, K. & TRAUTWEIN, W. (1979). Acetylcholine-induced potassium current fluctuation in the rabbit sinoatrial node. *Pflugers Arch.*, **381**, 255–262.

NOMA, A., YANAGIHARA, K. & IRISAWA, H. (1977). Inward membrane currents in the rabbit sinoatrial node cell. *Pflugers Arch.*, **372**, 43–51.

OZAKI, H., ISHIHARA, H., KOHAMA, K., NONOMURA, Y., SHIBATA, S. & KARAKI, H. (1987a). Calcium-independent phosphorylation of smooth muscle myosin light chain by okadaic acid isolated from black sponge (Halichondria okadai). *J. Pharmacol. Exp. Ther.*, **243**, 1167–1173.

OZAKI, H., KOHAMA, K., NONOMURA, Y., SHIBATA, S. & KARAKI, H. (1987b). Direct activation of the contractile elements in the smooth muscle of guinea pig taenia coli. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **335**, 356–358.

REUTER, H. (1983). Calcium channel modulation by neurotransmitters, enzymes and drugs. *Nature*, **301**, 569–574.

SEYAMA, I. (1976). Characteristics of the rectifying properties of the sinoatrial node cell of the rabbit. *J. Physiol.*, **225**, 379–397.

SHIBATA, S. (1987). Unique vasocontraction of okadaic acid isolated from black sponge, independent of extracellular Ca^{2+}. *Blood Vessel*, **24**, 104–107.

SHIBATA, S., ISHIDA, Y., KITANO, H., OHIZUMI, Y., HABON, J., TSUKITANI, Y. & KIKUCHI, H. (1982). Contractile effects of okadaic acid, a novel ionophore-like substance from black sponge, on isolated smooth muscles under the condition of Ca deficiency. *J. Pharmacol. Exp. Ther.*, **223**, 135–143.

STRAUSS, H.C., PRYSTOWSKI, E.W. & SCHEIMAN, M.M. (1977). Sinoatrial and atrial electrogenesis. *Prog. Cardiovasc. Dis.*, **19**, 385–404.

TACHIBANA, K., SCHEUER, P. J., TSUKITANI, Y., KIKUCHI, H., VAN ENGEN, E., CLARDY, J., GOPICHAND, Y. & SCHMITZ, F.J. (1981). Okadaic acid, a cytotoxin polyether from two marine sponge of the genus Halichondria. *J. Am. Chem. Soc.*, **103**, 2469–2471.

TSIEN, R.W. (1983). Calcium channels in excitable membranes. *Ann. Rev. Physiol.*, **45**, 341–358.

YANAGIHARA, K., NOMA, A. & IRISAWA, H. (1980). Reconstruction of sino-atrial node pace-maker potential based on the voltage clamp experiments. *Jpn. J. Physiol.*, **30**, 841–857.

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The effects of neuropeptide Y and its fragments upon basal and electrically stimulated ion secretion in rat jejunum mucosa

H.M. Cox & A.W. Cuthbert

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ

1 The effects of neuropeptide Y (NPY) and a range of C terminal fragments were investigated both on basal short circuit current (s.c.c.) and electrical field stimulated responses in voltage clamped preparations in rat jejunal mucosa.

2 Most of the NPY fragments tested had direct effects upon the mucosa, reducing baseline s.c.c. with EC_{50} values of 1 μM or more. NPY was 30 times more effective than any of the fragments tested and the order of potency was: NPY $\gg$ NPY (11–36) $\geqslant$ (12–36) $\geqslant$ (13–36) $\geqslant$ (14–36). NPY (15–36), (16–36), (20–36) and (22–36) were still less effective and complete concentration-response curves could not be constructed. NPY (26–36), des amido NPY and the C-terminal flanking peptide of NPY (CPON) were all inactive and did not significantly alter responses to NPY.

3 Electrical field stimulation (EFS) of mucosal preparations elicited rapid transient secretory responses in the presence of hexamethonium and atropine. NPY and fragments attenuated these secretory responses and where concentration-response relationships could be compared at a given time point the following order of potency was obtained: NPY $\gg$ NPY (11–36) > NPY (13–36). Again NPY (26–36), des amido NPY and CPON were ineffective, while at single concentrations (300 nM) a graded attenuation of EFS responses was obtained with NPY (14–36) $\geqslant$ NPY (15–36) > NPY (16–36) $\geqslant$ NPY (20–36) > NPY (22–36).

4 The attenuation of EFS responses by these peptides was not dependent upon the basal secretory state. Pretreatment of tissues with piroxicam reduced s.c.c. and attenuated further reductions in s.c.c. by NPY, but had no effect upon NPY-mediated inhibition of electrically-stimulated secretory responses.

5 NPY fragments attenuated both basal and EFS generated secretion. Since fragments are effective these receptors must, by definition be Y_2-like. NPY (11–36) and NPY (13–36) at 300 nM and 1 μM did not significantly attenuate secretory responses to either carbachol (CCh) or substance P (SP). A 1 μM concentration of either fragment was equivalent in effect to 30 nM NPY upon basal current, but NPY at this concentration significantly reduced both CCh- and SP-induced secretion. The reduced spectrum of fragment activity together with the different order and potency ratios obtained with these three peptides indicates a presynaptic action for NPY and the fragments.

Introduction

Neuropeptide (NPY) is located in subpopulations of both myenteric and submucous enteric neurones in the rat small intestine (Ekblad *et al.*, 1987). In addition extrinsic perivascular fibres also contain NPY, these being sympathetic in origin (Sundler *et al.*, 1983). Intrinsic submucous and myenteric NPY-positive neurones also contain vasoactive intestinal polypeptide (VIP) (Ekblad *et al.*, 1984; 1987) and whilst colocalisation of these two peptides is also observed in other systems of both rat and man (Leblanc *et al.*, 1987; Wattchow *et al.*, 1987), it does not appear to be a common pairing in the guinea-pig (Furness *et al.*, 1985). In the rat small intestine, VIP/NPY positive neurones in the myenteric ganglia issue short (2 mm) anal projections, whilst submucous VIP/NPY neurones project longer distances (4 mm) in an oral direction and it is these fibres that predominantly innervate the mucosa (Ekblad *et al.*, 1987). The abundant innervation of smooth muscle, vascular elements and mucosa by NPY-containing intrinsic neurones implicate this neuropeptide as a modulator of several enteric functions.

Both pre- and postjunctional NPY effects have been observed at sympathetic neuroeffector junctions (Wahlestedt *et al.*, 1986). In addition to direct vasoconstriction, NPY and its structural analogue peptide YY (PYY) also potentiated the postjunctional effect of noradrenaline (NA), as well as attenuating NA release. PYY derives from a different precursor to NPY, but like NPY it is 36 amino acids long and exhibits 70% sequence homology with NPY. The prejunctional inhibition of NA release was also observed with the C-terminal fragment PYY (13–36) which had no effect postjunctionally. Thus, the presence of two different NPY receptors was postulated, Y_1 being postjunctional and accepting only full length NPY and PYY, and Y_2 prejunctional receptors that could also be activated by fragments of NPY and PYY (Wahlestedt *et al.*, 1987). However, in the splenic vascular bed, NPY (13–36) also exerted postjunctional vasoconstrictor activity (Lundberg *et al.*, 1988) and shorter fragments NPY (16–36) and NPY (19–36) also exhibited direct vasoconstrictor effects in the guinea-pig coronary circulation *in vitro* (Rioux *et al.*, 1986). Thus in these two systems Y_2-like receptors appear to be located within postjunctional membranes. The use of a subdivided classification of NPY receptors is becoming widespread. However, Y_1-receptors, which will only accept full length NPY, are not always postjunctional and Y_2-receptors, which will tolerate loss of N terminal residues of NPY, are not necessarily prejunctional.

In the rat small intestine NPY is a potent antisecretory agent, inhibiting both basal Cl secretion (Cox *et al.*, 1988) and attenuating secretory responses generated by a range of neuropeptides and neurotransmitters (Cox & Cuthbert, 1988). Early preliminary data appeared to suggest that NPY (13–36) did not alter basal short circuit current (s.c.c.) while human pancreatic polypeptide was found to be considerably less potent than NPY and PYY (Cox *et al.*, 1988). Under voltage clamped conditions, mucosal preparations can be electrically stimulated (Hubel, 1978; Cooke *et al.*, 1983) and the resultant secretory responses (predominantly due to Cl secretion) are tetrodotoxin (TTX)-sensitive but not entirely cholinergic in character (Hubel, 1984; Cooke, 1984; Keast *et al.*, 1985). Much of this work has utilized rabbit and guinea-pig small intestine with little reference to the rat intestine. Nevertheless,

candidates for the atropine resistant component of electrically-stimulated secretory responses include substance P (Keast *et al.*, 1985) and possibly VIP (Gaginella *et al.*, 1981), though the pharmacological tools to verify this are still lacking.

The aim of this study was to establish what effects NPY had specifically upon non-cholinergic, electrically-stimulated secretory responses in rat jejunum mucosal preparations, containing intact submucous ganglia. The effects of a range of NPY fragments were also investigated upon both basal and electrically-stimulated epithelial anion secretion, in an attempt to identify the receptors and their location.

Methods

Electrical field stimulation studies

Epithelial sheets of jejunum were prepared from male Sprague-Dawley rats (200–300 g) as described previously (Cox *et al.*, 1988). Overlying smooth muscle layers were removed by dissection and the remaining mucosal sheets were placed between two halves of perspex Ussing chambers. The chambers accommodated two silver sheet electrodes both of which contacted the serosal surface of the mucosa. The exposed area of tissue was 0.6 cm^2 and preparations were automatically voltage clamped with WPI DVC 1000 voltage clamps. Under these conditions changes in short circuit current (s.c.c.) were recorded automatically. Four preparations from adjacent areas were routinely set up from a single jejunum, and all exhibited comparable basal s.c.c. and potential differences. Tissues were bathed in Krebs-Henseleit solution with the following composition (mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24.8, KH_2PO_4 1.2 and glucose 11.1, and were gassed with 95% O_2/5% CO_2.

The electrodes were attached to the output of a Tektronix pulse generator which delivered rectangular pulses of 0.6 ms duration and frequency of 5 Hz. Trains of 5 stimuli were delivered every 7 min throughout the experiment once a steady baseline had been achieved. After 4 trains had been delivered hexamethonium, 10 μM (Hex) and atropine, 10 μM (Atr) were added and a further 4 trains delivered. The mean value of these latter four responses was denoted as 100% for that preparation. Because of the variation in size of electrical field stimulation (EFS) responses between tissues, values are quoted as percentages of control responses obtained in a particular preparation. Trains of stimuli were continued at 7 min intervals following serosal application of either NPY or one of its fragments. Recording was continued until responses returned to the original, 100%, control levels. Unless otherwise stated EFS responses are given as a percentage of the control response obtained in the presence of Hex and Atr. Changes in baseline s.c.c. are quoted as μA 0.6 cm^{-2}, mean ± 1 s.e.mean. When the effects of peptides upon the secretory responses generated by EFS are compared, experimental values are quoted as percentages of controls, again as mean ± s.e.mean. Unpaired Student's *t* test was used for the statistical analysis of data (unless otherwise stated) and a *P* value of less than 0.05 was considered statistically significant.

Materials

Full length NPY (1–36) together with NPY (13–36), (16–36), (20–36), (22–36), (26–36) and the C-terminal flanking peptide of NPY (CPON) were purchased from Peninsula Laboratories Europe Ltd. Other NPY fragments namely NPY (11–36), (12–36), (13–36), (14–36) and (15–36) were gifts from Prof. Rolf Håkanson, Department of Pharmacology, Lund University. Hexamethonium, atropine, piroxicam and TTX were obtained from Sigma, Poole and all other reagents were of analytical grade.

Results

The effects of NPY and the C-terminal fragments of NPY upon baseline s.c.c. are shown in Figure 1. Clearly full length

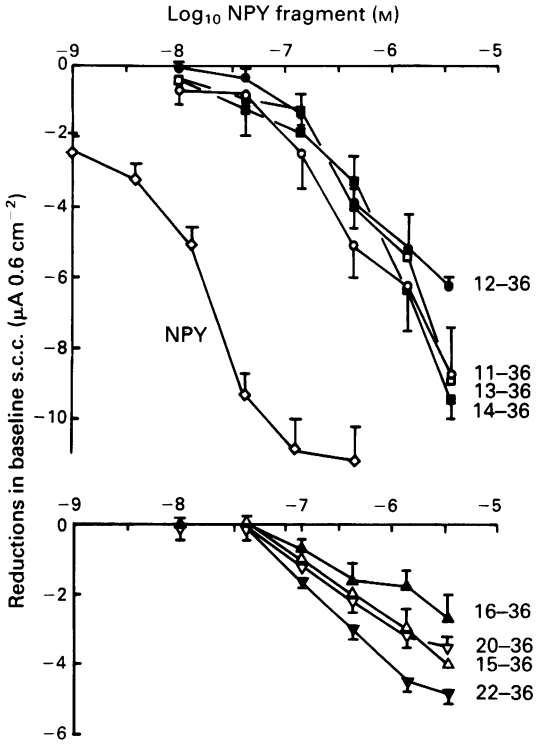

Figure 1 Antisecretory effects of neuropeptide (NPY) and fragments upon baseline short circuit current (s.c.c.). All peptide additions were made to the basolateral surface in a cumulative fashion and reductions in s.c.c. were recorded as μA 0.6 cm^{-2}. Each point represents the mean with vertical lines indicating 1 s.e.mean (some errors are omitted for reasons of clarity). The number of observations for each fragment are as follows: NPY, 3–11; NPY (11–36), 3–9; NPY (12–36), 2–9; NPY (13–36), 2–11; NPY (14–36), 2–9; NPY (15–36), 2–7; NPY (16–36), 2–7; NPY (20–36), 3–7; NPY (22–36), 3–7.

NPY is considerably more potent than any of the fragments tested with an EC_{50} of 13 nM. Shorter fragments of NPY were also antisecretory but were less potent; NPY (11–36), (12–36), (13–36) and (14–36) had threshold concentrations of 10–30 nM and approximate EC_{50} values of 2 μM. These fragments would appear to be full agonists. NPY (15–36), (16–36), (20–36) and (22–36) were less potent still with threshold concentrations of 30–100 nM. At the highest possible concentration (3 μM) tested these four fragments reduced baseline current by between −3.0 and −5.0 μA 0.6 cm^{-2}. Thus it was not possible to determine whether these fragments were full or only partial agonists. NPY (26–36), des amido NPY and CPON were without effect on baseline s.c.c. and had no significant effect upon NPY antisecretory responses. In the presence of either 300 nM NPY (26–36), des amido NPY or CPON, 30 nM NPY reduced s.c.c. by −6.5 ± 1.3 μA 0.6 cm^{-2} ($n = 6$), −7.9 ± 1.4 μA 0.6 cm^{-2} ($n = 8$) and −6.9 ± 0.6 μA 0.6 cm^{-2} ($n = 6$) respectively, compared with controls of −8.6 ± 1.5 μA 0.6 cm^{-2} ($n = 5$).

EFS (5 Hz, 0.6 ms for 1 s) elicited rapid transient increases in s.c.c. that returned to the baseline within 3 min. These secretory responses were constant when stimuli were applied at 7 min intervals and were attenuated 50% by simultaneous basolateral addition of Hex and Atr (from controls of 7.6 ± 0.6 μA 0.6 cm^{-2} ($n = 79$) to 3.8 ± 0.3 μA 0.6 cm^{-2} ($n = 79$)). Subsequent application of 100 nM TTX inhibited EFS responses further to 0.6 ± 0.1 μA 0.6 cm^{-2}, $n = 12$.

Following pretreatment of tissues with Hex and Atr either NPY or one of the NPY fragments was applied and changes in the baseline current and EFS responses recorded. NPY (3 nM and 10 nM) reduced the baseline s.c.c. as expected (Figure 2 and Cox *et al.*, 1988) and also attenuated secretory EFS responses in a concentration-dependent manner. NPY, 1 nM did not significantly inhibit either the basal s.c.c. or EFS responses. The apparent EC_{50} for NPY was 3 nM by inter-

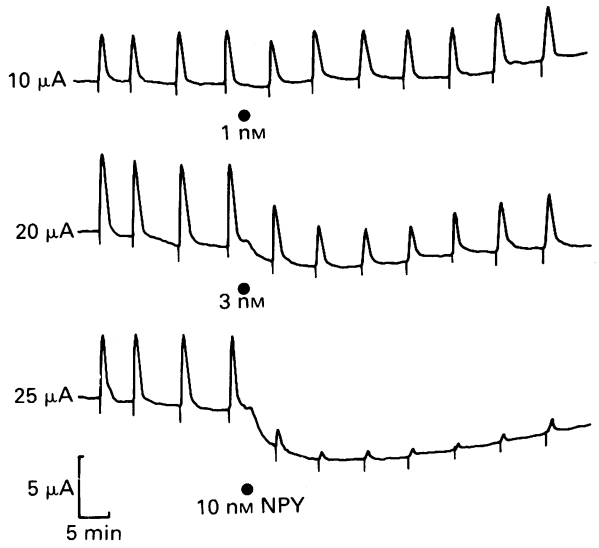

Figure 2 The effect of neuropeptide (NPY) upon electrical field stimulated (EFS) responses in the presence of hexamethonium and atropine (each 10 μM). Stimuli (5 Hz, 0.6 ms for 1 s) were delivered at 7 min intervals. Application of hexamethonium and atropine reduced EFS responses 40% (not shown) and the remaining resistant secretory responses were inhibited in a concentration-dependent manner by NPY (applied basolaterally). Baseline currents are given on the left and these three traces were obtained from adjacent sections of jejunum.

polation of the results at 14 min (as shown in Figure 3). Pooled data for NPY are compared with that of NPY (13–36) and des amido NPY in Figure 3. Higher concentrations (i.e. 100 nM) of NPY (13–36) were necessary to affect an inhibition of EFS responses similar to 3 nM NPY. NPY (13–36) (30 nM) did not significantly alter basal s.c.c. (-0.4 ± 0.3 μA 0.6 cm^{-2}, $n = 4$), while 100 nM NPY (13–36) significantly reduced s.c.c. by -1.4 ± 0.6 μA 0.6 cm^{-2} ($n = 7$, $P < 0.05$). Interpolation of data in the same way as for NPY, gave an approximate EC_{50} of 200 nM for NPY (13–36). A graded inhibition of EFS responses was also obtained with increasing NPY (11–36) concentrations from 10–300 nM. Fourteen minutes after addition of this fragment EFS responses were attenuated by 30%, 57% 83% and 89% with 10 nM, 30 nM, 100 nM and 300 nM NPY (11–36) respectively, allowing an EC_{50} estimation of 40 nM. After 56 min responses had returned to controls in 10 and 30 nM NPY (11–36)-treated preparations, but they were still reduced (by 15% and 89%) in tissues that had received 100 and 300 nM NPY (11–36). Loss of the C-terminal amino group of NPY, i.e. des amido NPY, resulted in total loss of activity, there being no change in either basal or EFS generated s.c.c.

The same range of fragments was tested for their ability to attenuate secretory responses generated by EFS. A concentration of 300 nM was chosen in each case, because measurable changes in baseline s.c.c. were recorded at this concentration and the responses obtained were on the linear portion of each respective concentration-response relationship (Figure 1). Figure 4a and b show the time-dependent attenuation of EFS responses in the presence of each NPY fragment tested. NPY (12–36), (13–36), (14–36) and (15–36) appeared to be equipotent with each other (at 300 nM), reducing field stimulated secretion by 60% 7–21 min after fragment addition; responses recovering within the experimental period (Figure 4a). NPY (16–36) and NPY (20–36) were slightly less effective, reducing EFS responses to $70.3 \pm 4.8\%$ ($n = 4$) and $55.2 \pm 6.0\%$ ($n = 4$) of controls respectively after 7 min (Figure 4b). With NPY

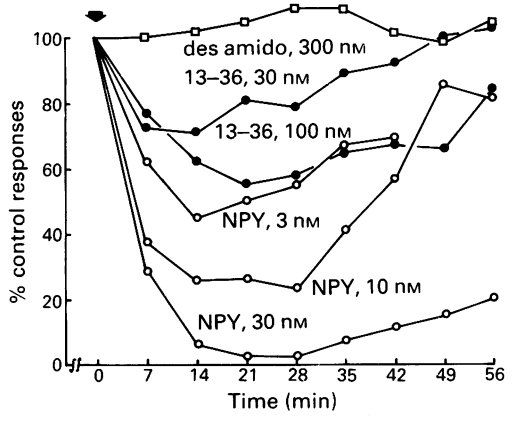

Figure 3 Pooled data comparing the inhibitory effects of neuropeptide Y (NPY), NPY (13–36) and des amido NPY upon electrical field stimulated (EFS) responses in the presence of hexamethonium and atropine. Control non-cholinergic responses were obtained in each tissue before the application of NPY and designated 100% (4.5 ± 0.8 μA 0.6 cm^{-2} $n = 12$ for 'NPY' controls; 3.1 ± 0.6 μA 0.6 cm^{-2}, $n = 4$ for 'des amido NPY' controls and 2.2 ± 0.3 μA 0.6 cm^{-2}, $n = 11$ for NPY (13–36) controls). One of the three peptides was then added basolaterally and all subsequent secretory EFS responses were calculated as a percentage of their respective tissue controls. Concentrations are given as nM and n values were 3, 4 and 3 for 3, 10 and 30 nM NPY; $n = 4$ and 7 for 30 and 100 nM NPY (13–36) and $n = 4$ for 300 nM des amido NPY, respectively. All points represent the mean and errors are not shown, though they were 5–10% of respective means.

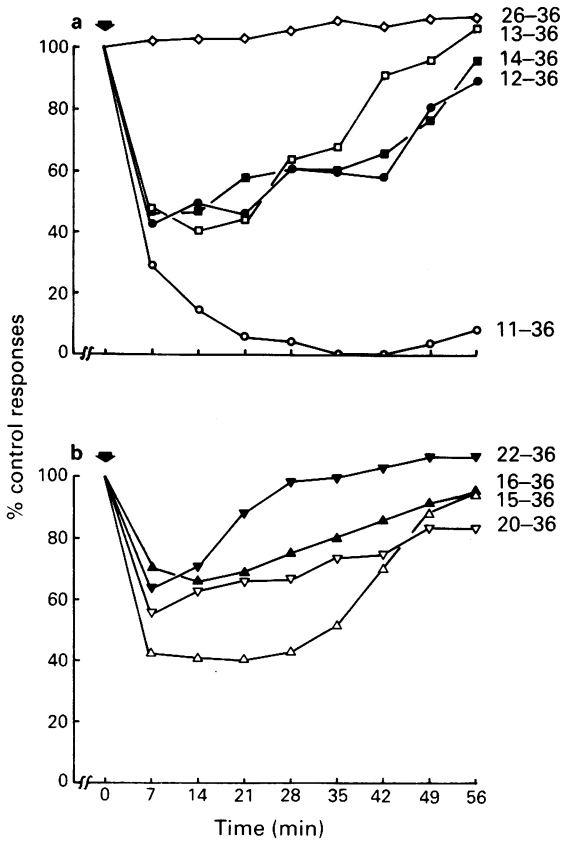

Figure 4 The effect of a range of neuropeptide Y (NPY) fragments upon hexamethonium/atropine-resistant electrical field stimulated (EFS) responses. Control secretory responses were obtained in each tissue in the presence of hexamethonium and atropine before application of NPY fragments (at 300 nM throughout). The figure is split into (a) and (b) for reasons of clarity. Control EFS responses were denoted as 100% and all subsequent increases in s.c.c. after EFS were calculated as a percentage of each tissue control. Control EFS responses as μA 0.6 cm^{-2} were as follows with n values in parentheses: 2.9 ± 0.4 (4) for NPY (11–36); 1.7 ± 0.2 (3) for NPY (12–36); 1.8 ± 0.2 (4) for NPY (13–36); 3.4 ± 0.8 (4) for NPY (14–36); 5.5 ± 0.8 (4) for NPY (15–36); 6.5 ± 0.5 (4) for NPY (16–36); 4.7 ± 0.3 (4) for NPY (20–36); 6.9 ± 1.7 (4) for NPY (22–36) and 6.6 ± 0.8 (4) for NPY (26–36). All points and values are quoted as means and the errors are not shown in the figure for reasons of clarity, but were not greater than 10%.

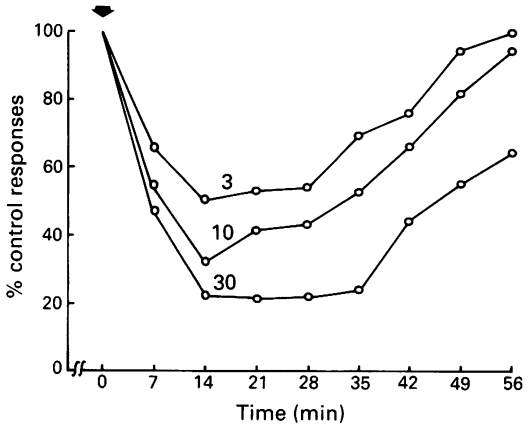

Figure 5 The effect of 3, 10 and 30 nM neuropeptide Y (NPY) (1–36) upon electrical field stimulated (EFS) responses in the absence of hexamethonium and atropine. Control responses to field stimulation were obtained initially in each tissue before basolateral application of NPY. Controls were $8.0 \pm 2.6\,\mu A\ 0.6\,cm^{-2}$ (3), $9.5 \pm 1.7\,\mu A\ 0.6\,cm^{-2}$ (3) and $4.7 \pm 1.1\,\mu A\ 0.6\,cm^{-2}$ (3) for 3, 10 and 30 nM NPY respectively. Values are quoted as means ± s.e.mean with n values in parentheses. Points in the figure are means but errors are not shown for reasons of clarity, though they were routinely 10–15% of respective mean values.

(22–36) inhibition was of short duration, the responses were reduced to 63.1 ± 6.2% ($n = 4$) of controls after 7 min and returned to control size within 28 min. As before with baseline s.c.c. changes, NPY (26–36) was inactive, eight consecutive trains of stimuli were not altered in size compared with those obtained before fragment addition.

Neither NPY (11–36) nor NPY (13–36) at concentrations of 300 nM, significantly altered secretory responses generated by exogenous carbachol (CCh). In the absence of either fragment 10 μM CCh (a submaximal concentration) increased s.c.c. by $57.5 \pm 6.4\,\mu A\ 0.6\,cm^{-2}$ ($n = 12$) compared with $43.7 \pm 4.4\,\mu A\ 0.6\,cm^{-2}$ in the presence of NPY (13–36) ($n = 11$, $P < 0.1$) and $49.4 \pm 9.2\,\mu A\ 0.6\,cm^{-2}$ with NPY (13–36) ($n = 4$, $P < 0.5$). NPY (11–36) (300 nM) did not alter submaximal substance P (SP 100 nM) secretory responses either (controls were $24.3 \pm 4.3\,\mu A\ 0.6\,cm^{-2}$, $n = 6$ and with NPY (11–36) $15.7 \pm 4.3\,\mu A\ 0.6\,cm^{-2}$, $n = 6$, $P < 0.3$). Paired t test analysis did not yield significant differences between CCh and SP groups in the absence or presence of either NPY (11–36) or NPY (13–36) respectively. Only the inhibition afforded by NPY (13–36) upon CCh secretory responses was significant ($P < 0.05$) by one tailed, paired t test. The effects of 1 μM NPY (13–36) and 10 nM NPY pretreatment (concentrations chosen because they cause similar reductions in basal s.c.c., Figure 1) on SP (100 nM) responses were also compared. Control secretory responses to SP were $29.6 \pm 3.6\,\mu A\ 0.6\,cm^{-2}$ ($n = 10$), while in the presence of NPY (10 nM) they were significantly reduced to $17.6 \pm 1.9\,\mu A\ 0.6\,cm^{-2}$ ($n = 8$, $P < 0.05$), and in the presence of NPY (13–36) (1 μM) $19.2 \pm 3.7\,\mu A\ 0.6\,cm^{-2}$ ($n = 6$, $P < 0.1$).

In all the field stimulation experiments described above the tissues were pretreated with Hex and Atr in order to establish what effects NPY and its fragments had exclusively upon non-cholinergic-mediated ion secretion. In a separate series the effects of NPY upon combined cholinergic and non-cholinergic EFS responses were investigated in the absence of blocking agents (Figure 5). There was no apparent difference in the extent to which 3, 10 and 30 nM NPY attenuated EFS responses either in the presence or the absence of cholinoceptor antagonists. The only significant difference was observed at 28 min after addition of 30 nM NPY. In the presence of the antagonists EFS responses were 2.5 ± 2.5% of controls ($n = 3$), while in the absence of Hex and Atr responses were 22.0 ± 0.3% ($n = 3$, $P < 0.01$) and there appeared to be a more rapid recovery of EFS responses in the latter group.

To investigate whether the lowering of baseline s.c.c. determined the size of the EFS responses, 5 μM piroxicam (a cyclooxygenase inhibitor) was added to both tissue surfaces (Figure 6) with a resultant reduction in s.c.c. of $-15.0 \pm 1.0\,\mu A\ 0.6\,cm^{-2}$ ($n = 7$; from $32.8 \pm 3.7\,\mu A\ 0.6\,cm^{-2}$ to $18.8 \pm 3.9\,\mu A\ 0.6\,cm^{-2}$). EFS responses (in the absence of Hex and Atr) were not changed following piroxicam ($6.3 \pm 1.2\,\mu A\ 0.6\,cm^{-2}$, $n = 7$) compared with those obtained before piroxicam pretreatment ($7.1 \pm 1.2\,\mu A\ 0.6\,cm^{-2}$, $n = 7$). Application of NPY subsequent to piroxicam did not further reduce the s.c.c. (as seen previously, Cox *et al.*, 1988 and Cox & Cuthbert, 1988). However, EFS responses were attenuated, by 16.4 ± 2.6% ($n = 2$), 77.3 ± 4.9% ($n = 3$) and 85.4 ± 4.4% ($n = 3$), 14 min after application of 3, 10 and 30 nM NPY respectively. These data were similar to NPY inhibition in the absence of piroxicam, at 14 min, 3, 10 and 30 nM NPY inhibited EFS responses by 49.4 ± 17.6% ($n = 3$), 68.0 ± 11.6% ($n = 3$) and 81.6 ± 9.3% ($n = 3$), respectively.

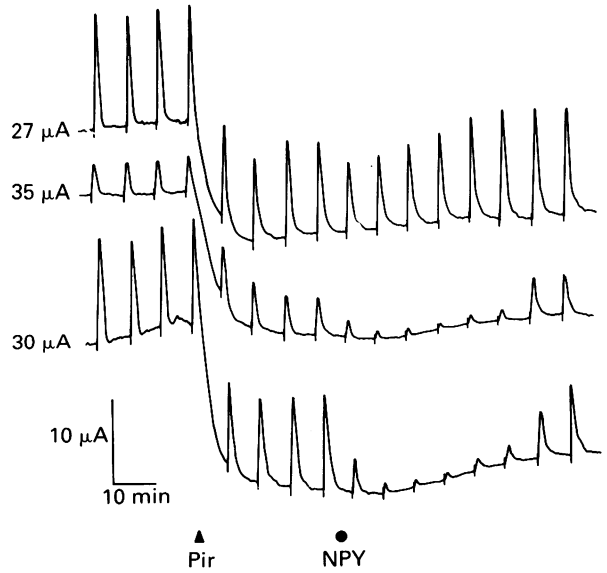

Figure 6 Effect of piroxicam and neuropeptide Y (NPY) upon electrical field stimulated (EFS) responses. All tissues received 5 μM piroxicam (Pir) both apically and basolaterally and once the baseline short circuit current had reached a steady level NPY 3 nM (upper trace), 10 nM (middle) and 30 nM (lower trace) was applied.

Discussion

In this study the effects of NPY and a range of NPY fragments were tested both on baseline jejunal s.c.c. and electrically stimulated secretory responses. In both cases full length NPY was more effective than any of the fragments as an inhibitor of both baseline and electrically stimulated s.c.c. The order of potency observed for attenuation of basal s.c.c. was NPY ≫ NPY (11–36), (12–36), (13–36), (14–36). The possibility that fragments (NPY) (15–36), (16–36), (20–36) and (22–36) may be partial agonists should not be overlooked in producing agonist orders of potency (Kenakin, 1983). However, within the concentration range tested the longer fragments NPY (11–36), (12–36), (13–36) and (14–36) all appeared to be full agonists, indicating that on epithelial membranes NPY receptors would appear to be of the Y_2 type. This is in agreement with biochemical studies of Servin *et al.* (1989) who, using epithelial cells from rat jejunum, found that C terminal fragments of NPY could inhibit specific [^{125}I]-PYY binding and attenuate VIP stimulated adenosine 3′:5′-cyclic monophosphate (cyclic AMP) production.

Residues 7–17 in the NPY molecule are thought to be important structurally, stabilizing the peptide in a conformation favourable for receptor interaction (Krstenansky *et al.*, 1989), while the C terminal region of NPY, 14–30 exhibits the potential to form an amphiphilic α-helix (Krstenansky &

Buck, 1987). Thus loss of amino acids from this region may well result in disruption and unfolding of the molecule thereby further reducing peptide efficacy. Amino acids 11, 12, 13 and 14 form the core sequence holding together the N terminal polyproline helix and amphiphilic α-helix of the C terminal (as shown in Fuhlendorff *et al.*, 1990). Sequential loss of these residues should therefore not greatly alter the tertiary structure of the remaining peptide. Although NPY (11–36), (12–36), (13–36) and (14–36) were found to be significantly less potent than NPY itself, these four fragments were equipotent at reducing baseline s.c.c. However, NPY fragments shorter than these i.e. NPY (15–36), (16–36), (20–36) and (22–36) were less effective again, indicating the importance of the full α-helix in peptide-receptor recognition.

The C terminal decapeptide, NPY (26–36), plus des amido NPY and CPON were all inactive, affecting neither the baseline s.c.c. nor EFS responses and not altering NPY responses in any way. Although a product of the same precursor as NPY, CPON appears to have little or no biological activity in other systems (Potter *et al.*, 1989). Equally, loss of the C-terminal amide group from full length NPY results in a loss of biological activity as shown above and elsewhere (Wahlestedt *et al.*, 1986; Potter *et al.*, 1989; MacKerell *et al.*, 1989). A noticeable difference in the ability to reduce s.c.c. and to inhibit EFS was seen with NPY (11–36). This peptide was equipotent with NPY (12–36), (13–36) and (14–36) at reducing basal s.c.c. (with an approximate EC_{50} of 2 μM). However, it was significantly more effective than the shorter fragments at inhibiting EFS responses (EC_{50} of 40 nM).

The size of EFS responses was not dependent upon the basal s.c.c., since piroxicam had no significant effect upon EFS secretory responses. However, subsequent application of NPY still attenuated EFS responses to a similar extent as in the absence of piroxicam. NPY attenuated both cholinergic and non-cholinergic components of EFS secretory response in rat small intestine. Direct prejunctional inhibition of both cholinergic and non-cholinergic induced contraction of small intestinal circular smooth muscle has been observed in the guinea-pig (Holzer *et al.*, 1987), whereas in guinea-pig colon longitudinal muscle NPY attenuates cholinergic transmission via an α-adrenergic pathway (Wiley & Owyang, 1987). McCulloch *et al.* (1987) observed NPY attenuation of field stimulated and muscarinic agonist-stimulated chloride secretion in guinea-pig distal colon. However, in this preparation NPY had no effect upon the basal secretory state of the tissue. In the rat jejunum NPY attenuates not only secretory responses generated electrically but also those observed following addition of exogenous CCh, SP and VIP (Cox & Cuthbert, 1988). Both CCh and SP stimulate Cl secretion via Ca^{2+}-dependent mechanisms, while VIP stimulates intracellular cyclic AMP generation thereby increasing Cl secretion. NPY (and presumably the gastrointestinal hormone and structural analogue, PYY) significantly attenuated CCh and VIP responses and abolished SP-induced secretion. Thus, the possibility that NPY fragments might similarly alter epithelial responses via postjunctional receptors had to be investigated.

Conventional statistical analysis (unpaired Student's *t* test) showed that 300 nM NPY (11–36) and NPY (13–36) did not significantly alter either CCh or SP secretory responses. Even when data were paired from each group (which is not routinely done) and analysed by two-tailed *t* tests, no significant differences were found. The only marginal difference ($P < 0.05$) was seen with 300 nM NPY (13–36), which with a single-tailed paired analysis was shown to attenuate CCh responses significantly. A comparison was also made between the effects of 10 nM NPY and 1 μM NPY (13–36), both of which attenuated basal s.c.c. by approximately 50%. Whereas pretreatment of preparations with NPY (10 nM) significantly attenuated SP-mediated secretion, application of 1 μM NPY (13–36) did not significantly reduce SP responses. Given this additional statistical information, the reduced spectrum of activity observed with these and presumably the other fragments may indicate the presence of prejunctional NPY receptors with Y_2-like characteristics.

According to the classification of Wahlestedt *et al.* (1986) there are two types of NPY receptors at sympathetic neuroeffector junctions; Y_1-receptors requiring the full length of NPY and Y_2-receptors which recognise truncated forms of NPY. The former appeared to be postjunctional, while the latter were found to be prejunctional in these vascular tissues. Whilst this subdivision of NPY receptors is becoming widely accepted, the preferential location of Y_1 and Y_2 to post- and prejunctional membranes respectively does not necessarily follow. Both types of NPY receptors have been observed in a range of human neuroblastoma cell lines and the pheochromocytoma cell line, PC12 (Sheikh *et al.*, 1989a,b). Y_2-like receptors have been identified in postjunctional membranes such as those of pig spleen (Lundberg *et al.*, 1988) and rabbit proximal tubule epithelia (Sheikh *et al.*, 1989c).

The antisecretory effects of NPY in rat jejunum would appear to be mediated by Y_2-receptors given that NPY (11–36), (12–36), (13–36) and (14–36) and shorter fragments will, albeit at high concentrations, produce direct reductions in basal s.c.c. From all the foregoing data it was found that by expressing the EC_{50} for NPY (11–36) and NPY (13–36) with respect to NPY, ratios of 13.0 and 67.0 were obtained respectively for EFS responses, while ratios of 154.0 for both in respect of basal current were obtained. This together with the potency order NPY ≫ NPY (11–36) = NPY (13–36) for basal and NPY > NPY (11–36) > NPY (13–36) for EFS, indicate subtle differences between pre- and postjunctional actions of NPY and its fragments. The classification of subdivisions of receptors with agonists is notoriously difficult (especially as some of the less potent, shorter fragments may be partial agonists), and the possibility of a combination of Y_1 and Y_2-like receptors existing on both surfaces cannot be ruled out at this stage. Only when selective agonists (Fuhlendorff *et al.*, 1990) and, more importantly, NPY antagonists become available will the subclassification of these receptors be clarified. For the moment NPY receptors with Y_2-like properties appear to be present within prejunctional, submucous neurones and postjunctional epithelial membranes in the rat small intestine.

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References

COOKE, H.J. (1984). Influence of enteric cholinergic neurons on mucosal transport in guinea-pig ileum. *Am. J. Physiol.*, **246**, G263–G267.

COOKE, H.J., SHONNARD, K. & WOOD, J.D. (1983). Effect of neuronal stimulation on mucosal transport in guinea-pig ileum. *Am. J. Physiol.*, **245**, G290–G295.

COX, H.M. & CUTHBERT, A.W. (1988). NPY antagonises secretagogue evoked chloride transport in rat jejunal epithelium. *Pflügers Arch.*, **413**, 38–42.

COX, H.M., CUTHBERT, A.W., HÅKANSON, R. & WAHLESTEDT, C. (1988). The effect of NPY and PYY on electrogenic ion transport in rat intestinal epithelia. *J. Physiol.*, **398**, 65–80.

EKBLAD, E., HÅKANSON, R. & SUNDLER, F. (1984). VIP and PHI coexist with a NPY-like peptide in intramural neurones of the small intestine. *Reg. Peptides*, **10**, 47–55.

EKBLAD, E., WINTHER, C., EKMAN, R., HÅKANSON, R. & SUNDLER, F. (1987). Projections of peptide-containing neurones in rat small intestine. *Neuroscience*, **20**, 169–188.

FURNESS, J.B., COSTA, M., GIBBINS, I.L., LLEWELLYN-SMITH, I.J. & OLIVER, J.R. (1985). Neurochemically similar myenteric and submucous neurones directly traced to the mucosa of the small intestine. *Cell Tissue Res.*, **241**, 155–163.

FUHLENDORFF, J., GETHER, U., AAKERLUND, L., LANGELAND-JOHANSEN, N., THØGERSEN, H., MELBERG, S.G., BANG OLSEN,

U., THASTRUP, O. & SCHWARTZ, T.W. (1990). [Leu^{31}, Pro^{34}] Neuropeptide Y: a specific Y_1 receptor agonist. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 182–186.

GAGINELLA, T.S., O'DORISIO, T.M. & HUBEL, K.A. (1981). Release of vasoactive intestinal peptide by electric field stimulation of rabbit ileum. *Reg. Peptides*, **2**, 165–174.

HOLZER, P., LIPPE, I.TH., BARTHO, L. & SARIA, A. (1987). NPY inhibits excitatory enteric neurons supplying the circular muscle of the guinea pig small intestine. *Gastroenterology*, **92**, 1944–1950.

HUBEL, K.A. (1978). The effects of electric field stimulation and tetrodotoxin on ion transport by the isolated rabbit ileum. *J. Clin. Invest.*, **62**, 1039–1047.

HUBEL, K.A. (1984). Electrical stimulus-secretion coupling in rabbit ileal mucosa. *J. Pharmacol. Exp. Ther.*, **231**, 577–582.

KEAST, J.R., FURNESS, J.B. & COSTA, M. (1985). Investigations of nerve populations influencing ion transport that can be stimulated electrically, by serotonin and by a nicotinic agonist. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **331**, 260–266.

KENAKIN, T.P. (1983). Receptor classification by selective agonists: coping with circulatory and circumstantial evidence. *Trends Pharmacol. Sci.*, **4**, 291–295.

KRSTENANSKY, J.L. & BUCK, S.H. (1987). The synthesis, physical characterisation and receptor binding affinity of NPY. *Neuropeptides*, **10**, 77–85.

KRSTENANSKY, J.L., OWEN, T.J., BUCK, S.H., HAGAMAN, K.A. & McLEAN, L.R. (1989). Centrally truncated and stabilised porcine neuropeptide Y analogs: design, synthesis and mouse brain receptor binding. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 4377–4381.

LEBLANC, G.G., TRIMMER, B.A. & LANDIS, S.C. (1987). NPY-like immunoreactivity in rat cranial parasympathetic neurons: coexistence with VIP and choline acetyl transferase. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 3511–3515.

LUNDBERG, J.M., HEMSEN, A., LARSSON, O., RUDEHILL, A., SARIA, A. & FREDHOLM, B.B. (1988). NPY receptor in pig spleen; binding characteristics, reduction of cAMP formation and calcium antagonist inhibition of vasoconstriction. *Eur. J. Pharmacol.*, **145**, 21–29.

MacKERELL, A.D., HEMSEN, A., LACROIX, J.S. & LUNDBERG, J.M. (1989). Analysis of structure-function relationships and neuropeptide Y using molecular dynamics simulations and pharmacological activity and binding measurements. *Reg. Peptides*, **25**, 295–313.

McCULLOCH, C.R., KUWAHARA, A., CONDON, C.D. & COOKE, H.J. (1987). Neuropeptide modification of chloride secretion in guinea pig distal colon. *Reg. Peptides*, **19**, 35–43.

POTTER, E.K., MITCHELL, L., McCLOSKEY, M.J.D., TSENG, A., GOODMAN, A.E., SHINE, J. & McCLOSKEY, D.I. (1989). Pre- and postjunctional actions of NPY and related peptides. *Reg. Peptides*, **25**, 167–171.

RIOUX, F., BACHELARD, H., MARTEL, J.C. & ST PIERRE, S. (1986). The vasoconstrictor effect of NPY and related peptides in the guinea pig isolated heart. *Peptides*, **7**, 27–31.

SERVIN, A.L., ROUYER-FESSARD, C., BALASUBRAMANIAM, A., SAINT PIERRE, S. & LABURTHE, M. (1989). Peptide YY and neuropeptide Y inhibit VIP-stimulated cAMP production in rat small intestine: structural requirements of peptides for interacting with peptide YY preferring receptors. *Endocrinology*, **124**, 692–700.

SHEIKH, S.P., HÅKANSON, R. & SCHWARTZ, T.W. (1989a). Y_1 and Y_2 receptors for neuropeptide Y. *FEBS Lett.*, **245**, 209–214.

SHEIKH, S.P., O'HARE, M.M.T., TORTORA, O. & SCHWARTZ, T.W. (1989b). Binding of monoiodinated neuropeptide Y to hippocampal membranes and human neuroblastoma cell lines. *J. Biol. Chem.*, **264**, 6648–6654.

SHEIKH, S.P., SHEIKH, M.I. & SCHWARTZ, T.W. (1989c). Y_2-type receptors for peptide YY on renal proximal tubular cells in the rabbit. *Am. J. Physiol.*, **257**, F978–F984.

SUNDLER, F., MOGHIMZADEH, E., HÅKANSON, R., EKELUND, M. & EMSON, P.C. (1983). Nerve fibres in the gut and pancreas of the rat displaying NPY immunoreactivity. *Cell Tissue Res.*, **230**, 487–493.

WAHLESTEDT, C., YANAIHARA, N. & HÅKANSON, R. (1986). Evidence for different pre- and postjunctional receptors for NPY and related peptides. *Reg. Peptides*, **13**, 307–318.

WAHLESTEDT, C., EDVINSSON, L., EKBLAD, E. & HÅKANSON, R. (1987). Effects of NPY at sympathetic neuroeffector junctions: existence of Y_1 and Y_2 receptors. In *Neuronal Messengers in Vascular Function*, ed. Arnedo-Nobin, B., Nobin, A. & Owman, C.L. (Fernström) Symposia No 8, pp. 231–242. Amsterdam: Elsevier.

WATTCHOW, D.A., FURNESS, J.B., COSTA, M., O'BRIEN, P.E. & PEACOCK, M. (1987). Distribution of neuropeptides in the human esophagus. *Gastroenterology*, **93**, 1363–1371.

WILEY, J. & OWYANG, C. (1987). NPY inhibits cholinergic transmission in the isolated guinea pig colon: mediation through α-adrenergic receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 2047–2051.

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Inhibition of human platelet activation by polymorphonuclear leukocytes

[1]Mirta A. Schattner, Jorge R. Geffner, Martin A. Isturiz & Maria A. Lazzari

Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, Pacheco de Melo 3081, (1425) Buenos Aires, Argentina

1 The effect of unstimulated human polymorphonuclear leukocytes (PMNs) on platelet activation was examined.

2 Human platelet aggregation and adenosine 5'-triphosphate (ATP) release induced by collagen (1–2 $\mu g\,ml^{-1}$); thrombin (0.01–0.02 u ml^{-1}) or arachidonic acid (AA) (0.1–0.2 mM) were markedly inhibited when conducted in the presence of unstimulated PMNs.

3 Platelet inhibition induced by PMNs was dependent on the number of PMNs and on the incubation time of the mixed cell suspension.

4 Platelet inhibition was not reversed in time when PMNs were depleted from the mixed-cell suspension.

5 PMN-mediated platelet-inhibition was not mediated by AA metabolites, oxygen reactive intermediates, nitric oxide or proteases.

6 The factor(s) accounting for the platelet inhibition mediated by PMNs are not yet characterized.

Introduction

Platelet interaction with polymorphonuclear leukocytes (PMNs) occurs at many steps in the inflammatory response, including chemotaxis, adhesion, secretion, degranulation and activation of the respiratory burst (Turner *et al.*, 1977; Marcus *et al.*, 1981; Rhee *et al.*, 1986). Substances that are released by PMNs and which affect platelet behaviour include oxygen radicals (Levine *et al.*, 1986), enzymes (Chignard *et al.*, 1986), arachidonic acid (AA) metabolites (Mehta *et al.*, 1986) and platelet activating factor (PAF) (Lotner *et al.*, 1980).

In a previous study, Zoucas *et al.* (1985) demonstrated that a soluble and filtrable factor(s) released by latex-stimulated PMNs in plasma suspension, impaired platelet aggregation induced by adenosine diphosphate (ADP) and AA. Furthermore, they showed that unstimulated PMNs did not inhibit platelet aggregation. In the present study, we reexamined whether unstimulated PMNs were able to supress platelet responses.

Methods

Blood samples

Blood samples were obtained from healthy donors who had taken no medication for at least 10 days before the day of sampling. In some experiments, blood samples were obtained from donors who had ingested 500 mg of acetylsalicylic acid (ASA) 24 h before the day of study. Blood was obtained by venepuncture of the forearm vein and it was drawn directly into plastic tubes containing 3.8% sodium citrate (1:9 v/v).

Preparation of polymorphonuclear leukocytes

Peripheral blood PMNs were isolated from citrated human blood samples, as described previously (Geffner *et al.*, 1987) by Ficoll-Hypaque centrifugation and sedimentation in dextran. Contaminating erythrocytes were removed by hypotonic lysis. After washing, the cells were resuspended at a final concentration of 5×10^6 PMNs ml^{-1} in Tyrode albumin buffer (TA buffer). The cell suspension contained 95–98% neutrophils.

[1] Author for correspondence.

Preparation of platelet rich plasma (PRP)

PRP was prepared by centrifugation of the citrated blood samples at 180 *g* for 10 min. The number of cells was adjusted to $5 \times 10^8\,ml^{-1}$ by addition of autologous platelet poor plasma obtained by centrifugation (1500 *g* for 30 min) of the remaining blood sample after removal of the PRP.

Preparation of washed platelets

PRP was centrifuged in the presence of EDTA (5 mM) for 15 min at 1500 *g*. Then, platelets were washed twice (1500 *g* for 20 min) with Tris saline buffer (TS buffer) and finally resuspended in TA buffer at a concentration of 5×10^8 platelets ml^{-1}.

Platelet aggregation and adenosine 5'-triphosphate (ATP) release

Platelet aggregation and ATP release were simultaneously measured with a Lumi-aggregometer (Chrono-Log) at 900 r.p.m. and 37°C; 200 μl of PMNs ($5 \times 10^6\,ml^{-1}$) or TA buffer were added to 200 μl of washed platelets (plus 0.5 mg ml^{-1} of fibrinogen) or PRP. When ATP release was measured, 5 μl of luciferin-luciferase (40 mg ml^{-1}) was also added. After 1 min of incubation, platelet agonists were added and aggregation response and ATP release were recorded during 3 min.

In some experiments, mixed-cell suspensions were centrifuged at 160 *g* for 1 min to spin down and eliminate all of the PMNs, as observed by light microscopy.

Materials

Buffers: Tris saline buffer (TS buffer) consisted of: 445 ml of 0.15 M NaCl; 50 ml of Tris HCl 0.15 M, pH = 7.4; 0.5 g of glucose; 10 ml of EDTA 0.10 M and 250 mg of bovine serum albumin (BSA). Tyrode albumin buffer (TA buffer) (pH = 7.4) consisted of (mM): NaCl 137, KCl 2.7, $NaHCO_3$ 11.9, $NaPO_4H_2$ 0.42, $MgCl_2$ 1, $CaCl_2$ 1, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 5, glucose 1 gl^{-1} and BSA 0.35%. PMNs and platelets suspended in TA buffer were used in all the experiments.

Drugs: the following were used (sources in parentheses) *p*-bromophenacyl bromide (BPB), N-tosyl-1-phenylalanine chloromethyl ketone (TPCK), phenylmethanesulphonyl fluoride (PMSF), superoxide dismutase (SOD), catalase, cytochrome C, methaemoglobin, methylene blue, AA (all Sigma), collagen (Hormon-Chemie), and human thrombin (Parke-Davis). Haemoglobin (Sigma) was dissolved in normal saline to produce a 1 mM solution. The haemoglobin was then fully reduced by the method of Martin *et al.* (1985). Briefly, sodium dithionite ($Na_2S_2O_4$) (10 mM) was added to the haemoprotein solution and it was then removed by dialysis against 500 volumes of distilled water for 3 h at 4°C. Aliquots of this solution were then stored at −20°C until used.

Statistics

Results are expressed as mean ± s.e.mean for n separate experiments. Student's paired t test was used to determine the significance of differences between means and $P < 0.05$ was taken as statistically significant.

Results

Effect of PMNs on platelet aggregation

The addition of 200 µl of a cell suspension containing PMNs (5×10^6 ml^{-1}) to 200 µl of washed platelets (5×10^8 ml^{-1}) completely suppressed the aggregation response to collagen (1 µg ml^{-1}) (Figure 1a). The inhibition was completely overcome by increasing the collagen concentration 2–8 fold (Figure 1a). The antiaggregatory activity of PMNs was dependent upon both the number of PMNs in the cell suspension (Figure 1b) and the incubation time of platelets with PMNs (Table 1). Whatever the platelet stimulus employed, 100% inhibition was achieved after 20 min of incubation (Table 1). At this time, platelet-mediated ATP release induced by collagen (1–2 µg ml^{-1}), AA (0.1–0.2 mM) or thrombin (0.01–0.02 u ml^{-1}) was also completely suppressed (% inhibition = 94 ± 5, $n = 3$). The suppression of the platelet response induced by PMNs did not require the presence of PMNs during the platelet aggregation assay. In fact, platelets that were preincubated with PMNs and then depleted of them remained inhibited for at least 2 h (Table 2).

Table 1 Time-dependent platelet inhibition induced by polymorphonuclear leutocytes

Agonist	n	*Platelet inhibition (%) at different incubation times* (min)			
		1	5	10	2
Collagen	5	58 ± 17	93 ± 7	100	100
AA	3	31 ± 9	83 ± 17	91 ± 9	100
Thrombin	3	40 ± 5	55 ± 12	91 ± 9	100

Aliquots (200 µl) of washed platelets (5×10^8 ml^{-1}) were incubated with 200 µl of PMN suspension (5×10^6 ml^{-1}) or 200 µl of TA buffer as control samples, for different periods of time at room temperature. Then, platelet aggregation was induced by the addition of collagen, arachidonic acid (AA) or thrombin at concentrations leading under control conditions to 70 to 80% increase in light transmission. These concentrations varied between 1–2 µg ml^{-1}, 0.1–0.2 mM and 0.01–0.02 u ml^{-1} for collagen, AA and thrombin, respectively, according to the donors. The results are expressed as a % inhibition compared to controls. Each value is the mean ± s.e. of n separate experiments.

Table 2 The effect of preincubation and subsequent depletion of polymorphonuclear leukocytes on platelet activation

Treatment	*Platelet aggregation (%) at different times* (min)		
	0	60	120
Untreated platelets	79 ± 3	75 ± 5	70 ± 4
Centrifuged platelets[a]	71 ± 3	65 ± 3	63 ± 2
Platelet depleted of PMNs by centrifugation[b]	5 ± 2	7 ± 3	10 ± 4

One ml of washed platelets (5×10^8 ml^{-1}) were incubated with 1 ml of PMNs (5×10^6 ml^{-1}) (b) or Tyrode albumin buffer (a) for 15 min at room temperature. Then, both cell suspensions were centrifuged at 160 *g* for 1 min. After centrifugation, 800 µl of each supernatant (containing only platelets) was removed and platelet aggregation was induced by collagen (1–2 µg ml^{-1}) at the indicated times. The centrifugation procedure spun down all PMNs, as observed by light microscopy. Untreated platelets represent washed platelets which were not centrifuged. The results are expressed as % of platelet aggregation. Each value is the mean ± s.e. of 3 separate experiments.

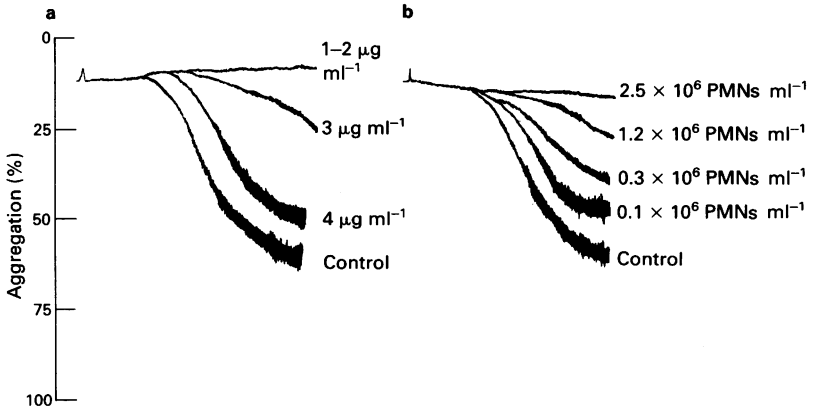

Figure 1 Inhibition of platelet aggregation by polymorphonuclear leukocytes (PMNs). (a) Aliquots (200 µl) of washed platelets (5×10^8 ml^{-1}) were incubated with 200 µl of PMN suspension (5×10^6 ml^{-1}) or 200 µl of Tyrode albumin (TA) buffer (control) for 10 min at room temperature. Then, aggregation was induced by the indicated collagen concentrations. Control samples were stimulated by collagen (1 µg ml^{-1}). (b) Aliquots (200 µl) of washed platelets (5×10^8 ml^{-1}) were incubated with 200 µl of PMN suspension containing different PMN concentrations, as indicated in the figure, or with 200 µl of TA buffer (control). After 10 min of incubation at room temperature, platelet aggregation was induced by collagen (1 µg ml^{-1}). The figures shows typical tracings of aggregation representative of five separate experiments.

Table 3 Effect of some inhibitors on the antiaggregatory activity of polymorphonuclear leukocytes

Treatment		*Platelet aggregation (%)* *Platelets*	*Platelets plus PMNs*	n
None		78 ± 2	35 ± 3	12
ASA*		—	33 ± 4	6
BPB**	10 μM	—	36 ± 6	5
SOD	30 u ml^{-1}	78 ± 5	16 ± 3	5
Cythocrome C	200 μg ml^{-1}	82 ± 4	15 ± 3	5
Catalase	1000 u ml^{-1}	80 ± 4	43 ± 3	5
PMSF	10 μM	74 ± 6	36 ± 5	4
TPCK	10 μM	77 ± 2	43 ± 4	4
$FeSO_4$	1 μM	72 ± 4	36 ± 5	4
Methaemoglobin	5 μM	71 ± 4	37 ± 2	4
Haemoglobin	10 μM	63 ± 7	32 ± 6	4
Methylene blue	10 μM	70 ± 4	31 ± 2	4

Aliquots (200 μl) of washed platelets (5×10^8 ml^{-1}) were incubated with 200 μl of PMN suspension (5×10^6 ml^{-1}) at room temperature for 1 min, in the presence of the different compounds mentioned above. Then, platelet aggregation was induced by the addition of collagen (1–2 μg ml^{-1}). The results are expressed as % of aggregation. Each value is the mean ± s.e. of n separate experiments.

* PMNs were obtained from donors who had ingested 500 mg acetylsalicylic acid (ASA) 24 h before the day of study. Platelets were obtained from donors who had not ingested ASA.

** PMNs were treated with p-bromophenacyl bromide (BPB) for 15 min at 37°C. After washing PMNs were added to washed platelets, which were not treated with BPB. Treated vs non-treated: $P < 0.0005$.

The antiaggregatory activity of PMNs was not mediated by a stable releasable factor(s) since supernatants obtained from PMNs or from mixed cell suspensions (PMNs plus platelets) did not modify platelet aggregation induced by collagen (1–2 μg ml^{-1}) ($n = 4$).

The antiaggregatory activity of PMNs was also observed in PRP. When 200 μl of PMNs (5×10^6 ml^{-1}) were incubated with 200 μl of PRP (5×10^8 ml^{-1}) for 20 min at room temperature, a marked inhibition of the platelet aggregation response to collagen (1 μg ml^{-1}) was observed (% inhibition = 74 ± 6, $n = 4$).

Mechanisms involved in PMN-mediated platelet inhibition

The activation of phospholipid metabolism and the respiratory burst play an important role in most of the biological responses mediated by PMNs (Maridonneau-Parini *et al.*, 1986). In order to determine whether any of the metabolites derived from these pathways account for the antiaggregating activity of PMNs, the effect of several inhibitors was assayed.

Since prostacyclin (PGI_2) is a potent platelet inhibitor (Ubatuba *et al.*, 1979), some experiments were done with PMNs obtained from normal donors who have ingested ASA (500 mg) the day before sampling. As shown in Table 3, there were no differences in the inhibitory effect induced by non-treated or ASA-treated PMNs. Moreover, the antiaggregatory activity was not affected by treatment of the PMNs with the phospholipase inhibitor BPB (Blackwell & Flower, 1983) (Table 3).

Superoxide anion and hydrogen peroxide are well known cytotoxic species released by PMNs. The role of these compounds in PMN-induced platelet inhibition was investigated. SOD (30 u ml^{-1}) and cytochrome C (200 μg ml^{-1}), two superoxide anion scavengers, were not only unable to decrease the anti-aggregating activity of PMNs but rather they significantly enhanced it (Figure 2 and Table 3). On the other hand, the inhibition of the platelet response was not modified by the hydrogen peroxide scavenger, catalase (Table 3).

To investigate whether the platelet antiaggregatory effect was related to the release of nitric oxide (NO) from PMNs (Rimele *et al.*, 1988), several inhibitory compounds of NO activity were assayed. $FeSO_4$ (1 μM), methaemoglobin (5 μM), haemoglobin (10 μM) and methylene blue (10 μM) were unable to reverse platelet inhibition induced by PMNs (Table 3).

Finally, no loss of antiaggregating activity was observed when the mixed-cell suspensions were incubated with the protease inhibitors TPCK (10 μM) and PMSF (10 μM) (Kitagawa *et al.*, 1980).

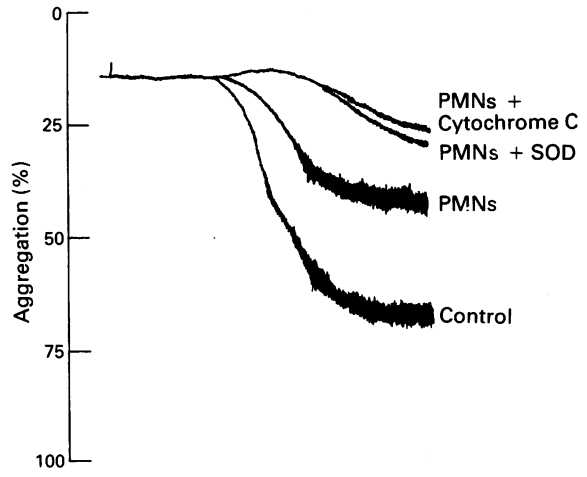

Figure 2 Platelet inhibition by polymorphonuclear leukocytes (PMNs) is increased by superoxide dismutase (SOD) and cytochrome C. Aliquots (200 μl) of washed platelets (5×10^8 ml^{-1}) were incubated with 200 μl of PMN suspensions (5×10^6 ml^{-1}) for 10 min at room temperature in the presence of SOD (30 u ml^{-1}) or cytochrome C (200 μg ml^{-1}). Then, aggregation was induced by collagen (4 μg ml^{-1}). Control: 200 μl of platelets (5×10^8 ml^{-1}) were incubated with 200 μl of Tyrode albumin buffer for 10 min at room temperature and then stimulated by collagen (4 μg ml^{-1}). The figure shows typical tracings of aggregation representative of five experiments.

Discussion

In this study, we have demonstrated that PMNs inhibit aggregation of washed human platelets and ATP release induced by several agonists. The antiaggregatory activity of PMNs is dependent on the number of PMNs and on the incubation time of the mixed-cell suspension. In addition, although inhibited platelets do not respond when activated by low agonist concentrations, they normally do at higher ones. Platelet inhibition by PMNs is also observed in platelet-rich-plasma. However, the effect seems to be less effective since the suppression obtained never reached 100%.

Zoucas *et al.* (1985) have previously reported that latex-stimulated PMNs impaired platelet aggregation through the

release of a soluble factor(s). Moreover, they showed that unstimulated PMNs were unable to inhibit the platelet response. On the contrary, our data demonstrate that unstimulated PMNs markedly suppress platelet aggregation. Controversial results may be explained considering that different experimental conditions were employed in each case. It is important to point out that in the system of Zoucas *et al.* (1985), PMNs were enclosed in dialysis tubes whereas in our experimental conditions, PMNs and platelets were in close cell-to-cell contact. The fact that platelet inhibitory activity is expressed by unstimulated PMNs suggests that this activity is normally present in PMNs. However, we cannot rule out a baseline stimulation of PMNs due to the procedures employed during their purification. Moreover, specific PMN activation by the agonists employed seems not to play a major role in platelet inhibition since platelets preincubated with unstimulated PMNs and then depleted of them remained inhibited when activated by AA, collagen or thrombin.

Our results showing that supernatants from PMNs or PMNs plus platelets do not possess antiaggregatory activity suggest: (1) that there is no PMN releasable factor(s), therefore the inhibition could be related to close cell-to-cell contact; (2) that there is a PMN releasable factor(s) with a very short half life. Interestingly, the presence of PMNs is not permanently required for inhibiting platelet responses since, as was previously mentioned, platelets which were preincubated with PMNs and then depleted of them remained inhibited. Therefore, it could be possible that exposure of platelets to factor(s) released by PMNs or to component(s) of the PMN's membrane are involved in the onset of the reaction.

With regard to the mechanisms implicated in platelet inhibition mediated by PMNs, our results suggest that neither AA metabolites, reactive oxygen intermediates nor proteases released by PMNs are responsible for platelet inhibition. These conclusions are supported by the following findings: (1) PGI_2, which is a very potent platelet inhibitor (Ubatuba *et al.*, 1979) does not account for the PMN-induced inhibition since similar results were obtained with non-treated and ASA-treated PMNs. Moreover, the treatment of PMNs with BPB, a phospholipase inhibitor (Blackwell & Flower, 1983) did not prevent platelet inhibition; (2) PMSF and TPCK, two protease inhibitors did not modify the inhibitory activity of PMNs and (3) SOD and cytochrome C, two superoxide anion scavengers enhanced the ability of PMNs to inhibit platelet responses while catalase had no effect.

Endothelial derived relaxing factor (EDRF), now characterized as nitric oxide (NO) (Palmer *et al.*, 1987) is a potent vasodilator and platelet inhibitor with a very short half life. Superoxide anions destroy this substance and Fe^{2+}, methaemoglobin, haemoglobin and methylene blue interfere with its activity (Gryglewski *et al.*, 1986). Recently, it was indicated that stimulated and non-stimulated PMNs release NO (Rimele *et al.*, 1988). Our results showing that the antiaggregatory activity of PMNs is: (1) enhanced by SOD and cytochrome C; (2) not found in the supernatants of PMNs plus platelets suspensions and (3) more active in washed platelets than in PRP fit with the biological properties described for NO (Moncada *et al.*, 1988). However, NO seems not to be responsible for the antiaggregatory activity of PMNs since it was not reversed by Fe^{2+}, methaemoglobin, haemoglobin or methylene blue. Further studies are required to characterize the nature of the inhibitory mechanism(s) involved in this interesting model of cell interaction.

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References

BLACKWELL, G.J. & FLOWER, R.J. (1983). Inhibition of phospholipase. *Br. Med. Bull.*, **39**, 260–264.

CHIGNARD, M., SELAK, M.A. & SMITH, J.B. (1986). Direct evidence for the existence of a neutrophil-derived platelet activator (neutrophilin). *Proc. Natl. Acad. Sci., U.S.A.*, **83**, 8609–8613.

GEFFNER, J.R., GIORDANO, M., PALERMO, M., PRAT, A., SEREBRINSKY, G. & ISTURIZ, M.A. (1987). Neutrophil-mediated cytotoxicity triggered by immune complexes: the role of reactive oxygen metabolites. *Clin. Exp. Immunol.*, **69**, 668–675.

GRYGLEWSKI, R.J., PALMER, R.M.J. & MONCADA, S. (1986). Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature*, **320**, 454–456.

KITAGAWA, S., TAKAKU, F. & SAKAMOTO, S. (1980). Evidence that proteases are involved in superoxide production by human polymorphonuclear leukocytes and monocytes. *J. Clin. Invest.*, **65**, 74–81.

LEVINE, P.H., WEINGER, R.S., SIMON, J., SCOON, K.L. & KRINSKY, H.I. (1986). Leukocytes-platelet interaction. Release of hydrogen peroxide by granulocytes as a modulator of platelet reactions. *J. Clin. Invest.*, **57**, 955–963.

LOTNER, G.Z., LYNCH, J.M., BETZ, S.J. & HENSON, P.M. (1980). Human neutrophil-derived platelet activating factor. *J. Immunol.*, **124**, 676–684.

MARCUS, A.J., SAFIER, L.B., ULLMAN, H.L., WONG, K.T., BROKNAN, M.J., WEKSLER, B.B. & KAPLAN, K.L. (1981). Effects of acetylglyceryletherphosphorylcholine on human platelet functions in vitro. *Blood*, **58**, 1027–1031.

MARIDONNEAU-PARINI, I., TRINGALE, S.M. & TAUBER, A.I. (1986). Identification of distinct activation pathways of the human neutrophil NADPH-oxidase. *J. Immunol.*, **137**, 2925–2929.

MARTIN, W., VILLIANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, F. (1985). Blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation of rabbit aorta by certain ferrous hemoproteins. *J. Pharmacol. Exp. Ther.*, **233**, 679–685.

MEHTA, P., MEHTA, J. & LAWSON, D. (1986). Leukotrienes potentiate the effects of epinephrine and thrombin on human platelet-aggregation. *Thromb. Res.*, **41**, 731–738.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1988). The discovery of Nitric Oxide as the endogenous nitrovasodilator. *Hypertension*, **12**, 365–372.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

RHEE, B.G., HALL, E.R. & McINTIRE, L.V. (1986). Platelet modulation of polymorphonuclear leukocyte shear induced aggregation. *Blood*, **67**, 240–246.

RIMELE, T.J., STURM, R.J. & ADAMS, L.M. (1988). Interaction of neutrophils with vascular smooth muscle: identification of a neutrophil-derived relaxing factor. *J. Pharmacol. Exp. Ther.*, **245**, 102–111.

TURNER, S.R., TAINER, J.A. & LYNN, W.S. (1977). Biogenesis of chemotactic molecules by the arachidonate lipoxygenase system of platelets. *Nature*, **257**, 680–682.

UBATUBA, F.B., MONCADA, S. & VANE, J. (1979). The effect of prostacyclin (PgI_2) on platelet behaviour, thrombus formation in vivo and bleeding time. *Thromb. Haemost.*, **41**, 425–435.

ZOUCAS, E., FALDT, R. & ANKERST, J. (1985). Effect of latex-stimulated granulocytes on platelet aggregation in man. *Haemostasis*, **15**, 176–181.

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Role of epithelium in agonist-induced contractile responses of guinea-pig trachealis: influence of the surface through which drug enters the tissue

C.F. Iriarte, *R. Pascual, M.M. Villanueva, M. Román, [1,†]J. Cortijo & E.J. Morcillo

Department of Physiology and Pharmacology, Faculty of Medicine, University of Alcalá, *Laboratorios Liade, Alcalá de Henares, Madrid and †Department of Pharmacology, University of Valencia, Avenida Blasco Ibañez, No 15, 46010, Valencia, Spain

1 A method has been used in guinea-pig isolated tracheal rings to achieve selective drug entry from the adventitial or mucosal surface. A study has been made of the effects of epithelium removal on responses to spasmogens entering the tissue solely from the adventitial or the mucosal surface.

2 Cumulative concentration-response curves for KCl (1 to 100 mM), acetylcholine (0.1 μM to 10 mM) and histamine (1 μM to 1 mM) were constructed in intact and epithelium-denuded tracheal rings in circumstances where drug entry was unrestricted or restricted to the adventitial or mucosal surface.

3 Epithelium removal did not alter the responsiveness or sensitivity of tracheal rings to KCl either when drug entry was unrestricted or when drug entry was restricted to the adventitial or mucosal surface.

4 When acetylcholine entered from the mucosal or adventitial surfaces of intact tracheal rings its concentration-response curve was displaced to the right with respect to that obtained for unrestricted drug entry. A greater rightward shift was observed for mucosal drug entry than for adventitial drug entry. Epithelium removal potentiated acetylcholine entering from the mucosal surface to a greater extent (27.5 fold) than it potentiated acetylcholine entering from both surfaces (4 fold). Epithelium removal did not potentiate effects of acetylcholine entering from the adventitial surface alone.

5 In intact tracheal segments, concentration-response curves for histamine entering from the mucosal surface were displaced to the right compared with those for histamine entering in an unrestricted fashion or from the adventitial surface alone. This displacement was absent in epithelium-denuded preparations. Epithelium removal potentiated (2–3 fold) histamine entering from the mucosal surface or entering in an unrestricted way. It did not potentiate histamine entering from the adventitial surface alone.

6 Our findings suggest that the epithelium does not modulate tracheal responses to KCl. Its ability to modulate responses to acetylcholine and histamine is observed when these spasmogens enter the tissue from the mucosal surface but not when they enter from the adventitial surface. The mechanism by which epithelium removal preferentially potentiates acetylcholine and histamine entering from the mucosal rather than the adventitial surface remains to be determined.

Introduction

Bronchial hyperreactivity is a characteristic feature of human asthma which may be related to epithelial damage as suggested by morphological, experimental and clinical observations (Hogg & Eggleston, 1984; Laitinen *et al.*, 1985; Seltzer *et al.*, 1986). Mechanical removal of the epithelium enhances the responsiveness and sensitivity to a variety of spasmogens in airways preparations isolated from several animal species, including man (for reviews see: Cuss & Barnes, 1987; Fedan *et al.*, 1988; Vanhoutte, 1988). If mechanisms such as alterations in drug metabolism/disposition or the loss of a passive diffusion barrier can be discounted, the increased reactivity found *in vitro* after epithelium removal is suggestive of the existence of an inhibitory signal generated by the epithelium (Flavahan *et al.*, 1985). Results from bioassay experiments (Ilhan & Sahin, 1986; Guc *et al.*, 1988; Fernandes *et al.*, 1989) and modified 'sandwich' preparations (Tschirhart & Landry, 1986; Hay *et al.*, 1987) further support this concept.

The existence of epithelial-derived contracting factors has also been proposed (Jongste *et al.*, 1987; Stuart-Smith & Vanhoutte, 1988). However, the precise epithelial cell type(s) producing these factors as well as their chemical nature, biological half-life and mechanism(s) of action remain to be determined (Fedan *et al.*, 1988). It can be proposed that such factors are released towards the submucosa and diffuse through the tissues to reach the deeper-lying smooth muscle where their modulatory role is exerted. Nerve fibres, microvessels, secretory cells, neuroendocrine cells and inflammatory cells within the airway wall should probably also be considered as targets for the actions of epithelium-derived modulatory factors.

The effect of epithelium removal on airways reactivity has usually been studied, with few exceptions (Munakata *et al.*, 1988, 1989), under conditions of unrestricted drug entry to the airway wall. However, for optimal demonstration of the influence of the epithelium, airway responses should be measured in circumstances where drug enters the airway wall from the mucosal surface alone or from the adventitial surface alone. A procedure previously developed to restrict drug entry to the intimal or adventitial surface of rabbit isolated aorta (Pascual & Bevan, 1979; 1980; Iriarte *et al.*, 1985; Pascual *et al.*, 1989) has now been applied to guinea-pig isolated trachea. The aim of the present work was to determine whether the effects of the epithelium in modulating the sensitivity of airways smooth muscle to spasmogens is dependent on the route by which the spasmogen gains entry to the tissue.

Methods

Male albino guinea-pigs weighing 300 to 450 g were killed by stunning and bleeding. Tracheae were excised, cleaned of adhering connective and adipose tissue, and cut into rings of approximately 4 mm width. At least six rings were obtained from each trachea. These rings were randomly allocated to

[1] Author for correspondence.

provide paired preparations (epithelium-intact and epithelium-denuded) for either unrestricted drug entry or for drug entry from the adventitial or mucosal surface alone (see below). Where appropriate the epithelium was removed by gently rubbing the mucosal surface with a wet filter paper. At the end of each experiment epithelial removal was assessed by histological examination (light microscopy, haematoxylin-eosin staining). Tracheal rings were suspended under a resting tension of 2 g (progressively imposed by small increments of tension over 30 min) in 50 ml tissue baths containing Krebs bicarbonate solution bubbled with 95% O_2 and 5% CO_2 at 37°C (pH = 7.4). An equilibration period of 60 min elapsed before any drug addition to the bath. Tension was measured with Grass FT03 isometric transducers and recorded on a model 7 Grass polygraph.

The technique used to determine the surface by which drug entered the tracheal wall was similar to that described for rabbit isolated aorta (Pascual *et al.*, 1989). In brief, Vaseline was applied to cover completely either the adventitial or the mucosal surface of the ring, thus restricting drug entry to the mucosal or the adventitial surface, respectively. Preliminary experiments demonstrated the effectiveness of the barrier in that covering both surfaces of the preparation with Vaseline resulted in complete loss of drug-induced response.

Experimental protocol

After the equilibration period, all preparations were exposed to a single priming concentration of acetylcholine (0.1 mM). After washout and recovery of baseline, cumulative concentration response curves to either KCl (1 to 100 mM), acetylcholine (0.1 μM to 10 mM) or histamine (1 μM to 1 mM) were constructed. With the exception of KCl, only one concentration-response curve to an agonist was obtained from each ring. For KCl a second concentration-response curve was generated after washout and a rest period of 45 min. The application of Vaseline to determine the route of drug entry was carried out before the second concentration-response curve to KCl or before the concentration-response curves for acetylcholine or histamine. Comparisons were made with paired preparations not subjected to Vaseline application. There was no significant difference between the resting tension values of control (intact rings, unrestricted drug entry) and test (epithelium-denuded, adventitial or mucosal drug entry) tracheal rings at the time of constructing the concentration-response curves to the agonists.

Efficacy (estimated as E_{max}) and potency (assessed as $-\log_{10}$ molar EC_{50}) of the agonists was calculated from individual concentration-response curves. Differences in sensitivity to the agonists under different conditions (with/without epithelium; unrestricted/restricted drug entry) was quantified by measurement of concentration-ratios (CR) at the EC_{50} level and expressed as $\log_{10}$ CR. The results are given as means ± s.e.mean. Statistical analysis of the results was performed by analysis of variance (ANOVA), followed by Duncan's tests. Unless otherwise stated the significance levels quoted are for these tests. Differences were considered significant when $P < 0.05$.

Drugs and solutions

Drugs used were acetylcholine chloride and histamine hydrochloride (Sigma). KCl was of analytical grade. Drugs were prepared and diluted in distilled water and added to the tissue bath in small volumes. With the exception of KCl (where the stated concentration was that in excess of KCl provided by the Krebs solution) drug concentrations are expressed as final bath concentrations (molar). The composition of the Krebs solution used in this study was (mM): NaCl 115, KCl 4.6, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 25, EDTA 0.02 and glucose 11.1.

Results

Responses to KCl

KCl (1–100 mM) produced concentration-dependent contractions of intact, untreated (i.e. unrestricted drug entry) preparations (first curves in Table 1). Repetition of KCl administration in intact, untreated rings after a 45 min interval resulted in concentration-response curves (second curves in Table 1) with characteristics not significantly different from those obtained after its first addition to the bath. Irrespective of whether this second concentration-response curve was generated with KCl selectively entering the tissue via the mucosal or the adventitial surface, it did not significantly differ from the second curve obtained in paired, time-matched, rings where drug entry was unrestricted or from the initial curve generated in the same ring when drug entry was unrestricted (Table 1). First and second concentration-response curves for KCl constructed in epithelium-denuded rings did not differ from those obtained, under similar experimental conditions (unrestricted or selective drug entry), in intact paired rings (Table 1, Figure 1a,b).

Responses to acetylcholine

Acetylcholine (0.1 μM–1 mM) produced concentration-dependent contraction of intact, untreated tracheal rings. When acetylcholine entry was restricted to either the mucosal or the adventitial surface of the trachea, concentration-response curves for this agent were displaced to the right compared with those generated in untreated rings (Table 2). Concentration-response curves for acetylcholine entering from the mucosal surface were more displaced to the right than those for acetylcholine entering from the adventitial surface. The concentration-response curve for mucosal acetylcholine entry therefore lay to the right of that obtained after adventitial drug entry (CR = 11.75). The maximal effects of acetylcholine entering in an unrestricted way, or by the adventitial route, or by the mucosal route, did not differ significantly.

Removal of the epithelium produced a 4.07 fold leftward shift of the concentration-response curves to acetylcholine

Table 1 Effect of KCl in guinea-pig trachea for unrestricted or selective (mucosal or adventitial) drug entry: maximal effects (E_{max}) and log(EC_{50}) values are given for the first and second concentration-response curves (CRC) constructed to KCl in intact (E+) or epithelium-denuded (E−) tracheal rings

		$-log\ EC_{50}$			E_{max} (g)	
1st CRC	*Unrestricted*	*Unrestricted*	*Unrestricted*	*Unrestricted*	*Unrestricted*	*Unrestricted*
E+	2.23 ± 0.08	2.16 ± 0.06	2.13 ± 0.07	1.54 ± 0.22	1.81 ± 0.19	2.11 ± 0.23
E−	2.14 ± 0.12	1.97 ± 0.09	1.99 ± 0.09	1.26 ± 0.11	1.59 ± 0.34	1.73 ± 0.29
2nd CRC	*Unrestricted*	*Mucosal*	*Adventitial*	*Unrestricted*	*Mucosal*	*Adventitial*
E+	2.11 ± 0.09	1.99 ± 0.09	2.00 ± 0.09	1.73 ± 0.27	1.67 ± 0.20	1.89 ± 0.26
E−	2.03 ± 0.15	1.87 ± 0.08	1.87 ± 0.11	1.43 ± 0.14	1.77 ± 0.43	1.70 ± 0.22

Data are means ± s.e.mean of 7 experiments in each group. No significant difference among groups was found.

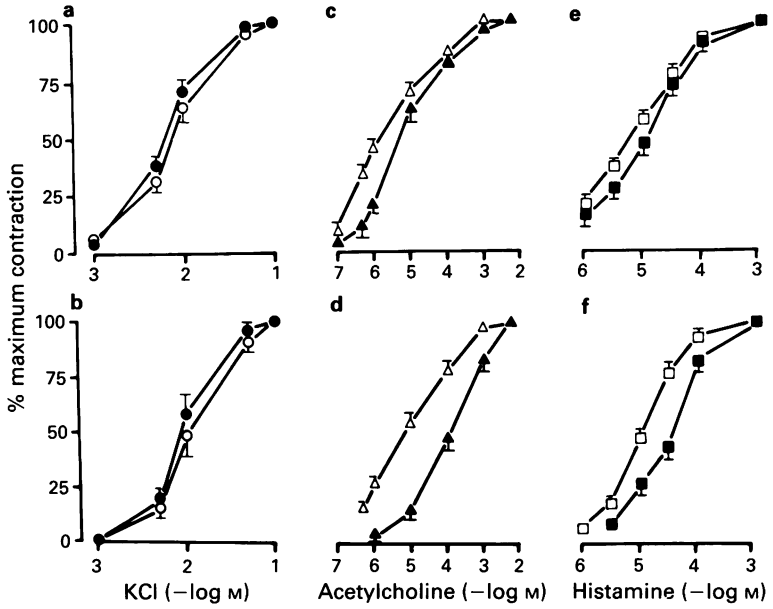

Figure 1 Log concentration-response curves to KCl (a, b), acetylcholine (c, d) and histamine (e, f) in guinea-pig isolated trachea. Responses were obtained in intact (closed symbols) or epithelium-denuded (open symbols) preparations for unrestricted (upper panel, i.e. a, c and e) or mucosal (lower panel, i.e. b, d and f) entry of drugs. Data are expressed as percentage of maximal response to each agonist (see Tables 1, 2 and 3) and are shown as means of 7 experiments for KCl, 6 for acetylcholine and 8 for histamine; s.e.mean shown by vertical bars.

entering in an unrestricted way without affecting its maximal effect (Table 2, Figure 1c). Removal of the epithelium did not alter the concentration-response curve for acetylcholine entering from the adventitial surface of the preparation. In contrast, the concentration-response curve obtained in epithelium-denuded preparations for acetylcholine entering from the mucosal surface was shifted to the left (27.54 fold) and the maximal effect was increased compared with the corresponding curve observed in intact preparations (Table 2, Figure 1d). The degree of leftward displacement produced after epithelium removal was greater in rings prepared for mucosal access of acetylcholine (CR = 27.54) than in the untreated preparations (CR = 4.07). In the epithelium-denuded preparations, the concentration-response curves for acetylcholine entering from the mucosal or adventitial surfaces lay superimposed although they exhibited a rightward shift compared with that for unrestricted drug entry (Table 2). This indicates that the removal of the epithelium eliminated the separation observed in intact preparations between the concentration-response curve for acetylcholine entering by the mucosal route and the corresponding curve for entry by the adventitial route (Table 2).

Responses to histamine

Histamine (1 μM–1 mM) produced concentration-dependent contraction of intact, untreated tracheal rings. When histamine entry was restricted to the adventitial surface of the trachea its concentration-response curve was not significantly different from that observed with unrestricted drug entry. In contrast, when histamine entry was restricted to the mucosal surface its concentration-response curve was displaced to the right compared with that for unrestricted drug entry or entry from the adventitial surface (Table 3). The concentration-response curve for histamine entering from the mucosal surface lay to the right of that obtained for adventitial drug entry (CR = 2.69). The separation between concentration-response curves for histamine entering from the adventitial surface alone and that for mucosal entry was smaller than that observed for acetylcholine ($P < 0.05$, Table 2 and Table 3). The maximal effects of histamine entering in an unrestricted way, or from the adventitial surface or from the mucosal surface did not differ significantly. Removal of the epithelium produced a 2.04 fold leftward shift of the concentration-response curve for histamine entering in an unrestricted way,

Table 2 Effect of acetylcholine in guinea-pig trachea for unrestricted or selective (mucosal or adventitial) drug entry: maximal effects (E_{max}) and log(EC_{50}) values are given for concentration-response curves constructed in intact (E+) or epithelium-denuded (E−) tracheal rings

	$-log_{10}$ EC_{50}			E_{max} (g)		
	Unrestricted	*Mucosal*	*Adventitial*	*Unrestricted*	*Mucosal*	*Adventitial*
E+	5.18 ± 0.10	3.74 ± 0.06◆	4.81 ± 0.13◆†	1.45 ± 0.15	1.04 ± 0.19	1.29 ± 0.08
E−	5.79 ± 0.12*	5.18 ± 0.16*◆	4.95 ± 0.26◆	1.57 ± 0.22	1.75 ± 0.16*	1.41 ± 0.18

	log_{10} concentration-ratio				
Unrestricted E− *vs.* E+	*Mucosal* E− *vs.* E+	*Adventitial* E− *vs.* E+	*With epithelium (E+) adventitial vs. mucosal*	*Without epithelium (E−) adventitial vs. mucosal*
−0.61 ± 0.10	−1.44 ± 0.11◆	−0.13 ± 0.16◆†	−1.07 ± 0.09	−0.24 ± 0.11*

Shift in curves is expressed as log_{10} concentration-ratio (a negative sign signifies a leftward shift) at the EC_{50} level.
Data are means ± s.e.mean of 6 experiments in each group. * $P < 0.05$ from rings with epithelium. ◆ $P < 0.05$ from values for unrestricted drug entry. † $P < 0.05$ from values for mucosal drug entry.

Table 3 Effect of histamine in guinea-pig trachea for unrestricted or selective (mucosal or adventitial) drug entry: maximal effect (E_{max}) and log(EC_{50}) values are given for concentration-response curves constructed in intact (E+) or epithelium-denuded (E−) tracheal rings

	$-log_{10}$ EC_{50}			E_{max} (g)		
	Unrestricted	*Mucosal*	*Adventitial*	*Unrestricted*	*Mucosal*	*Adventitial*
E+	4.92 ± 0.06	4.44 ± 0.07◆	4.87 ± 0.08†	0.77 ± 0.06	0.06 ± 0.07	0.85 ± 0.20
E−	5.23 ± 0.10*	4.97 ± 0.08*◆	4.93 ± 0.11◆	0.83 ± 0.19	0.89 ± 0.18*	0.79 ± 0.15

log_{10} concentration-ratio				
Unrestricted E− vs. E+	*Mucosal* E− vs. E+	*Adventitial* E− vs. E+	*With epithelium (E+)* adventitial vs. mucosal	*Without epithelium (E−)* adventitial vs. mucosal
−0.31 ± 0.11	−0.53 ± 0.13	−0.05 ± 0.04◆†	−0.43 ± 0.11	−0.04 ± 0.08*

Shift in curves is expressed as log_{10} concentration-ratio (a negative sign signifies a leftward shift) as the EC_{50} level.
Data are means ± s.e.mean of 6 experiments in each group. * $P < 0.05$ from rings with epithelium. ◆ $P < 0.05$ from values for unrestricted drug entry. † $P < 0.05$ from values for mucosal drug entry.

without affecting its maximal effect (Table 3, Figure 1e). Removal of the epithelium did not alter the concentration-response curve for histamine entering from the adventitial surface of the preparation. In contrast, the concentration-response curve observed in epithelium-denuded preparations for histamine entering from the mucosal surface was displaced leftward 3.39 fold with respect to the corresponding curve obtained in intact preparations (Table 3, Figure 1f). Although this displacement was greater than that observed in untreated preparations, it did not reach statistical significance. As for acetylcholine, the separation existing between the concentration-response curves for histamine entering by the adventitial and mucosal routes in intact rings was abolished by epithelium removal (Table 3). Epithelium removal produced significantly greater leftward displacement of concentration-response curves for unrestricted or mucosal acetylcholine entry than it did for histamine under the same circumstances ($P < 0.05$, Table 2 and Table 3).

Discussion

The results of *in vitro* pharmacomechanical and bioassay experiments generally suggest that a factor released from the airway epithelium modulates airway smooth muscle reactivity (Cuss & Barnes, 1987; Fedan *et al.*, 1988; Vanhoutte, 1988). However, some authors (e.g. Lundblad & Persson, 1988) have been unable to obtain data in support of this suggestion. In most studies of the effects of the epithelium on airways smooth muscle responsiveness, agonists added to the tissue bath entered the isolated airway preparation both from the mucosal and from the adventitial surfaces simultaneously. However, study of the influence of the epithelium is probably better approached by determining the consequences of epithelium removal in circumstances where the agonist enters the tissue from the mucosal surface alone or from the adventitial surface alone. A procedure by which drug access to isolated vascular smooth muscle was restricted to the intimal or adventitial surface (Pascual & Bevan, 1979; 1980; Iriarte *et al.*, 1985; Pascual *et al.*, 1989), has been adapted for use in guinea-pig tracheal rings. The technique involves coating the appropriate tissue surface with Vaseline and preliminary experiments showed that this did not cause overt damage to the epithelial or smooth muscle layers. The technique was applied to study the role of the epithelium in modulating tracheal responses to KCl, acetylcholine and histamine.

In the epithelium-intact guinea-pig tracheal tubes, hyperosmolar KCl applied to the mucosal surface suppresses the spasm evoked by the application of hyperosmolar KCl to the adventitial surface (Munakata *et al.*, 1988). Furthermore, in the same type of preparation, epithelium removal potentiates KCl as a spasmogen applied to the mucosal surface (Munakata *et al.*, 1989). These findings are consistent with the proposal that KCl may release an inhibitory factor from the airways epithelium. It is therefore curious that, in the present study, epithelium removal failed to modulate responses to KCl irrespective of whether KCl entry to the trachealis was unrestricted or was from the adventitial or mucosal surface alone. Nevertheless, our findings confirm those of earlier workers (e.g. Goldie *et al.*, 1986; Hay *et al.*, 1986; Holroyde, 1986; Fedan *et al.*, 1988) who have studied the action of KCl in opened segments of trachea and bronchus under conditions of unrestricted drug entry. The present data suggest that the epithelium acts neither as a diffusion barrier for KCl nor as a site of loss for KCl. In contrast to the results of the experiments with perfused tracheal tubes (Munakata *et al.*, 1988; 1989), the present data suggest either that hyperosmolar KCl does not liberate a relaxant factor from the airways epithelium or that the spasmogenic effects of KCl on the airways smooth muscle cells (which involve depolarization and Ca^{2+} influx – Foster *et al.*, 1983) cannot be functionally antagonized by such a factor.

In the present study concentration-response curves for acetylcholine and histamine obtained in epithelium-denuded preparations with unrestricted drug entry, exhibited about 4 fold and 2 fold leftward shifts, respectively, compared with the corresponding curves obtained in intact tracheal rings. Although Goldie *et al.* (1986) reported that stripping of epithelium did not alter responses to acetylcholine, other authors have found that removal of epithelium produces a leftward shift of concentration-response curves to acetylcholine in the guinea-pig (Holroyde, 1986) and other animal species (Flavahan *et al.*, 1985; Stuart-Smith & Vanhoutte, 1987; 1988) including man (Aizawa *et al.*, 1988). The concentration-ratios reported in these studies ranged from 2.7 to 12.5. Concentration-response curves for histamine are also displaced to the left (concentration-ratios ranging from 1.99 to 8.2) in epithelium-denuded tracheal preparations from the guinea-pig (Goldie *et al.*, 1986; Hay *et al.*, 1986; Holroyde, 1986; Tschirhart *et al.*, 1987), and other animal species (Flavahan *et al.*, 1985; Aizawa *et al.*, 1988; Stuart-Smith & Vanhoutte, 1988). As stated in recent reviews (Cuss & Barnes, 1987; Fedan *et al.*, 1988; Vanhoutte, 1988), these effects may be explained in terms of the epithelium acting as a passive diffusion barrier, as a site for the uptake and metabolism of drugs or as a source of a relaxing factor.

In the present experiments with acetylcholine and histamine concentration-response curves for selective drug entry lay (with the sole exception of histamine entry from the adventitial surface) to the right of those obtained when the drug entry was unrestricted. Furthermore, concentration-response curves for acetylcholine and histamine entering from the mucosal surface were consistently displaced to the right of those observed for drug entry from the adventitial surface.

When applied to the trachea *in vitro*, acetylcholine and histamine have to diffuse through various tissue layers before reaching the smooth muscle. They are subjected to metabolic degradation (hydrolysis and oxidative deamination, respectively) and to unspecific binding and uptake processes before reaching their specific receptors. This situation creates an inwardly-directed concentration gradient for these drugs. It is therefore understandable that when drug entry was

restricted to only one surface of the preparation, the sensitivity of the trachea to acetylcholine and histamine was diminished (Figure 1, Tables 2 and 3).

This loss of sensitivity was most pronounced in epithelium-intact preparations where drug entry through the adventitial surface was prevented. In other words, restricting entry to the mucosal surface caused a greater reduction in potency than restricting drug entry to the adventitial surface. That the epithelium was mainly responsible for limiting the potency of drugs entering from the mucosal surface is suggested by the fact that epithelium removal rendered the tissue equisensitive to drugs entering via the adventitial or mucosal surfaces (Tables 2 and 3).

Removal of the epithelium produced a greater leftward displacement of concentration-response curves to acetylcholine and histamine entering from the mucosal surface than that observed in preparations where drug entry was unrestricted, although this difference only reached statistical significance for acetylcholine. In the case of acetylcholine, therefore, the modulatory role of the epithelium is greater when the drug enters from the mucosal surface than when it enters from the adventitial surface.

Tissue sensitivity to acetylcholine and histamine entering from the adventitial surface was similar in intact and epithelium-free preparations respectively. This may suggest that very little of the agonist reaches the epithelium by this route and/or that agonist contact with the adventitial side of the epithelium is not able to cause the release of epithelium-derived relaxing factor.

In spite of methodological differences, the present results for acetylcholine and histamine are consistent with those reported by Munakata *et al.* (1989) although the degree of potentiation of acetylcholine or histamine entering from the mucosal surface caused by epithelium removal was smaller in the present work. An additional similarity between the present results and those of Munakata *et al.* (1989) is the greater influence of epithelium removal on sensitivity to acetylcholine than on sensitivity to histamine. Possible explanations for this might include the epithelium acting as a more powerful site of loss for acetylcholine compared with histamine or acetylcholine having the greater ability to stimulate the release of an epithelium-derived relaxing factor. The possibility that epithelium-derived relaxant factor might functionally antagonize the action of acetylcholine on the trachealis muscle, but not that of histamine, should also be considered. Carbachol, a cholinomimetic resistant to cholinesterases, is not potentiated by epithelium removal in the guinea-pig trachea (Tschirhart *et al.*, 1987; Lundblad & Persson, 1988). In contrast, methacholine and bethanechol are potentiated by epithelium removal in guinea-pig (Hay *et al.*, 1986) and rabbit (Butler *et al.*, 1987) tracheal preparations, respectively. Inhibition of cholinesterase in canine bronchus showed that the activity of this enzyme is irrelevant to the effect of epithelium removal on acetylcholine-induced responses (Flavahan *et al.*, 1985). Dixon *et al.* (1989) have demonstrated that the guinea-pig tracheal epithelium does not represent a site of metabolic loss for acetylcholine. As far as we are aware, the presence and distribution of enzymes degrading histamine in the airway wall have not been examined in any detail.

The augmentation, after epithelium removal of the maximal response to acetylcholine entering from the mucosal surface (Table 2) suggests that the epithelium modulates contraction to acetylcholine by mechanisms other than those with a dispositional basis (Kalsner, 1974). This finding is compatible with acetylcholine releasing an epithelium-derived relaxing factor. In contrast, the corresponding maximal effect for histamine was not changed by epithelium removal. Other studies add weight to this difference between acetylcholine and histamine. Thus, acetylcholine (10 μM–1 mM), but not histamine (same concentration-range), released a relaxant factor from guinea-pig trachea in bioassay experiments (Ilhan & Sahin, 1986). Holroyde (1986) showed, in superfusion studies, that histamine (up to 10 mM) failed to release any relaxant factor from guinea-pig trachea. Recently, Fernandes *et al.* (1989) provided direct evidence for the production of an epithelium-derived relaxing factor from the guinea-pig trachea in response to methacholine and histamine, but much greater concentrations of the latter were needed for the same releasing effect.

We conclude that the epithelium does not modulate tracheal responses to hyperosmolar KCl. Its ability to modulate responses to acetylcholine and histamine is greatest when these agents enter the tissue from the mucosal rather than the adventitial surface. The mechanism by which epithelium removal preferentially potentiates acetylcholine and histamine entering from the mucosal rather than the adventitial surface remains to be determined. However, it could result from these agents causing the release of an epithelium-derived relaxant factor by acting on the luminal surface of the epithelial cells. In addition, *in vitro* studies using selective (mucosal *vs.* adventitial) drug entry may be pertinent in comparisons with *in vivo* studies (Holtzman *et al.*, 1983; Fennessy *et al.*, 1986) where different effects of drugs were found after their administration by inhalation or by the intravenous route.

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References

AIZAWA, H., MIYAZAKI, N., SHIGEMATSU, N. & TOMOOKA, H. (1988). A possible role of airway epithelium in modulating hyperresponsiveness. *Br. J. Pharmacol.*, **93**, 139–145.

BUTLER, G.B., ADLER, K.B., EVANS, J.N., MORGAN, D.W. & SZAREK, J.L. (1987). Modulation of rabbit airway smooth muscle responsiveness by respiratory epithelium. *Am. Rev. Respir. Dis.*, **135**, 1099–1104.

CUSS, F.M. & BARNES, P. (1987). Epithelial mediators. *Am. Rev. Respir. Dis.*, **136**, S32–S35.

DIXON, J.S., GOOD, D.M., GOSLING, J.A. & SMALL, R.C. (1989). Effects of epithelium removal and cholinesterase inhibition on responses of guinea-pig trachealis to muscarinic agonists. *Br. J. Pharmacol.*, **97**, 552P.

FEDAN, J.S., HAY, D.W.P., FARMER, S.G. & RAEBURN, D. (1988). Epithelial cells: Modulation of airway smooth muscle reactivity. In *Asthma: Basic Mechanisms and Clinical Management*. ed. Barnes, P.J., Rodger, I.W. & Thomson, N.C. pp. 143–162. Academic Press: London.

FENNESSY, M.R., STEWART, A.G. & THOMPSON, D.C. (1986). Aerosolized and intravenously administered leukotrienes: effects on the bronchoconstrictor potency of histamine in the guinea-pig. *Br. J. Pharmacol.*, **87**, 741–749.

FERNANDES, L.B., PATERSON, J.W. & GOLDIE, R.G. (1989). Co-axial bioassay of a smooth muscle relaxant factor released from guinea-pig tracheal epithelium. *Br. J. Pharmacol.*, **96**, 117–124.

FLAVAHAN, N.A., AARHUS, L.L., RIMELE, T.J. & VANHOUTTE, P.M. (1985). Respiratory epithelium inhibits bronchial smooth muscle tone. *J. Appl. Physiol.*, **58**, 834–838.

FOSTER, R.W., SMALL, R.C. & WESTON, A.H. (1983). The spasmogenic action of potassium chloride in guinea-pig trachealis. *Br. J. Pharmacol.*, **80**, 553–559.

GOLDIE, R.G., PAPADIMITRIOU, J.M., PATERSON, J.W., RIGBY, P.J., SELF, H.M. & SPINA, D. (1986). Influence of the epithelium on responsiveness of guinea-pig isolated trachea to contractile and relaxant agonists. *Br. J. Pharmacol.*, **87**, 5–14.

GUC, M.O., ILHAM, M. & KAYAALP, S.O. (1988). The rat anococcygeus muscle is a convenient bioassay organ for the airway epithelium-derived relaxant factor. *Eur. J. Pharmacol.*, **148**, 405–409.

HAY, D.W.P., FARMER, S.G., RAEBURN, D., ROBINSON, V.A., FLEMING, W.W. & FEDAN, J.S. (1986). Airway epithelium modulates the reactivity of guinea-pig respiratory smooth muscle. *Eur. J. Pharmacol.*, **129**, 11–18.

HAY, D.W.P., MUCCITELLI, R.M., HORSTEMEYER, D.L., WILSON, K.A. & RAEBURN, D. (1987). Demonstration of the release of an

epithelium-derived inhibitory factor from a novel preparation of guinea-pig trachea. *Eur. J. Pharmacol.*, **136**, 247–250.

HOGG, J.C. & EGGLESTON, P.A. (1984). Is asthma an epithelial disease? *Am. Rev. Respir. Dis.*, **129**, 207–208.

HOLROYDE, M.C. (1986). The influence of epithelium on the responsiveness of guinea-pig isolated trachea. *Br. J. Pharmacol.*, **87**, 501–507.

HOLTZMAN, M.J., McNAMARA, M.P., SHEPPARD, D., FABBRI, L.M., HAHN, H.L., GRAF, P.D. & NADEL, J.A. (1983). Intravenous versus inhaled atropine for inhibiting bronchoconstrictor responses in dogs. *J. Appl. Physiol.*, **54**, 134–139.

ILHAN, M. & SAHIN, P. (1986). Tracheal epithelium releases a vascular smooth muscle relaxant factor: Demonstration by bioassay. *Eur. J. Pharmacol.*, **131**, 293–296.

IRIARTE, C.F., PASCUAL, R., MORCILLO, E. & BEVAN, J.A. (1985). Responses of rabbit aorta to tyramine in relation to the surface of drug entry. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **329**, 24–29.

KALSNER, S. (1974). A new approach to the measurement and classification of forms of supersensitivity of autonomic effector responses. *Br. J. Pharmacol.*, **51**, 427–434.

LAITINEN, L.A., HEINO, M., LAITINEN, A., KAVA, T. & HAAHTELA, T. (1985). Damage of the airway epithelium and bronchial reactivity in patients with asthma. *Am. Rev. Respir. Dis.*, **131**, 599–606.

LUNDBLAD, K.A.L. & PERSSON, C.G.A. (1988). The epithelium and the pharmacology of guinea-pig tracheal tone *in vitro*. *Br. J. Pharmacol.*, **93**, 909–917.

MUNAKATA, M., HUANG, I., MITZNER, W. & MENKES, H. (1989). Protective role of epithelium in the guinea-pig airway. *J. Appl. Physiol.*, **66**, 1547–1552.

MUNAKATA, M., MITZNER, W. & MENKES, H. (1988). Osmotic stimuli induce epithelial-dependent relaxation in the guinea-pig trachea. *J. Appl. Physiol.*, **64**, 466–471.

PASCUAL, R. & BEVAN, J.A. (1979). Variation in contractile response characteristics of rabbit aorta strips with surface of drug entry. *J. Pharmacol. Exp. Ther.*, **210**, 51–55.

PASCUAL, R. & BEVAN, J.A. (1980). Asymmetry of consequences of drug disposition mechanisms in the wall of the rabbit aorta. *Circ. Res.*, **46**, 22–28.

PASCUAL, R., VILLANUEVA, M., IRIARTE, C.F. & MORCILLO, E. (1989). Action of methylene blue and haemoglobin in rabbit aorta in relation to the surface of drug entry. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **339**, 340–347.

SELTZER, J., GEFFROY, B., STULBARG, M., HOLTZMAN, M.J., NADEL, J.A. & BOUSHEY, H.A. (1986). Ozone induced change in bronchial reactivity to methacholine and airway inflammation in human subjects. *J. Appl. Physiol.*, **60**, 1321–1326.

STUART-SMITH, K. & VANHOUTTE, P.M. (1987). Heterogeneity in the effects of epithelium removal in the canine bronchial tree. *J. Appl. Physiol.*, **63**, 2510–2515.

STUART-SMITH, K. & VANHOUTTE, P.M. (1988). Airway epithelium modulates the responsiveness of porcine bronchial smooth muscle. *J. Appl. Physiol.*, **65**, 721–727.

TSCHIRHART, E., FROSSARD, N., BERTRAND, C. & LANDRY, Y. (1987). Arachidonic acid metabolites and airway epithelium-dependent relaxant factor. *J. Pharmacol. Exp. Ther.*, **243**, 310–316.

TSCHIRHART, E. & LANDRY, Y. (1986). Airway epithelium releases a relaxant factor: demonstration with substance P. *Eur. J. Pharmacol.*, **132**, 103–104.

VANHOUTTE, P.M. (1988). Editorial. Epithelium derived relaxing factor: myth or reality? *Thorax*, **43**, 665–668.

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Thermal trauma alters myocardial cyclic nucleotides and protein content in mice

[1]John F. Tomera & J.A. Jeevendra Martyn

Department of Anaesthesiology, Harvard Medical School, and Clinical Pharmacology Laboratory, Anesthesia Services, Massachusetts General Hospital and Shriners Burns Institute, Boston, Massachusetts, MA02114, U.S.A.

1 This study tested the hypothesis that the systemic effects of burn include altered metabolic activity in the heart. Metabolic activity was studied by measuring alterations in cyclic nucleotide levels and protein concentrations in atrial and ventricular muscle in mice at 14 and 22 days after a 20% body surface area (BSA) burn. Thermal injury was produced on the dorsal surface of anesthetized male CD mice by immersion in water at 95°C for 8 s. This resulted in a full-thickness, 3° scald burn. In atrial and ventricular tissues, levels of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) were analyzed by ^{125}I-radioimmunoassay.

2 The protein content (mg prot g^{-1} dry wt) increased in the atria. The cyclic AMP content (nmol g^{-1} dry wt) was significantly increased fourfold and ninefold at 14 and 22 days, respectively, in atria from burned animals compared to controls. The cyclic AMP/cyclic GMP ratios were similarly increased.

3 In the ventricle, the protein content and cyclic AMP levels were not altered, but the cyclic AMP/cyclic GMP ratios (nmol g^{-1} dry wt) were increased at both 14 and 22 days. These changes both in atria and ventricles were less prominent when cyclic nucleotide concentrations or ratios were expressed as pmol mg^{-1} protein.

4 The data confirm the hypothesis that a 20% BSA thermal injury evokes effects in sites remote from burn injury such as in the atria and ventricles. These effects include total body weight loss, elevated cyclic AMP, cyclic AMP/cyclic GMP ratios, and protein levels in the atria, and elevated cyclic AMP/cyclic GMP ratios in both atrial and ventricular tissues at 2 and 3 weeks after thermal injury. To prevent underestimation of cyclic nucleotide levels such changes should preferably be expressed on a prot g^{-1} dry weight basis.

Introduction

Thermal injury to man and animals evokes myocardial dysfunction (Baxter *et al.*, 1966; Aikawa *et al.*, 1978; Martyn *et al.*, 1980). This dysfunction is apparent not only in the acute resuscitation phase but also in the hypermetabolic (recovery) phase of thermal injury (Martyn *et al.*, 1980; 1986). Dysfunction was indicated by an increase in end-diastolic volume and/or decrease in ejection fraction (Martyn *et al.*, 1980; 1981; 1986). This ventricular dysfunction following trauma is thought to increase morbidity and mortality due to the inability of the ventricle to maintain adequate cardiac output (Martyn *et al.*, 1980; Hoffman *et al.*, 1983). The decreased contractility of the myocardium in the hypermetabolic phase is an unexpected finding since circulating catecholamines are elevated in the burned patient (Wilmore *et al.*, 1974). Catecholamines, by altering cyclic nucleotide levels, augment the amplitude and shorten the duration of cardiac contraction (Rasmussen *et al.*, 1972). These events are governed through activation of a variety of protein kinases capable of phosphorylating proteins that regulate Ca^{2+} in the myoplasm. Despite the documented elevations in catecholamines following burn trauma, whether cyclic nucleotides in the myocardium respond appropriately is unknown, although the systemic effects of burn on skeletal muscle cyclic nucleotides have been examined (Tomera & Martyn, 1988; 1989b; Tomera *et al.*, 1988). We have previously reported the effects of infection (*viz*, endotoxicosis) on cyclic nucleotides of the heart when compared to skeletal muscle (Tomera & Martyn, 1990). Infection, a consequence of immunosuppression, is an underlying complication of burn trauma.

In the present study cyclic nucleotides within atrial and ventricular tissues were measured in mice at two and three weeks after a 20% body surface area (BSA) thermal injury. This was done to test the hypothesis that even a small thermal injury induces changes in cyclic nucleotides, which may account for the inotropic and chronotropic changes observed. Two major findings of the present report were that cyclic AMP/cyclic GMP ratios increase in atria and ventricles and protein levels increase in atria at two and three weeks after thermal trauma. This must be considered when expressing changes in cyclic nucleotides to avoid underestimating the effects of thermal trauma.

[1] Author for correspondence at: Anesthesia Services, Massachusetts General Hospital, 51 Fruit Street, Boston, MA 02114, U.S.A.

Methods

Animals

Male CD mice, 9–10 months, were obtained from Charles River Breeding Laboratories (Wilmington, MA) and maintained on a standard laboratory diet, with water provided *ad libitum*.

Murine thermal injury model

This model was described previously in studies on hypermetabolism, immunosuppression and, more recently, skeletal neuromuscular function (Tomera *et al.*, 1988). All investigations involving mice were conducted in compliance with the guidelines for experimental procedures set forth in the NIH Guide for the Care and Use of Laboratory Animals (Dept. of Health, Education and Welfare No. 80-23, Office of Science and Health Reports, DRR/NIH, Bethesda, MD, U.S.A.). The project was also reviewed and approved by the Animal Studies Committee of the Shriners Burns Institute and the Massachusetts General Hospital. Mice were deeply anaesthetized with pentobarbitone (40 mg kg^{-1} i.p., Lemmon Company, Sellersville, PA, U.S.A.) prior to shaving; anaesthesia was considered adequate when the mice did not show a reflex withdrawal response when their toes were compressed

with haemostats. The anaesthetized mice were shaved in an area corresponding to 20% BSA, calculated for individual mice according to the equation $A = K(Wt)^{2/3}$, where A = area, Wt = weight, and K = proportionality constant (12.3 for mice), (Schildt & Nilsson, 1970). This predefined BSA was then immersed in 95°C water for 8 s and immediately removed. No mortality using this size of BSA injury was encountered. Control mice did not suffer thermal injury but were prepared in a similar fashion and immersed in lukewarm water.

Subsequent to scalding, a full-thickness 3° burn injury having sharp margins resulted. While recovering from the anaesthesia and trauma animals were kept warm in an ambient environmental temperature of 26°C. All animals were resuscitated with 0.9% saline (4 mg kg^{-1}/% burn, i.p.) and received no additional anaesthesia. The burn wound was treated with applications of silver sulphadiazine (1%, Silvadene Creme, Marion Laboratories, St. Louis, MO, U.S.A.), and gauze dressings were applied. After 4 h the animals were returned to their cages and given 0.9% saline as drinking water with food *ad libitum* for 1 week after burn injury. After this first week they were returned to normal tap water. No antibiotics were administered since burn injury including resulting complications was the aim of the study.

Protein content

Replicate heart tissues, paired to those used for cyclic nucleotide determinations, were used to assay changes in total protein content. Tissue digests were made in 1 M NaOH (Lowry *et al.*, 1951). Actual samples were derived from mice killed on Days 14 and 22. Protein content was expressed by the following equation:

$$\underbrace{\text{total protein}}_{\text{mg prot g}^{-1}\text{ dry wt.}} = \underbrace{\text{Lowry assay}}_{\text{mg prot g}^{-1}\text{ wet wt.}} \times \underbrace{\text{wet/dry wt. ratio}}_{\text{g wet g}^{-1}\text{ dry wt.}}$$

Cyclic nucleotide determinations

Hearts were excised from mice that were killed by cervical dislocation. Left ventricular and atrial tissues were rinsed free of blood in a cold sodium acetate buffer (0.05 M, pH 6.2), then immediately frozen in liquid nitrogen and stored at −70°C. Levels of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) in heart muscle were measured with ^{125}I-radioimmunoassay (RIA) kits purchased from New England Nuclear Corporation (Boston, MA, U.S.A.). On the day of the assay, the frozen muscles were thawed in trichloroacetic acid (6% TCA) and homogenized to a tissue slurry. All samples were analyzed by RIA in duplicate (Steiner *et al.*, 1972).

Cyclic nucleotide levels were expressed in terms of pmol mg^{-1} prot. To account for any change in myocardial protein, they were alternatively expressed on a nmol g^{-1} dry wt. basis by the following equation:

$$\underbrace{\text{total cyclic AMP}}_{\text{nmol g}^{-1}\text{ dry wt.}} = \underbrace{\text{cyclic AMP by RIA}}_{\text{nmol mg}^{-1}\text{ prot}} \times \underbrace{\text{Lowry assay}}_{\times\text{ mg pro g}^{-1}\text{ wet wt.}} \times \underbrace{\text{wet/dry st. ratio}}_{\times\text{ g wet/g dry wt.}}$$

where nmol g^{-1} dry wt. is the product of several procedures performed in duplicate on tissue from the same animal. ^{125}I-RIA was performed on the supernatant, and the mg prot was measured on tissue pellets of previously homogenized and centrifuged samples to obtain nmol mg^{-1} prot, mg prot g^{-1} wet wt. was calculated from samples previously weighed and digested for Lowry determination and from wet/dry wt. ratios. Actual sample sizes ranged between 100–300 mg wet wt.

Total body weights

All mice were weighed throughout this study at the various time intervals defined within this paper.

Statistical analyses

All values depict mean ± s.e.mean. Statistical significance was determined from treatment of the data by analysis of variance (ANOVA). Comparisons were made for each of the thermally injured groups relative to time-matched controls.

Results

Total body weight changes

Changes in total body weight loss in response to the 20% BSA burn are shown in Table 1 for Days 14 and 22 post-burn. All burned animals showed a weight loss that was significantly greater than their time-matched controls. This weight loss ranged between 6–9% of total body weight. It should be noted that control mice of this age do not typically gain weight at 10 months (Tomera & Martyn, 1989a).

Atria

Muscle protein and cyclic nucleotide changes The myocardial tissues in each group were assayed for total protein content in pre-weighed wet wt. tissue samples, and the tissue wet/dry wt. ratios were also determined. The wet/dry wt. ratios for both the control and the thermally-injured groups did not differ significantly in either the atria or the ventricles. Therefore, the wet/dry wt. ratios for the control and thermally-injured groups were 4 ± 1 ($n = 47$, in atria $n = 5$ for each group of controls and burned mice at Days 14 and 22; and in ventricles at Day 14, controls = 8 and burned mice = 7, at Day 22 controls = 6 and burned mice = 6). This ratio was used in estimating cyclic nucleotides on a nmol g^{-1} dry wt. basis. Figure 1 shows a threefold increase in protein (mg protein g^{-1} dry wt.) in the atria at Days 14 ($P < 0.05$) and 22 ($P < 0.001$), respectively, as a consequence of thermal injury. The cyclic AMP changes expressed as pmol mg^{-1} protein at 14 and 22 days after burn and in controls are shown in Figure 2. At 22 days after thermal injury, a significant increase in cyclic AMP content in the atria was noted; no changes were observed at 14 days. When the cyclic AMP data were re-expressed as nmol g^{-1} dry wt to take into account the burn-induced increases in atrial protein concentrations, significant increases in cyclic AMP content were noted at both 14 and 22 days after burn. A disparity in the attainment of significant changes in cyclic AMP levels existed when expressed as pmol mg^{-1} prot versus nmol g^{-1} dry wt. At Day 14 after thermal injury, a significant fourfold increase ($P < 0.05$) in cyclic AMP levels (nmol g^{-1} dry wt.) was observed, while if expressed alternatively as pmol g^{-1} prot, no change was discerned. At Day 22 a ninefold increase ($P < 0.001$) in cyclic AMP was observed when expressed as nmol g^{-1} dry wt., compared to a twofold increase ($P < 0.001$) when expressed as pmol mg^{-1} prot, which did not consider protein increases. Thus, these results show greater differences in the atrial cyclic AMP levels of

Table 1 Effect of burn injury on total body weight (g)†

Groups (*n*)	*Postburn days* Day 14	Day 22
Controls (5)	44.26 ± 0.39	44.92 ± 0.31
20% BSA burn ($n = 5$)	40.82 ± 0.38*	41.86 ± 0.87*

Values are mean ± s.e.mean.
* $P < 0.01$, ANOVA.
† Time-matched controls.

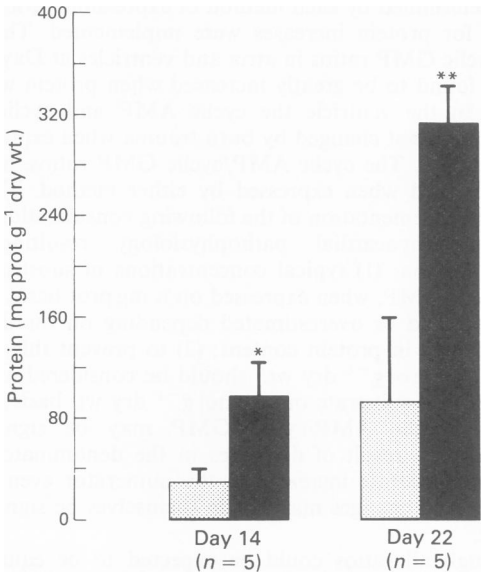

Figure 1 Increased protein levels within the atria at Days 14 and 22 after thermal injury. Stippled columns, control; solid columns, burn. Columns show mean values in terms of mg prot g^{-1} dry wt.; (number of observations), s.e.mean shown by vertical bars. * $P < 0.05$, ** $P < 0.025$.

thermally injured mice at both Days 14 and 22 when converted to a nmol g^{-1} dry wt. basis. The cause and importance of these differences is discussed in the following section. No significant changes were observed in cyclic GMP levels when assessed by either method (Table 2).

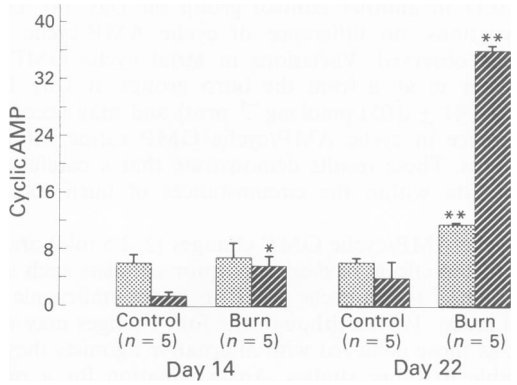

Figure 2 Increasing atrial cyclic AMP levels at Days 14 (nmol g^{-1} dry wt) and 22 (pmol mg^{-1} prot, nmol g^{-1} dry wt.) after thermal injury. Stippled columns, pmol mg^{-1} prot; hatched columns, nmol g^{-1} dry wt. Columns show mean values expressed by both methods (number of observations); vertical bars show s.e.mean. Day 14 levels were not increased when expressed as pmol mg^{-1} prot, * $P < 0.05$, ** $P < 0.001$.

Relationships of atrial protein to total body weight Because there was at least a threefold increase in atrial protein between the second and third week after burn, these data were tested for any form of systemic effect that might prove responsible. When atrial protein (mg prot g^{-1} dry wt.) data were correlated to total body weight, a non-linear relationship was observed (i.e. $y = 125x^2 - 10890x + 238127$, $R^2 = 0.79$, $n = 15$). This relationship indicated that as total body weight loss occurred, levels of protein content within the atria increased.

Cyclic AMP/cyclic GMP ratios Figure 3 shows the expression of cyclic AMP/cyclic GMP in the atria by both previously discussed methods. At Day 14 the cyclic AMP/cyclic GMP ratio was increased threefold ($P < 0.05$, ANOVA two-way) in the thermally injured group when protein changes were considered (i.e., when expressed on a nmol g^{-1} dry wt. basis). No change was observed when expressed in pmol mg^{-1} prot; this observation confirms the importance of accounting for protein changes when changes in cyclic nucleotides are considered. However, at Day 22 the ratios expressed by both methods had increased equally, by twofold ($P < 0.001$).

Relationship of atrial cyclic AMP/cyclic GMP ratios to total body weight To examine the effect of total body weight loss on atrial cyclic nucleotide ratios, a non-linear regression analysis was performed. A relationship ($y = 80.31x^2 - 2678x + 59405$, $R^2 = 0.50$, $n = 20$) indicated that as total body weight decreased, the cyclic AMP/cyclic GMP ratio increased. This relationship was only observed when the data of the cyclic AMP/cyclic GMP ratio were derived from nucleotide concentrations expressed as nmol g^{-1} dry wt.

Ventricles

Muscle protein No significant changes in ventricular protein levels were detected at two and three weeks after thermal

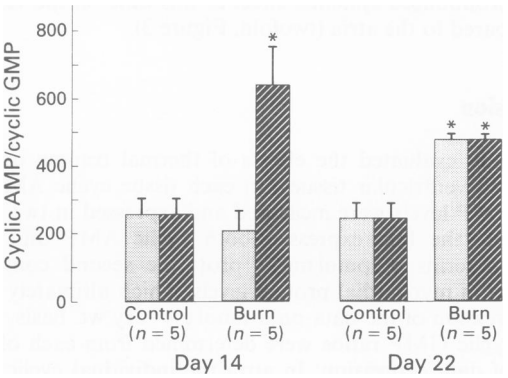

Figure 3 Increased atrial cyclic AMP/cyclic GMP ratios at Days 14 (nmol g^{-1} dry wt.) and 22 (pmol mg^{-1} prot, and nmol g^{-1} dry wt.) after thermal injury. Stippled columns, pmol mg^{-1} prot; hatched columns nmol g^{-1} dry wt. Columns show mean values expressed by both methods (number of observations); vertical bars show s.e.mean. * $P < 0.001$.

Table 2 Myocardial cyclic GMP levels at 2 and 3 weeks after thermal injury

	Time after thermal injury			
	Day 14		Day 21	
Tissues	Control	Thermally Injured	Control	Thermally Injured
Atria				
pmol mg^{-1} prot	0.030 ± 0.011 (5)	0.081 ± 0.051 (5)	0.032 ± 0.011 (5)	0.022 ± 0.001 (5)
nmol g^{-1} dry wt.	0.009 ± 0.005 (5)	0.003 ± 0.002 (5)	0.031 ± 0.030 (5)	0.070 ± 0.002 (5)
Ventricle				
pmol mg^{-1} prot	0.153 ± 0.071 (8)	0.012 ± 0.004 (7)	0.332 ± 0.181 (6)	0.012 ± 0.041 (6)
nmol g^{-1} dry wt.	0.013 ± 0.006 (8)	0.022 ± 0.011 (7)	0.042 ± 0.032 (6)	0.031 ± 0.012 (6)

Values are mean ± s.e.mean (number of observations).

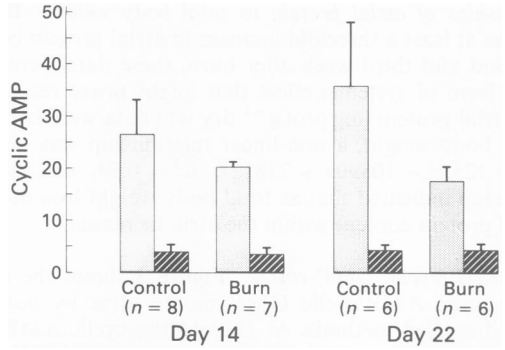

Figure 4 Ventricular cyclic AMP levels were not significantly different at Days 14 and 22 after thermal injury. Stippled columns, pmol mg^{-1} prot; hatched columns, nmol g^{-1} dry wt. Columns show mean values (number of observations) with s.e.mean shown by vertical bars.

injury. The values (mg prot g^{-1} dry wt.) obtained were: Day 14 (n = 7–8), control 150 ± 50, burn 150 ± 60; Day 22 (n = 6), control 140 ± 45, burn 230 ± 50.

Cyclic AMP and cyclic GMP Unlike atrial tissues, ventricular tissues did not show any significant change in either cyclic AMP (Figure 4) or cyclic GMP (Table 2) levels at two and three weeks after thermal injury when assessed by either method.

Cyclic AMP/cyclic GMP ratios These data, when expressed by either method, showed significant elevations in cyclic AMP/cyclic GMP ratios (Figure 5). In ventricular tissues a 17 fold ($P < 0.05$) increase was observed in the ratio at Day 14 when depicted either as pmol mg^{-1} prot or as nmol g^{-1} dry wt. By Day 22, the ventricular cyclic AMP/cyclic GMP ratio was still increased by eight to 11 fold. These observations indicate a pronounced systemic effect at this time on the ventricle as compared to the atria (twofold, Figure 3).

Discussion

This study evaluated the effects of thermal trauma on both atrial and ventricular tissues. In each tissue cyclic AMP and cyclic GMP levels were measured and expressed in two different ways: the first expressed both cyclic AMP and cyclic GMP in terms of pmol mg^{-1} prot; the second considered increases in myocardial protein levels, which ultimately led to the expression of the data on a nmol g^{-1} dry wt. basis. Cyclic AMP/cyclic GMP ratios were determined from each of these forms of data expression. In atria the individual cyclic AMP values determined by each method of expression varied if corrections for protein increases were implemented. The cyclic AMP/cyclic GMP ratios in atria and ventricles at Day 14 and 22 were found to be greatly increased when protein was considered. In the ventricle the cyclic AMP and cyclic GMP changes were not changed by burn trauma when expressed by either method. The cyclic AMP/cyclic GMP ratios, however, were increased when expressed by either method. This suggests the implementation of the following considerations when evaluating myocardial pathophysiology resulting from thermal trauma: (1) typical concentrations of substrates, e.g. pmol cyclic AMP, when expressed on a mg prot basis, may be underestimated or overestimated depending on the direction of the change in protein content; (2) to prevent this, protein changes (mg prot g^{-1} dry wt.) should be considered and used to express the substrate on a nmol g^{-1} dry wt. basis; and (3) ratios of cyclic AMP/cyclic GMP may be significantly increased as a result of decreases in the denominator along with commensurate increases in the numerator even though the individual changes may not in themselves be significantly different.

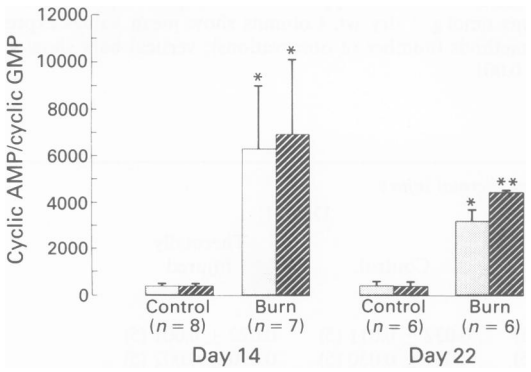

Figure 5 Increased ventricular cyclic AMP/cyclic GMP ratios at Days 14 and 22 after thermal injury. Stippled columns, pmol mg^{-1} prot; hatched columns, nmol g^{-1} dry wt. Columns show mean values expressed by both methods (number of observations); vertical bars show s.e.mean. $^{*}P < 0.05$, $^{**}P < 0.025$.

Although all ratios could be expected to be equal when based on cyclic nucleotide levels expressed as pmol mg^{-1} prot and/or pmol g^{-1} dry wt, this was not the case, as shown in Figure 3. The ratios cyclic AMP/cyclic GMP (Figures 3 and 5) differed when expressed per mg of protein and per g of dry weight. The absolute value of the individual nucleotide changed with each form of expression. Although the ratio was expected to remain the same, since both cyclic AMP and cyclic GMP pmol mg^{-1} prot were multiplied by the same factors (i.e. mg prot/wet wt. and wet/dry weight ratio, see Methods, *Cyclic nucleotide determinations*), it did not. This was thought to be due to the variation of data within the burn groups and the controls. This can be appreciated in observing cyclic GMP values (Table 2, cyclic GMP values (pmol mg^{-1} protein) in ventricle 0.332 ± 0.181 in controls on Day 21 and 0.153 ± 0.71 in another control group on Day 14). Despite such variations, no difference of cyclic AMP/cyclic GMP ratios was observed. Variations in atrial cyclic GMP were even greater in atria from the burn groups at Day 14 (i.e. Table 2, 0.081 ± 0.051 pmol mg^{-1} prot) and may account for the difference in cyclic AMP/cyclic GMP ratios within the burn groups. These results demonstrate that a careful evaluation of data within the circumstances of burn trauma is required.

The cyclic AMP/cyclic GMP changes (2–2.5 fold) are comparable to the effects of β-adrenoceptor agonists such as isoprenaline (10^{-6} M) on cyclic AMP in intact embryonic chick hearts (Linden, 1987). Although the fold changes may not be as great as those achieved with alternative agonists they were comparable to other studies. An explanation for a reduced change would include desensitization of adenylate cyclase by catecholamines (Aprille *et al.*, 1979). No studies of the effects of isoprenaline on atria and ventricles from burned animals have yet been performed.

Thus, the validity of the evaluation of burn-induced changes in biochemical parameters should be based on a nmol g^{-1} dry wt. basis to circumvent artifactual estimates. This is further attested to by control values (pmol mg^{-1} prot or nmol g^{-1} dry wt.), which fall within acceptable limits in spite of various methods currently available for tissue sampling. No mortality was encountered at burn injury to 20% BSA. However, this model shows that even small BSA dermal burns can provoke changes in other organ systems (e.g. heart) by burn-induced mediators that are systematically circulated throughout the body by the blood. Therefore, such organ systems, although distant from the focal area of burn injury, are still subjected to a trauma response, even for small BSA burns (i.e. 20%).

An increase in atrial protein concentrations was observed in burned animals despite a decrease in total body weight. It has been previously observed that there is also an increase in protein concentrations in skeletal muscle following burn trauma despite total body weight loss (Tomera & Martyn,

1988). Although total body protein turnover rates are increased following burn trauma (Shangraw & Turinsky, 1982), it is unclear why there is an increase in protein concentrations in the atria. This finding is not peculiar to burn trauma. In protein-calorie malnutrition, where the weight loss is mechanistically different from burn trauma (Shephard *et al.*, 1986), it has also been noted that a decrease in body weight is associated with an increase in atrial and ventricular weight when normalized to body weight (Benfey *et al.*, 1983). The fact that there was no change in wet/dry weight ratios in either atrial or ventricular tissue requires additional clarification. Although the wet dry weight ratios were unchanged, protein concentration increased substantially in the atria (Figure 1). It would appear reasonable that when protein concentration increases, the wet/dry weight ratio would decrease provided there was no oedema. Decreased wet/dry weight ratios were not observed. Therefore, it would appear that oedema in the wet tissues may prevent the wet/dry weight ratios from changing.

In the present study no changes in ventricular protein or cyclic AMP concentrations were observed. This disparate response between atrial and ventricular muscle with respect to protein and cyclic AMP concentrations is difficult to explain. Although no functional studies were performed, it is tempting to conclude that burn-induced ventricular dysfunction documented previously (Baxter *et al.*, 1966; Aikawa *et al.*, 1978; Martyn *et al.*, 1980; 1986) may be related to the inability of the ventricle to hypertrophy (to increase in protein concentration) and increase contractility (via increased cyclic AMP levels) in response to chronic hypermetabolic demands. The lack of increase in cyclic AMP levels in ventricular muscle is surprising since catecholamines (Wilmore *et al.*, 1974), which increase cyclic AMP levels via β-adrenoceptors, are increased in burn trauma. This finding, however, is consistent with the findings in endotoxicosis, where despite elevations of catecholamines, no increases in myocardial cyclic AMP levels are evident (Shephard *et al.*, 1986).

Inotropy in the ventricle is thought to be mediated via elevations of cyclic AMP levels (Entman, 1974). Despite a burn-induced increase in O_2 consumption (Tomera *et al.*, 1988) and an increased catecholamine secretion (Wilmore *et al.*, 1974; Aprille *et al.*, 1979), cyclic AMP levels in ventricular muscle were not elevated. Hearts of thermally injured patients may be working on the brink of insufficiency because of their inability to increase cyclic AMP levels. This inadequacy may be characteristic of the systemic effects of thermal injury on the heart despite the high plasma catecholamine levels in this state documented by others (Wilmore *et al.*, 1974; Aprille *et al.*, 1979). Sustained inotropy through β-adrenoceptor stimulation is difficult because of intense and repeated exposure to endogenous β-agonists, which can diminish both receptor population (down regulation) and agonist affinity (desensitization) (Motulsky & Insel, 1982). In this circumstance, use of exogenous agents that increase cyclic AMP via β-receptors would be futile.

It is interesting to note that by three weeks after thermal injury the eight to 11 fold increase in ventricular cyclic AMP/cyclic GMP ratios has declined from the 17 fold maxima attained at two weeks. This observation suggests that sympathetic compensatory mechanisms may be waning at three weeks. It further provides biochemical evidence (e.g. elevated cyclic AMP/cyclic GMP ratios) to support the previous observation (Hilton *et al.*, 1986) that direct myocardial depression did not occur. The overall significance of elevated cyclic AMP/cyclic GMP ratios as phosphorylation indices within the cardiovascular system has been previously established for other disease states (Amer, 1975). However, in light of the disparities in data obtained within cyclic AMP levels (Figure 2) when expressed on either a pmol mg^{-1} prot or a nmol g^{-1} dry wt. basis, it is difficult to say which, if any, of the parameters (cyclic AMP, cyclic GMP, cyclic AMP/cyclic GMP) is the most important index in monitoring myocardial performance as a consequence of thermal injury. Recent studies, have shown that the relationship between myocardial metabolism, in particular the myocardial status of energy-rich substrates and phosphates and myocardial function, is not as clearly defined as had been thought (Isselhard, 1988).

In future studies of thermal injury it would be beneficial to examine serially the relationships between cyclic nucleotides and their ratios with other factors (e.g. cardiac function, metabolism, and haemodynamics). Measurements of high-energy phosphate changes monitored by nuclear magnetic resonance (n.m.r.) and correlated to functional changes are planned. Applications of this work could utilize the suggestion of others (Schaub & Kunz, 1986) to combine cardiac glycosides with β-antagonists. The glycosides would (1) promote the availability of Ca^{2+} for contraction; (2) decrease phosphodiesterase interaction by Ca^{2+}-calmodulin interactions (Potter *et al.*, 1980); and (3) maintain inotropism through a mechanism not affected by β-antagonists.

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References

AIKAWA, N., MARTYN, J.A.J. & BURKE, J.F. (1978). Pulmonary artery catherization and thermodilution cardiac output determination in the management of critically burned patients. *Am. J. Surg.*, **135**, 881–887.

AMER, M.S. (1975). Cyclic nucleotides in disease: on the biochemical etiology of hypertension. *Life Sci.*, **17**, 1021–1038.

APRILLE, J.R., AIKAWA, N., BELL, T.C., BODE, H.H. & MALAMUD, D.F. (1979). Adenylate cyclase after burn injury: resistance to desensitization by catecholamines. *J. Trauma*, **19**, 812–818.

BAXTER, C.R., COOK, W. & SHIRES, G.T. (1966). Serum myocardial depressant factor of burn shock. *Surg. Forum*, **17**, 1–2.

BENFEY, B.G., VARMA, D.R. & YUE, T.L. (1983). Myocardial inotropic responses and adrenoreceptors in protein-deficient rats. *Br. J. Pharmacol.*, **80**, 527–531.

ENTMAN, M.L. (1974). The role of cyclic AMP in the modulation of cardiac contractility. In *Advances in Cyclic Nucleotide Research, Vol. 4.* ed. Greengard, P. & Robison, G.A. p. 163. New York: Raven Press.

HILTON, J.G., MCPHERSON, M.G. & MARULLO, D.S. (1986). Effects of thermal trauma on cardiac force of contraction. *Burns*, **12**, 167–171.

HOFFMAN, M.J., GREENFIELD, L.J. & SUGARMAN, H.J. (1983). Unsuspected right ventricular dysfunction in shock and sepsis. *Ann. Surg.*, **198**, 307–316.

ISSELHARD, W. (1988). Biochemistry: index of the functional state of the heart. *Br. J. Anaesth.*, **60**, 235–275.

LINDEN, J. (1987). Enhanced cAMP accumulation after termination of cholinergic action in the rat heart. *FASEB J.*, **1**, 119–124.

LOWRY, O.H., ROSENBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

MARTYN, J.A.J., SNIDER, M.T., SZYFELBEIN, S.K., BURKE, J.F. & LAVER, M.B. (1980). Right ventricular dysfunction in acute thermal injury. *Ann. Surg.*, **191**, 330–335.

MARTYN, J.A.J., SNIDER, M.T., FARAGO, L.F. & BURKE, J.F. (1981). Thermodilution right ventricular volume: a novel and better predictor of volume resuscitation in acute thermal injury. *J. Trauma*, **21**, 619–626.

MARTYN, J.A.J., WILSON, R.S. & BURKE, J.F. (1986). Right ventricular function and pulmonary hemodynamics during dopamine infusion in burned patients. *Chest*, **89**, 357–360.

MOTULSKY, H.J. & INSEL, P.A. (1982). Adrenergic receptors in man: direct identification of physiologic regulation and clinical alterations. *N. Engl. J. Med.*, **307**, 18–29.

POTTER, J.D., PIASCI, T., WISLER, P.L., ROBERTSON, S.P. & JOHNSON C.L. (1980). Calcium dependent regulation of brain and cardiac

muscle adenylate cyclase. *Ann. N.Y. Acad. Sci.*, **356**, 220–230.
RASMUSSEN, H., GOODMAN, D.B.P. & TENENHOUSE, A. (1972). The role of cyclic AMP and calcium in cell activation. *C.R.C. Crit. Rev. Biochem.*, Feb. 95–148.
SCHAUB, M.C. & KUNZ, B. (1986). Regulation of contraction in cardiac and smooth muscle. *J. Cardiovasc. Pharmacol.*, **8**, S117–S123.
SCHILDT, B. & NILSSON, A. (1970). Standardized burns in mice. *Burn Surg. Res.*, **2**, 23–33.
SHANGRAW, R.E. & TURINSKY, J. (1982). Effect of disease and thermal injury on protein turnover in skeletal muscle. *J. Surg. Res.*, **33**, 345–355.
SHEPHARD, R.E., McDONNOUGH, K.H. & BURNS, A.H. (1986). Mechanism of cardiac dysfunction in hearts from endotoxin treated rats. *Circ. Shock*, **19**, 371–384.
STEINER, A.L., PARKER, C.W. & KIPNIS, D.M. (1972). Radioimmunoassay for cyclic nucleotides. *J. Biol. Chem.*, **247**, 1106–1113.
TOMERA, J.F., HOAGLIN, D.C. & MARTYN, J.A.J. (1988). Neuromuscular dysfunction in burns and its relationship to burn size, hypermetabolism, and immunosuppression. *J. Trauma*, **28**, 1499–1504.
TOMERA, J.F. & MARTYN, J.A.J. (1988). Systemic effects of single hindlimb burn injury on skeletal muscle function and cyclic nucleotide levels in the murine model. *Burns*, **14**, 216–218.
TOMERA, J.F. & MARTYN, J.A.J. (1989a). Intraperitoneal endotoxin but not protein malnutrition shifts d-tubocurarine dose-response curves in mouse gastrocnemius muscles. *J. Pharmacol. Exp. Ther.*, **250**, 216–220.
TOMERA, J.F. & MARTYN, J.A.J. (1989b). Mediators of burn-induced neuromuscular changes in mice. *Br. J. Pharmacol.*, **98**, 921–929.
TOMERA, J.F. & MARTYN, J.A.J. (1990). The effects of endotoxin infection on cyclic nucleotides of ventricular and gastrocnemius muscle. *Circ. Shock*, (in press).
WILMORE, D.W., LONG, J.M., MASON, A.D., SKREEN, R.W. & PRUITT, B.A. (1974). Catecholamines: mediators of the hypermetabolic response to thermal injury. *Ann. Surg.*, **180**, 653–659.

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Effects of morphine, H-Tyr-D-Arg-Phe-Lys-NH_2 (DALDA) and B-HT920 on non-cholinergic nerve-mediated bronchoconstriction in pithed guinea-pigs

M.P. Rechtman, A.L.A. Boura, [1]R.G. King, J.E. Olley & *P.W. Schiller

Department of Pharmacology, Monash University, Clayton, Victoria, 3168, Australia and *Institut de Recherches Cliniques de Montréal, 110 Avenue des Pins ouest, Montréal H2W 1R7, Canada

1 Electrical stimulation (1 ms, 5 Hz, 80 V) for 5 or 15 s at the level of C4–T1 in the spinal canal of artificially respired pithed guinea-pigs (which had received intravenously (i.v.) (+)-tubocurarine chloride 2 mg kg^{-1}, atropine sulphate 2 mg kg^{-1} and pentolinium tartrate 5 mg kg^{-1}) caused constriction of airways, indicated by increased insufflation pressure.

2 This non-cholinergic constriction was inhibited by morphine (1–3 mg kg^{-1}, i.v.), the peripherally acting μ-receptor agonist, H-Tyr-D-Arg-Phe-Lys-NH_2 (DALDA, 0.1–1 mg kg^{-1}, i.v.) or the α_2-adrenoceptor agonist B-HT920 (1–3 mg kg^{-1}, i.v.)

3 The effects of either morphine (3 mg kg^{-1}, i.v.) or DALDA (1 mg kg^{-1}, i.v.) were inhibited by naloxone (3 mg kg^{-1}, i.v.). Idazoxan (3 mg kg^{-1}, i.v.) inhibited the anti-constrictor effect of B-HT920 (3 mg kg^{-1}, i.v.), but not that of DALDA (0.1 mg kg^{-1}, i.v.).

4 Thus activation of peripheral μ-opioid receptors or α_2-adrenoceptors inhibits airways constriction induced by non-cholinergic nerve stimulation in the pithed guinea-pig. This preparation therefore provides a further method for the *in vivo* examination of the effects of drugs on non-cholinergic tracheobronchial constrictor nerve function.

Introduction

Transmural electrical stimulation of guinea-pig isolated bronchi causes both cholinergic and long-lasting non-cholinergic contractions, the latter being possibly due to the release of neuropeptides such as substance P (Andersson & Grundström, 1983). Such non-cholinergic contraction of guinea-pig bronchi is inhibited by μ-opioid agonists such as morphine and [D-Ala^2,NMePhe^4,Gly-ol^5]enkephalin (DAMGO, Frossard & Barnes, 1987), and also by stimulation of α_2-adrenoceptors (Grundström *et al.*, 1984). *In vivo* opioid receptor- and α_2-adrenoceptor-mediated inhibition of non-cholinergic guinea-pig airway constriction, induced by electrical stimulation of peripheral vagus nerves has also been demonstrated (Grundström & Andersson, 1985; Belvisi *et al.*, 1988). However, in previous *in vivo* studies of non-cholinergic bronchoconstriction in the guinea-pig, the central nervous system has been intact, thus allowing possible complications arising from central actions of drugs and the effects of anaesthesia.

The aim of the present study was to determine the feasibility of using the pithed guinea-pig to examine specific peripheral effects of drugs on constrictor responses of the airways to non-cholinergic nerve stimulation. Stimulation was at the level of C4–T1, and therefore probably involved either visceral or somatic afferent fibres, as ganglionic, cholinergic muscarinic, and skeletal neuromuscular transmission were inhibited by pentolinium, atropine and (+)-tubocurarine respectively. The usefulness of the preparation has been validated by use of the α_2-adrenoceptor agonist B-HT920 and the opioid receptor agonists morphine and H-Tyr-D-Arg-Phe-Lys-NH_2 (DALDA). DALDA is a recently synthesized μ-receptor selective polar dermorphin analogue which acts peripherally (Schiller *et al.*, 1989a,b).

Methods

Male Dunkin-Hartley guinea-pigs (250–710 g) were anaesthetized with 4% halothane in nitrous oxide (1 l min^{-1}) and oxygen (1 l min^{-1}). In each, the left carotid artery was tied and an identifying ligature looped around the right carotid artery. The trachea was cannulated. With the guinea-pig prone, a 2 cm midline incision was made through the skin of the scalp, and a 2 mm hole made with a punch in the sagittal fissure between the orbits. A pithing rod (with an inbuilt electrode at the tip, a modification to that described for use in the rat by Gillespie *et al.*, 1970) and similar to that used by Armstrong & Boura (1973) was inserted via the hole through the foramen magnum and down the full length of the spinal canal.

The tracheal cannula was connected to a ventilator (Ugo Basile 7025) and the animal ventilated with air (10 ml kg^{-1}; 60 strokes min^{-1}). Insufflation pressure was recorded (Grass polygraph model 7D) via a pressure transducer (Gould Statham P23) connected to a side arm of the tracheal cannula. This was used as an index of tracheobronchial resistance. The right carotid artery was cannulated for monitoring blood pressure via a pressure transducer (Gould Statham P23) connected to the polygraph recorder. Triggering of a rate meter by the pulse pressure enabled heart rate to be recorded.

When surgery was completed the rod was withdrawn to leave a length of 4.5–7.0 cm between the electrodes and the point of insertion into the skull. The electrodes then lay at the level of C4–T1. For intravenous administration of drugs a jugular vein was cannulated. Temperature was maintained at 35.5–37°C by placing the animal on a heated table, with radiant heat provided by an overhead lamp.

(+)-Tubocurarine chloride (2 mg kg^{-1}) and atropine sulphate (2 mg kg^{-1}) were administered intravenously to prevent stimulation of nicotinic receptors on skeletal muscle and to block muscarinic receptors respectively. Pentolinium tartrate (5 mg kg^{-1}) was also given intravenously to block nicotinic receptor-mediated ganglionic transmission and possible release of catecholamines from the adrenal medulla.

The cord was stimulated by the electrodes with pulses of 1 ms at 5 Hz and 80 V (Grass stimulator S88). These parameters were chosen to stimulate nerves at a supramaximal voltage to produce a submaximal response from which recovery readily occurred. In initial experiments stimulation was for 5 or 15 s duration, stimulation being repeated at 10 min intervals for a total time of 100 min. In further experiments, when effects on opioid receptors and α_2-adrenoceptors

[1] Author for correspondence.

were examined, only one duration of stimulation (15 s) was used, and the test period shortened to 1 h.

Statistical analysis was performed by Student's paired t test. Level of significance was taken as $P < 0.05$.

Drugs

Drugs were obtained as follows: (+)-tubocurarine chloride, atropine sulphate monohydrate (Koch Light Laboratories); pentolinium tartrate (May & Baker Ltd.); morphine hydrochloride, naloxone hydrochloride (Sigma); B-HT920 dihydrochloride (6-allyl-2-amino-5,6,7,8-tetrahydro- 4H-thiazolo-[4,5-*d*]azepine) (Boehringer Ingelheim); idazoxan hydrochloride (Reckitt & Colman); DALDA was synthesized as described previously (Schiller *et al.*, 1989b). All drugs were dissolved in saline (0.9 w/v) before use. Doses are given in terms of the salt used.

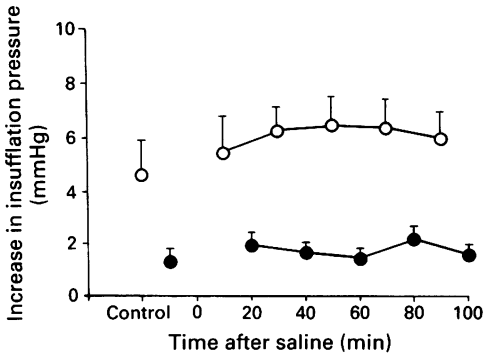

Figure 2 Non-cholinergic nerve-mediated increase in insufflation pressure to electrical stimulation (● 5 s; ○ 15 s) before and after saline administration (4 ml kg^{-1}, i.v. 0.9% w/v). Vertical lines indicate s.e.mean (n = 5–7).

Results

Electrical stimulation causing non-cholinergic airway constriction

The basal insufflation pressure 2 min before electrical stimulation was 15.5 ± 0.3 mmHg (n = 30), and remained constant until drug administration. Electrical stimulation at the level of C4–T1 (5 Hz, 80 V, 1 ms for 5 or 15 s duration alternately) increased the insufflation pressure. The increase in insufflation pressure is shown in Figure 2 and was significantly greater with 15 s stimulation compared with 5 s stimulation ($P < 0.05$). The duration of the constriction (3–5 min) considerably outlasted the period of cord stimulation (Figure 1). Stimulation of a higher frequency (10 Hz) for 15 s caused a greater and more long-lasting response than 5 Hz for 15 s, from which recovery did not occur for at least 10 min (data not shown). Therefore, in all further experiments stimulation was at 5 Hz. Intravenous administration of saline (4 ml kg^{-1}, 0.9% w/v) had no effect on responses which were maintained during repeated stimulation at 10 min intervals over a period of 100 min (Figure 2).

Effect of morphine on non-cholinergic airway constriction

As shown in Figure 3a, morphine (1 mg kg^{-1}, i.v.) significantly attenuated responses to the 5 s electrical stimuli for at least 100 min. However, with 15 s periods of stimulation, significant attenuation after this dose was maintained for less than 30 min. After a higher dose (3 mg kg^{-1}) in a further group of animals, responses to 15 s electrical stimuli were significantly attenuated ($P < 0.05$) for at least 60 min (Figure 3b). The inhibitory effect of the latter dose of morphine on 15 s stimuli was antagonized by the μ-receptor antagonist naloxone (3 mg kg^{-1}, i.v., a dose chosen to antagonize completely the effect of morphine), administered 15 min after morphine. Neither morphine (3 mg kg^{-1}, n = 12) nor naloxone (3 mg kg^{-1}, n = 4) had any significant effect on basal insufflation pressure measured 2 min after intravenous administration (data not shown).

Effect of DALDA on non-cholinergic airway constriction

DALDA (0.1 or 1.0 mg kg^{-1}, i.v.) significantly attenuated ($P < 0.05$) non-cholinergic airway constriction (Figure 4) to 15 s electrical stimulation for at least 60 min. This effect of 1 mg kg^{-1} DALDA was inhibited by naloxone (1 mg kg^{-1}, i.v.). Neither DALDA (1 mg kg^{-1}, n = 9) nor naloxone (1 mg kg^{-1}, n = 5) had any significant effect on basal insufflation pressure measured 2 min after intravenous administration (data not shown). Idazoxan (3 mg kg^{-1}) an α_2-adrenoceptor antagonist did not affect attenuation of non-cholinergic

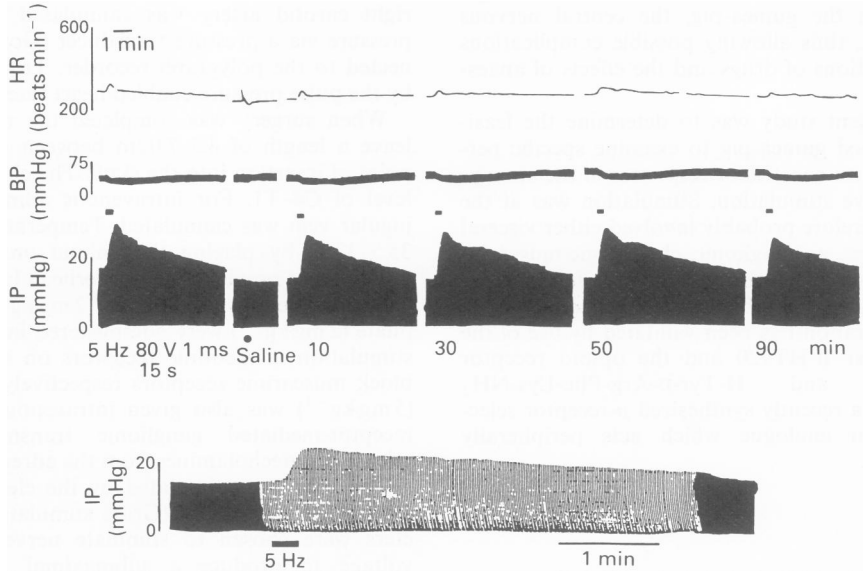

Figure 1 Non-cholinergic nerve-mediated increase in insufflation pressure to electrical stimulation, indicated by the horizontal bars in upper and lower traces, at the level of C4–T1 (5 Hz, 80 V, 1 ms for 15 s) in pithed guinea-pigs. The lower trace has been run on an expanded time scale and the period of stimulation is indicated by the horizontal bar, illustrating both latency and prolongation of the response. Heart rate (HR), blood pressure (BP), and insufflation pressure (IP) are shown.

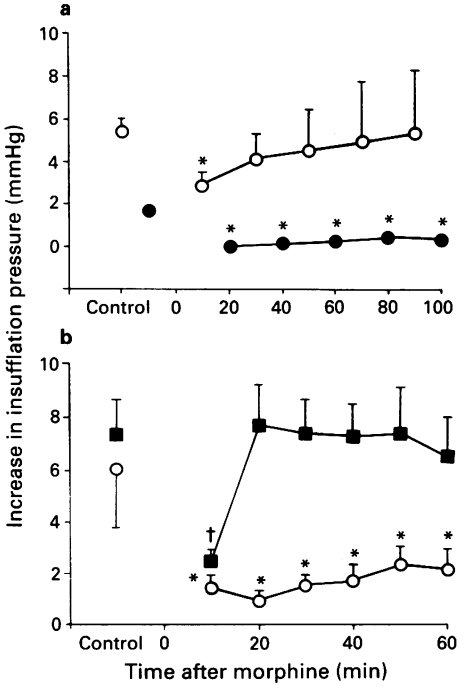

Figure 3 Non-cholinergic nerve-mediated increase in insufflation pressure to electrical stimulation before and after morphine administration. (a) The effects of morphine (1 mg kg^{-1}, i.v.) on responses to 5 s (●) and 15 s (○) stimuli. Error bars to responses at 5 s are smaller than the symbols. (b) The effect of morphine (3 mg kg^{-1}, i.v.) on responses to 15 s stimuli (○), and its antagonism by naloxone (3 mg kg^{-1}, i.v. ■) administered 15 min later. Vertical lines indicate s.e.mean ($n = 4$). * Significantly different from before morphine, $P < 0.05$; † Significantly different from control responses and those after naloxone, $P < 0.05$.

airway constriction caused by DALDA (0.1 mg kg^{-1}, i.v., $n = 4$, data not shown).

Effect of B-HT920 on non-cholinergic airway constriction

As shown in Figure 5, B-HT920 (1 mg kg^{-1}, i.v.) produced a significant ($P < 0.05$) inhibition of non-cholinergic airway constriction for 30 min, whereas a higher dose (3 mg kg^{-1}), resulted in a significant inhibition ($P < 0.05$) that was maintained for at least 60 min. The α_2-antagonist idazoxan (3 mg kg^{-1}, i.v.), administered 15 min after B-HT920 (3 mg kg^{-1}, i.v.), effectively inhibited the effect of the latter (Figure 5). Neither B-HT920 ($n = 9$) nor idazoxan ($n = 4$)

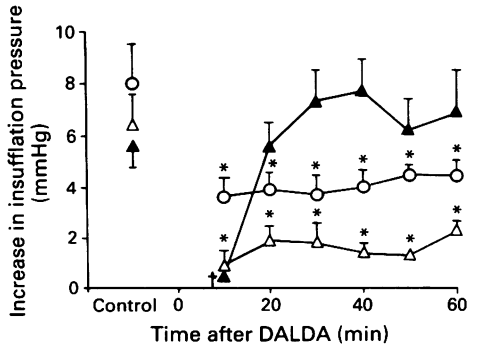

Figure 4 Non-cholinergic nerve-mediated increase in insufflation pressure to electrical stimulation before and after H-Tyr-D-Arg-Phe-Lys-NH$_2$ (DALDA) (○) 0.1 mg kg^{-1}, i.v. and (△) 1 mg kg^{-1}, i.v. The effect of naloxone (▲) 1 mg kg^{-1}, i.v. administered 15 min after 1 mg kg^{-1} DALDA is also shown, ($n = 4$–5). * Significantly different from before DALDA, $P < 0.05$. † Significantly different from control response and those also after naloxone, $P < 0.05$. Vertical lines indicate s.e.mean.

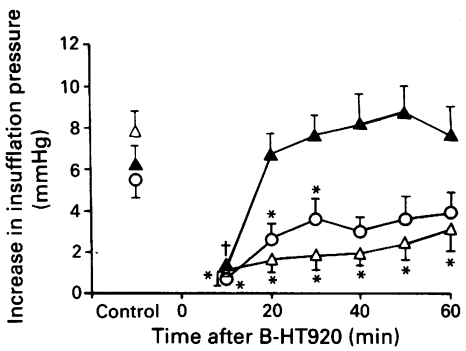

Figure 5 Non-cholinergic nerve-mediated increase in insufflation pressure to electrical stimulation before and after B-HT920 administration (○, 1 mg kg^{-1}, i.v.; △, 3 mg kg^{-1}, i.v.). Effect of idazoxan (▲, 3 mg kg^{-1}, i.v.) administered 15 min after 3 mg kg^{-1} B-HT920. Vertical lines indicate s.e.mean ($n = 4$–5). * Significantly different from before B-HT920, $P < 0.05$; † Significant difference between control responses and those after idazoxan, $P < 0.05$.

(both 3 mg kg^{-1} i.v.) had any significant effect on the basal insufflation pressure, measured 5 min after intravenous administration (data not shown). However, B-HT920 in contrast to the other agonists did cause a transient rise of basal insufflation pressure, which was significantly raised 2 min after injection (17 ± 0.7 mmHg, $P < 0.05$, compared with 15.2 ± 0.6 mmHg, 2 min prior to drug administration, $n = 9$).

Discussion

Many previous studies of the effects of drugs on neuronal non-cholinergic airways constriction have utilized isolated tissues (e.g. Grundström *et al.*, 1984; Karlsson *et al.*, 1984; Frossard & Barnes, 1987), and some have been performed in anaesthetized guinea-pigs, in which vagal afferent nerves were stimulated in retrograde fashion (e.g. Grundström & Andersson, 1985; Belvisi *et al.*, 1988). The present study demonstrated that non-cholinergic airways constriction can also be elicited by stimulation of the spinal cord in pithed guinea-pigs. Since ganglionic, cholinergic muscarinic, and skeletal neuromuscular transmission were blocked with pentolinium, atropine and (+)-tubocurarine, it is likely that either visceral or somatic afferent nerve fibres were involved in the observed responses. Also, it is unlikely that responses involved liberation of catecholamines from either postganglionic sympathetic efferent fibres or the adrenal medulla, since this would be prevented by pentolinium treatment. The magnitude of the constriction was dependent upon the duration and frequency of stimulation, and was repeatable at 10 min intervals for at least 100 min. The latency of the response *in vivo* has previously been demonstrated by Grundström & Andersson (1985). The airways constriction was inhibited by α_2-adrenoceptor stimulation with B-HT920, and μ-opioid receptor activation with either morphine or DALDA. The effect of B-HT920 was antagonized by idazoxan, and that of morphine or DALDA by naloxone. These results, therefore, suggest that, as *in vitro* (Grunström *et al.*, 1984; Frossard & Barnes, 1987), non-cholinergic guinea-pig airway constriction can be inhibited by stimulation of either α_2-adrenoceptors or μ-opioid receptors. However, from the results of the present study it is not possible to be certain that μ-opioid receptors were involved, as δ- and κ-opioid receptor agonists were not used, and naloxone is not totally specific for μ-receptors. The data are analogous to the results obtained by Belvisi *et al.* (1988), who stimulated retrogradely the afferent vagal nerve fibres in the anaesthetized guinea-pig. The site of these inhibitory effects was peripheral, since the spinal cord and brain stem had been destroyed by pithing. Moreover, DALDA is highly polar, and would be unlikely to cause central effects (Schiller *et al.*, 1989b). A likely site of action of the agonists

used in the present study is therefore at, or in the vicinity of, sensory nerve endings within the thoracic cavity. They may possibly be acting to inhibit the release of bronchoconstrictor agents, a release which is in turn mediated by non-cholinergic nerve stimulation.

In summary, results of the present study show that the pithed guinea-pig provides a suitable preparation for the study of the peripheral effects of drugs on function of the non-cholinergic nerve supply to the tracheo-bronchial tree.

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References

ANDERSSON, R.G.G. & GRUNDSTRÖM, N. (1983). The excitatory non-cholinergic, non-adrenergic nervous system of the guinea pig airways. *Eur. J. Respir. Dis.*, **64**, 141–157.

ARMSTRONG, J.M. & BOURA, A.L.A. (1973). Effects of clonidine and guanethidine on peripheral sympathetic nerve function in the pithed rat. *Br. J. Pharmacol.*, **47**, 850–852.

BELVISI, M.G., CHUNG, K.F., JACKSON, D.M. & BARNES, P.J. (1988). Opioid modulation of non-cholinergic neural bronchoconstriction in guinea pigs *in vivo*. *Br. J. Pharmacol.*, **95**, 413–418.

FROSSARD, N. & BARNES, P.J. (1987). μ-Opioid receptors modulate non-cholinergic constrictor nerves in guinea pig airways. *Eur. J. Pharmacol.*, **141**, 519–522.

GILLESPIE, J.S., MACLAREN, A. & POLLOCK, D. (1970). A method of stimulating different segments of the autonomic outflow from the spinal column to various organs in the pithed cat and rat. *Br. J. Pharmacol.*, **40**, 257–267.

GRUNDSTRÖM, N.S.I. & ANDERSSON, R.G.G. (1985). *In vivo* demonstration of alpha-2-adrenoceptor-mediated inhibition of the excitatory non-cholinergic neurotransmission in guinea pig airways. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **328**, 236–240.

GRUNDSTRÖM, N., ANDERSSON, R.G.G. & WIKBERG, J.E.S. (1984). Inhibition of the excitatory non-adrenergic, non-cholinergic neurotransmission in the guinea-pig tracheo-bronchial tree mediated by α_2-adrenoceptors. *Acta Pharmacol. Toxicol.*, **54**, 8–14.

KARLSSON, J.A., FINNEY, M.J.B., PERSSON, C.G.A. & POST, C. (1984). Substance P antagonists and the role of tachykinins in non-cholinergic bronchoconstriction. *Life Sci.*, **35**, 2681–2691.

SCHILLER, P.W., NGUYEN, T.M-D., CHUNG, N.N., DIONNE, G. & MARTEL, R. (1989a). Peripheral antinociceptive effect of an extremely μ-selective polar dermorphin analogue (DALDA). In *International Narcotics Research Conference (INRC) '89* ed. Jhamandas, K. & Quirion, R. New York: A.R. Liss (in press).

SCHILLER, P.W., NGUYEN, T.M-D., CHUNG, N.N. & LEMIEUX, C. (1989b). Dermorphin analogues carrying an increased positive net charge in their 'message' domain display extremely high μ-opioid receptor selectivity. *J. Med. Chem.*, **32**, 698–703.

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Effects of diltiazem on calcium concentrations in the cytosol and on force of contractions in porcine coronary arterial strips

Katsuya Hirano, [1]Hideo Kanaide, Shimako Abe & Motoomi Nakamura

Research Institute of Angiocardiology, Division of Molecular Cardiology and Cardiovascular Clinic, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan

1 Using front-surface fluorometry with fura-2-loaded porcine coronary arterial strips, we simultaneously measured effects of a Ca^{2+} antagonist, diltiazem, on cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) and on tension development.

2 In the presence of extracellular Ca^{2+} (1.25 mM), histamine concentration-dependently induced abrupt (the first component) and then sustained (the second component) elevations of $[Ca^{2+}]_i$. In the absence of extracellular Ca^{2+}, histamine induced transient elevations of $[Ca^{2+}]_i$, and the time course was similar to that of the first component observed in the presence of extracellular Ca^{2+}. Histamine caused a greater contraction for a given change in $[Ca^{2+}]_i$ than did potassium, at $[Ca^{2+}]_i$ over 300 nM.

3 Diltiazem, 10^{-8} M to 10^{-5} M, concentration-dependently inhibited the second component of $[Ca^{2+}]_i$ elevation and tension development induced by histamine (10^{-5} M). Only at higher concentrations (over 10^{-5} M) did diltiazem inhibit the first component of increases in $[Ca^{2+}]_i$ and tension development induced by histamine, both in the presence and absence of extracellular Ca^{2+}.

4 Diltiazem (10^{-6} M) inhibited increases in $[Ca^{2+}]_i$ and tension development induced by cumulative applications of extracellular Ca^{2+} during K^+-depolarization. The curve of $[Ca^{2+}]_i$ against tension of these Ca^{2+}-induced contractions obtained in diltiazem-treated strips overlapped with that obtained in untreated strips. This suggests that diltiazem has no direct effects on contractile elements.

5 In contrast, the histamine-induced Ca^{2+}-tension curve (second component) was shifted in parallel to the left by diltiazem.

6 We conclude that diltiazem, at therapeutic concentrations, specifically inhibits extracellular Ca^{2+}-dependent increases in $[Ca^{2+}]_i$, with no effects on the release of Ca^{2+} from intracellular store sites or on Ca^{2+}-sensitivity of the contractile elements involved in the contractions induced by elevations of $[Ca^{2+}]_i$.

Introduction

Changes in cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) play a central role in determining the force of contraction of vascular smooth muscles (Kamm & Stull, 1985; Sommerville & Hartshorne, 1986). However, the precise relationship between $[Ca^{2+}]_i$ and tension development in intact smooth muscles has not been clarified, because of the difficulty in making direct and simultaneous determinations of $[Ca^{2+}]_i$ and contractile force in intact cells. To assess the relationship between Ca^{2+} concentrations and tension development, mechanically or chemically skinned muscle strips have been used. In such cases, sarcolemmal signal transduction systems might be seriously damaged (Saida & Nonomura, 1978; Kuriyama *et al.*, 1982).

Recent developments in optical techniques in biology and in fluorescent-indicator dyes have made the direct determination of metabolic and ionic changes in intact cells feasible (Tsien *et al.*, 1982; Grynkiewicz *et al.*, 1985; Kobayashi *et al.*, 1985; Kanaide *et al.*, 1988). We developed front-surface NADH- fluorometry, of the whole heart, using optic fibres, the objective being to minimize optical artifacts which can arise from contractile movements (Kanaide *et al.*, 1982). Using the Ca^{2+}-indicator dye fura-2 and front-surface fluorometry, we measured simultaneously $[Ca^{2+}]_i$ and force during contractions of porcine coronary arteries induced by endothelin (Kodama *et al.*, 1989) or by okadaic acid (Hirano *et al.*, 1989).

Ca^{2+}-antagonists are potent vasodilators and inhibit coronary artery spasm and reduce high blood pressure (Fleckenstein 1977; Cauvin *et al.*, 1983). It was demonstrated in our laboratory that pretreatment with the Ca^{2+}-antagonist, diltiazem, inhibited the spasm of coronary artery induced by histamine in experimentally produced atherosclerotic lesions of miniature swine (Shimokawa *et al.*, 1983). In aortic smooth muscle cells in primary culture, we found that diltiazem not only specifically inhibited the histamine-induced influx of Ca^{2+} through the sarcolemma at low concentrations (IC_{50} 0.18 μM), but also inhibited the release of Ca^{2+} from intracellular store sites at high concentrations (IC_{50} 97.5 μM), resulting in a reduction in $[Ca^{2+}]_i$ (Kanaide *et al.*, 1988; Matsumoto *et al.*, 1989). In the present study, we simultaneously determined the effects of diltiazem on $[Ca^{2+}]_i$ and on tension development and examined the effects on the relationship between $[Ca^{2+}]_i$ and tension in fura-2-loaded, intact muscle strips of the porcine coronary artery.

Methods

Tissue preparation

Hearts from adult pigs of either sex were obtained from a local slaughterhouse immediately after the animals had been killed. These hearts were placed in ice-cold saline solution and brought to the laboratory. Left circumflex arteries were isolated and cut longitudinally, and then segments 2–3 cm from the origin were excised. To remove the endothelium, the luminal surface was rubbed off with a cotton swab. Following removal of the adventitia, medial preparations were cut into approximately 1 × 5 mm circular strips 0.1 mm thick.

Fura-2 loading

Vascular strips thus prepared were loaded with the Ca^{2+} indicator dye, fura-2 by incubating in medium containing 25 μM fura-2/AM (an acetoxymethyl ester form of fura-2) and 5% foetal bovine serum for 3–4 h at 37°C. The fura-2-loaded strips were washed with normal physiological salt solution (normal PSS) to remove dye in the extracellular space, and further incubated in normal PSS for 1 h before the initiation of mea-

[1] Author for correspondence.

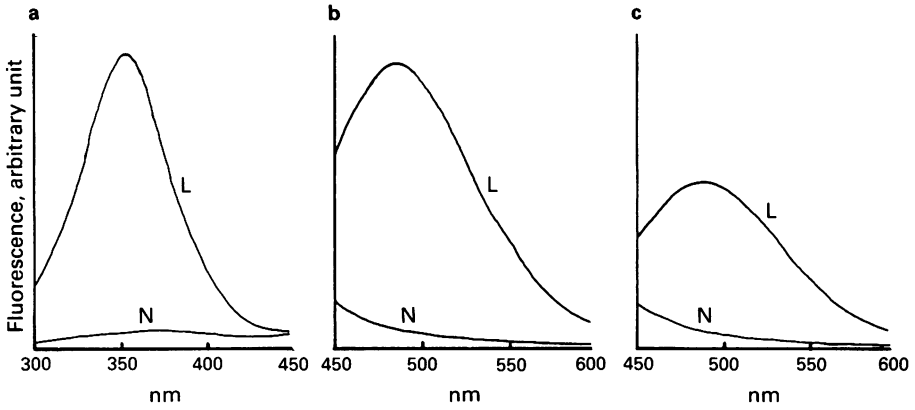

Figure 1 Fluorescence spectra of fura-2-loaded vascular strips. Excitation and emission spectra were recorded in the same strip, before and after fura-2-loading. Fluorescence spectra were recorded in normal PSS (1.25 mM Ca^{2+}) with a Hitachi fluorescence spectrophotometer (model 650-40). Excitation spectrum (a) was recorded at 500 nm emission. Emission spectrum were recorded either at 340 nm (b) or 380 nm (c) excitation. L, loaded strip; N, non-loaded strip.

surements. To examine whether strips were effectively loaded with fura-2, fluorescence excitation and emission spectra were determined in the same strips before and after fura-2-loading. A fluorescence spectrophotometer (model 650–40, Hitachi, Tokyo, Japan) was used.

Non-loaded strips showed no significant peak in the excitation spectrum when the fluorescence emission was monitored at 500 nm (Figure 1a). Fluorescence emission spectra also showed no peak at 340 nm or at 380 nm excitation (Figure 1b,c). In the fura-2-loaded strips, the intensity of the fluorescence markedly increased; 20 times at 340 nm excitation and 10 times at 380 nm excitation. The fura-2-loaded strip showed a fluorescence excitation spectrum with a peak at 340–350 nm (Figure 1a) and fluorescence emission spectra with a peak at 500 nm, both at 340 nm and at 380 nm excitation (Figure 1b,c). These characteristics indicated that fluorescence spectra obtained with fura-2 loaded strips were specific for this dye (Grynkiewicz *et al.*, 1985).

Measurement of tension development

Strips were mounted vertically in a quartz organ bath and a force-transducer (TB-612T, Nihon Koden, Japan) was used. During a one hour fura-2 equilibration period, the strips were stimulated with 60 mM K^+-depolarization every 15 min, and the resting tension was increased stepwise. After equilibration, the resting tension was adjusted to 250 mg. The responsiveness of each strip to 118 mM K^+-depolarization was recorded before the experimental protocol was started. The developed tension was expressed as %; the values in normal PSS (5.9 mM K^+) and 118 mM K^+ PSS were assumed to be 0% and 100%, respectively.

Front-surface fluorometry

Changes in the fluorescence intensity of fura-2-Ca^{2+} complex were monitored with a specifically designed front-surface fura-2 fluorometer (model CAM-OF-1) in collaboration with Japan Spectroscopic Co., (Tokyo, Japan). A block diagram of the front-surface fluorometer is shown in Figure 2. In brief, excitation light (340 nm and 380 nm) was obtained spectroscopically from a Xenon light source. Strips were illuminated by guiding the alternating (400 Hz) excitation light through quartz optic fibres arranged in a concentric inner circle (diameter = 3 mm). Surface fluorescence of strips was collected by glass optic fibres arranged in an outer circle (diameter = 7 mm) and introduced through a 500 nm band-pass filter into a photomultiplier. Special care was taken to keep the distance between a strip and the end of the optical fibres constant during measurement.

Ratio of the fluorescence intensities at 340 nm excitation to that at 380 nm excitation was monitored and expressed as %; the values in normal PSS (5.9 mM K^+) and 118 mM K^+ PSS were assumed to be 0% and 100%, respectively. The absolute value of $[Ca^{2+}]_i$ was calculated from the % ratio (R), from the following equation;

$$[Ca^{2+}]_i = K_d(R - R_{min})/(R_{max} - R)$$

where K_d is a dissociation constant and assumed to be 224 nM (Grynkiewicz *et al.*, 1985). R_{max} was determined by the addition of 25 μM ionomycin in normal PSS (1.25 mM Ca^{2+}) and R_{min} was determined in Ca^{2+}-free PSS (0 mM Ca^{2+}; 2 mM EGTA). Mean values ($n = 10$) of R_{max} and R_{min} were 156.3 ± 11.8% and −76.1 ± 22.9%, respectively. The mean values of ten different measurements of $[Ca^{2+}]_i$ at rest (0%) and during 118 mM K^+-depolarization (100%) were 108 ± 27 nM and 715 ± 103 nM, respectively.

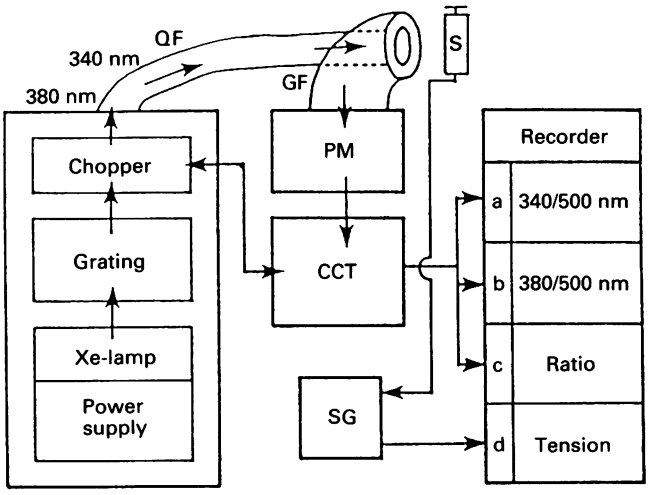

Figure 2 Block diagram of a front-surface fluorometer. Excitation light was obtained from a spectroscope (grating) from a Xenon light source. Strips (1 × 5 × 0.1 mm) were illuminated by guiding the alternating (400 Hz) 340 nm and 380 nm excitation light through quartz optic fibres arranged in a concentric inner circle (diameter = 3 mm). Surface fluorescence of strips was collected by glass optic fibres arranged in an outer circle (diameter = 7 mm) and introduced through a 500 nm band-pass filter into a photomultiplier. CCT, control circuit; GF, glass optic fibres; PM, photomultiplier; QF, quartz optic fibres; S, vascular strip; SG, strain gauge.

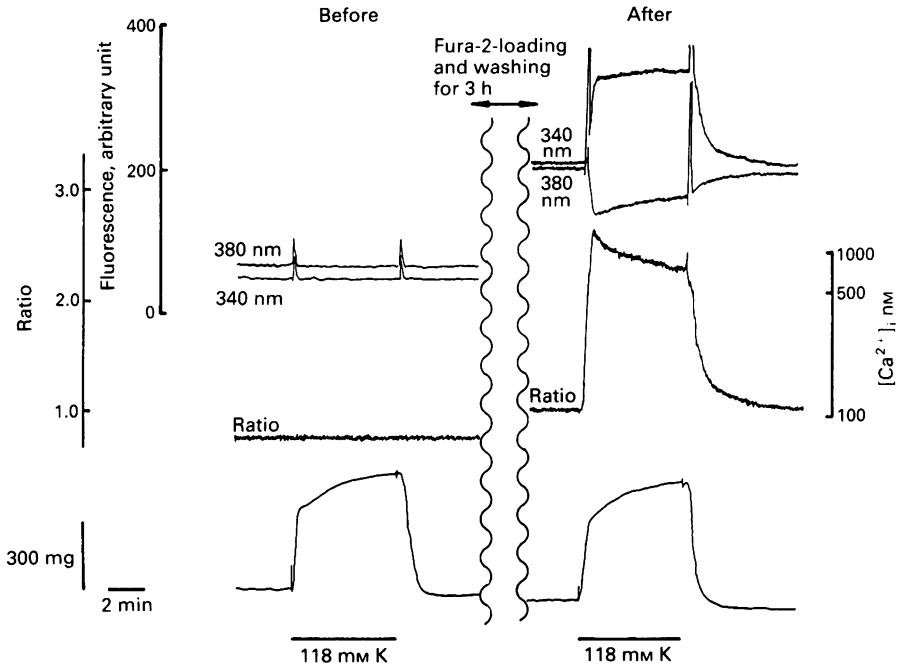

Figure 3 Effects of fura-2-loading on the contractility of porcine coronary arterial strips. The response to 118 mM K^+-depolarization was recorded in the same strip, before and after fura-2-loading. The first and the second traces from the top show traces in 500 nm-fluorescence intensities obtained at 340 nm and 380 nm excitations. The third trace shows changes in ratio of fluorescence at 340 nm excitation to that at 380 nm excitation and the lowest trace shows tension development.

Effects of fura-2-loading on contractility of porcine coronary arterial strips

Ca^{2+} indicator dyes have a Ca^{2+}-buffering action and hence, may decrease the contractility (Tsien *et al.*, 1982; Grynkiewicz *et al.*, 1985; Rink & Pozzan, 1985). To examine the effects of dye-loading on contractility of porcine coronary arterial strips, the responsiveness to 118 mM K^+-depolarization of the same strip was determined before and after loading with fura-2 (Figure 3). Before the strips were loaded with fura-2, 118 mM K^+-depolarization caused a rapid increase in tension and a maximum steady level was reached at 3 min. During this response there were no changes in fluorescence intensities at either 340 nm and or 380 nm excitations, or in the ratio of these fluorescence intensities. After the strips were loaded with fura-2, resting fluorescence intensities at 340 nm and at 380 nm excitations increased from 63 to 230 arbitrary units and from 77 to 222 arbitrary units, respectively, and the fluorescence ratio accordingly increased from 0.84 to 1.05. Loading with fura-2 did not affect the resting tension. After fura-2-loading, 118 mM K^+-depolarization caused the same extent of tension development with the same time course as that observed before loading. If the maximum developed tension during 118 mM K^+-depolarization before fura-2-loading is assumed to be 100%, that obtained after fura-2-loading was $96.0 \pm 10.7\%$ ($n = 3$). There were abrupt increases and decreases in 500 nm fluorescence intensities at 340 nm and at 380 nm excitations, respectively, and an increase in fluorescence ratio. When the bathing solutions were exchanged, optic artifacts in fluorescence intensities resulted both at 340 nm and at 380 nm excitations. However, these artifacts offset each other and disappeared. These results indicate that fura-2 loading produces no change in the contractility of vascular strips.

Drugs and solutions

Normal PSS was of the following composition (in mM): NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.25 and D-glucose 11.5. Ca^{2+}-free version of PSS (Ca^{2+}-free PSS) contained 2 mM ethyleneglycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) instead of 1.25 mM $CaCl_2$. High potassium PSS was identical to normal PSS, except for an equimolar substitution of KCl for NaCl. PSS was gassed with a mixture of 5% CO_2 and 95% O_2 (pH 7.4; 37°C). Diltiazem hydrochloride was kindly donated by Tanabe Seiyaku Co. (Osaka, Japan). Histamine dihydrochloride and fura-2/AM were purchased from Wako (Osaka, Japan) and Molecular Probes Inc., (Eugene, OR, U.S.A.), respectively.

Statistical analysis

Values are expressed as mean ± s.e.mean. Student's *t* test was used to determine the statistical significance. A *P* value less than 0.05 was considered to be significant. The $[Ca^{2+}]_i$-tension curve of contractions induced by cumulative applications of Ca^{2+} during K^+-depolarization was fitted to Hill's equation (Segel, 1976);

$$\log(T/(T_{max} - T)) = n(\log[Ca^{2+}]_i - \log K_d)$$

where T represents measured value of developed tension, expressed as %, and $[Ca^{2+}]_i$ represents value of cytosolic Ca^{2+} concentration calculated as described in the case of front-surface fluorometry. n and K_d are constant values. T_{max} represents estimated maximum tension development and was calculated by use of the following equation (Scatchard plot (Scatchard, 1949)) and least squares method.

$$T/[Ca^{2+}]_0 = (T_{max} - T)/K'$$

where $[Ca^{2+}]_0$ represents the extracellular Ca^{2+} concentration applied and K' is a constant.

Results

Effects of diltiazem on histamine-induced contractions

Figure 4 shows representative recordings of changes in fluorescence ratio and force induced by 10^{-5} M histamine in normal PSS (a) and Ca^{2+}-free PSS (b). When histamine (10^{-5} M) was applied in normal PSS, the fluorescence ratio abruptly elevated and reached the first peak (the first

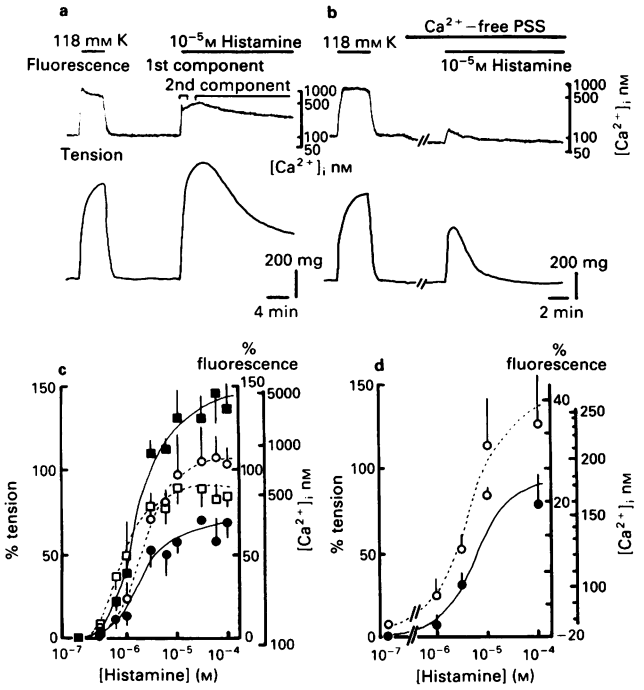

Figure 4 Changes in fluorescence ratio (340/380 nm excitation) and tension induced by histamine. (a and b) Representative time courses of fluorescence ratio and tension induced by 10^{-5} M histamine in normal PSS (a) and Ca^{2+}-free PSS (b). Histamine was applied after 15 min incubation in Ca^{2+}-free PSS. Note that the time scales differ between (a) and (b). (c and d) Dose-related responses of the first component and the second component induced by histamine in normal PSS (c) and Ca^{2+}-free PSS (d). In (c), (●, ■) indicate developed tension of the first (●) and the second (■) component and (○, □) indicate fluorescence ratio of the first (○) and the second (□) component. In (d), (○) and (●) indicate fluorescence ratio and developed tension, respectively. Data are mean of 6 different measurements; vertical lines show s.e.mean.

component) at 12 ± 3 s (n = 6). After a slight dip at 30 s, the fluorescence ratio reached the second peak at 2.4 ± 0.6 min (n = 6) and then began to decline gradually, but remained at levels higher than those seen before stimulation (the second component) (Figure 4a). The levels of $[Ca^{2+}]_i$ before stimulation, at the first peak (the first component) and 15 min after the application of histamine (the second component) were 108 ± 27 nM (n = 10), 413 ± 131 nM and 283 ± 47 nM (n = 6), respectively. Tension also developed rapidly and reached a maximum at 3.8 ± 0.7 min (n = 6), then declined gradually. The extent of tension developed at the maximum and after 15 min was 121 ± 3% and 62 ± 12% of that observed during 118 mM K^+-depolarization, respectively. In Ca^{2+}-free PSS, histamine induced a rapid and transient elevation of both fluorescence and tension. When vascular strips were exposed to Ca^{2+}-free PSS containing 2 mM EGTA, the fluorescence ratio gradually declined to reach a steady state (82 ± 4 nM) while the tension remained unchanged (Figure 4b). Application of 10^{-5} M histamine after 15 min incubation in Ca^{2+}-free PSS caused abrupt elevations in the fluorescence ratio and tension, which then rapidly declined to the pre-stimulation level within 10 min. The extent of the elevations of the first and second components of the fluorescence ratio and developed tension in normal and Ca^{2+}-free PSS were concentration-dependent (Figure 4c,d). Because of similarities between the time courses of the first components observed both in normal and Ca^{2+}-free PSS and of the lack of the second component in Ca^{2+}-free PSS, we propose that the first component is due to a release of Ca^{2+} from intracellular store sites (Somlyo, 1985) and that the second component depends on extracellular Ca^{2+}.

Figure 5 shows the effects of various concentrations of diltiazem on time courses of changes in fluorescence and tension development induced by histamine (10^{-5} M; the concentration

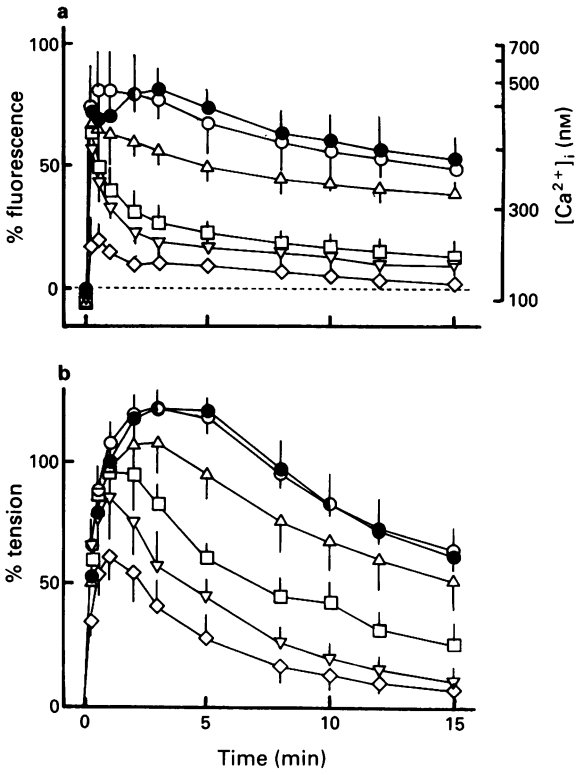

Figure 5 Effects of diltiazem on changes in fluorescence (a) and tension development (b) induced by 10^{-5} M histamine in normal PSS. Time courses of changes in fluorescence ratio (a) and tension development (b) induced by 10^{-5} M histamine in strips treated with 0 (●; control), 10^{-8} (○), 10^{-7} (△), 10^{-6} (□), 10^{-5} (▽) and 10^{-4} (◇) M diltiazem. Diltiazem was applied 15 min before the application of histamine. The abscissa scales indicate the time (in min) after the application of histamine. Both fluorescence and tension developed in PSS (5.9 mM K^+) and 118 mM K^+-PSS are assumed to be 0% and 100%, respectively. Dotted line in (a) indicates the fluorescence zero level. Data are mean of 6 different measurements; vertical lines show s.e.mean.

which gave maximum responses of fluorescence, 89.0 ± 10.2% of that observed during 118 mM K^+-depolarization) in normal PSS. Diltiazem was applied 15 min before and during the applications of histamine, because it has been shown that 15 min were required for equilibration at its binding sites (Green *et al.*, 1985). Diltiazem (over 10^{-6} M) significantly ($P < 0.05$) decreased the fluorescence level at rest (before histamine-stimulation), but did not affect the resting tension. In the range 10^{-8} M to 10^{-5} M, diltiazem concentration-dependently decreased fluorescence levels in the second component with no effects on the first component. The inhibitory effect of diltiazem on fluorescence levels gradually became apparent after the initiation of contractions. Diltiazem also inhibited the histamine-induced tension development in the second component and shortened the time to reach the maximum development of tension. The inhibitory effects of diltiazem on tension development gradually became apparent after the application of histamine (as in the case of fluorescence). Only very high concentrations of diltiazem ($> 10^{-5}$ M) inhibited both the first and second components of the fluorescence ratio and caused further decreases in tension development. Although we incubated the tissues with diltiazem for 15 min before applying histamine, there was practically no difference in changes in fluorescence ratio and tension development between the groups of 15 min, 10 min and 0 min of pre-incubation with diltiazem.

In Ca^{2+}-free PSS, elevations in fluorescence ratio and tension induced by 10^{-5} M histamine were little inhibited by diltiazem 10^{-6} to 10^{-5} M. Only very high concentrations of diltiazem (10^{-4} M) inhibited the fluorescence ratio and tension elevations ($P < 0.05$, Figure 6).

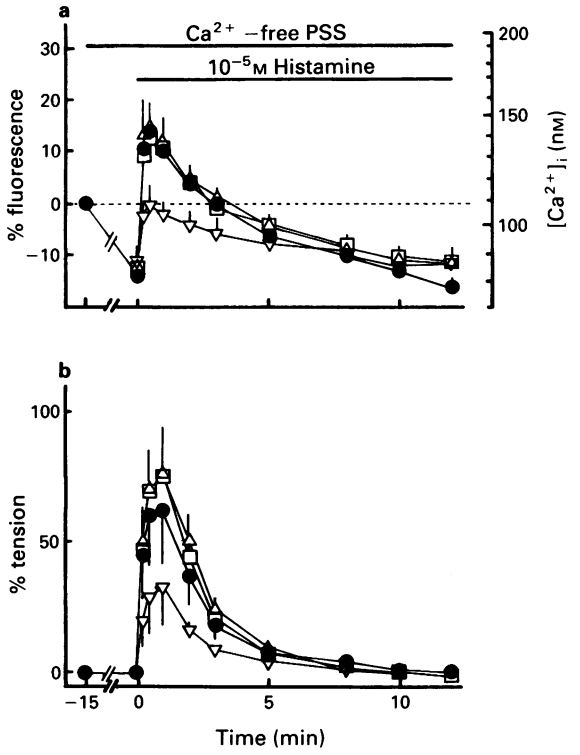

Figure 6 Effects of diltiazem on histamine-induced Ca^{2+}-release in Ca^{2+}-free PSS. Time courses of changes in fluorescence ratio (a) and tension development (b) induced by 10^{-5} M histamine in strips treated with 0 (●; control), 10^{-6} (△), 10^{-5} (□) and 10^{-4} (▽) M diltiazem. Diltiazem was applied at the time of exposure to Ca^{2+}-free PSS, i.e. 15 min before the application of histamine. Both fluorescence and tension developed in PSS (5.9 mM K^+) and 118 mM K^+-PSS were assumed to be 0% and 100%, respectively. Dotted line in (a) indicates the fluorescence zero level. Data are mean of 3 different measurements; vertical lines show s.e.mean.

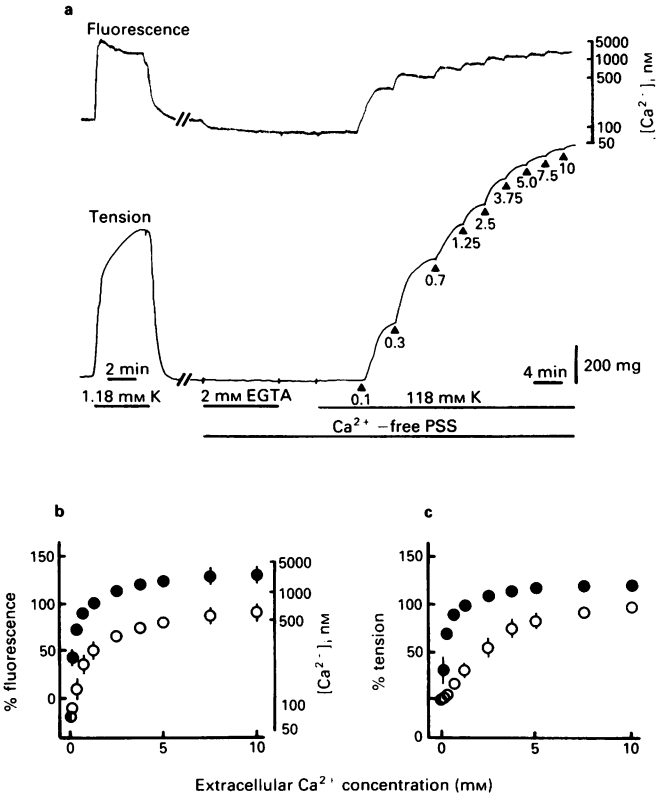

Figure 7 Effects of 10^{-6} M diltiazem on contractions induced by cumulative applications of extracellular Ca^{2+} during 118 mM K^+-depolarization. (a) Representative recording of the changes in fluorescence (upper panel) and tension (lower panel) induced by cumulative applications of extracellular Ca^{2+} during 118 mM K^+-depolarization. Numbers at each triangle (▲) indicate concentrations of extracellular Ca^{2+} (in mM). (b and c) Fluorescence ratio (b) and tension development (c) obtained from 6 different measurements, with (○) or without (●) treatment with 10^{-6} M diltiazem. Both fluorescence and tension developed in PSS (5.9 mM K^+) and 118 mM K^+-PSS are assumed to be 0% and 100%, respectively. Data are mean and vertical lines show s.e.mean.

Effects of diltiazem on Ca^{2+}-induced contractions

After 10 min incubation of tissues in Ca^{2+}-free PSS containing 2 mM EGTA, and then 5 min incubation in Ca^{2+}-free PSS without EGTA, the re-addition of extracellular Ca^{2+} in a cumulative manner (from 0 mM to 10 mM) during 118 mM K^+-depolarization led to increases in fluorescence and tension development (Figure 7b,c). With no exposure to diltiazem, the fluorescence increased from $-19.8 \pm 4.7\%$ at 0 mM extracellular Ca^{2+} to $131.8 \pm 8.9\%$ at 10 mM extracellular Ca^{2+} ($n = 6$). The tension increased from $2.6 \pm 1.6\%$ to $120.9 \pm 3.7\%$ ($n = 6$). Treatment with 10^{-6} M diltiazem 15 min before and during cumulative applications of extracellular Ca^{2+} significantly inhibited increases in fluorescence and tension development. In the diltiazem-treated strips, the fluorescence increased from $-18.2 \pm 3.8\%$ to $93.1 \pm 8.5\%$ and tension increased from $1.6 \pm 1.7\%$ to $99.1 \pm 5.7\%$ ($n = 6$) with cumulative applications of extracellular Ca^{2+} from 0 mM to 10 mM during 118 mM K^+-depolarization.

Effects of diltiazem on $[Ca^{2+}]_i$-tension relationship

Effects of diltiazem on the $[Ca^{2+}]_i$-tension relationship were evaluated from data in Figures 5 and 7. Absolute values of $[Ca^{2+}]_i$ were calculated, as described. The $[Ca^{2+}]_i$-tension relationship of the Ca^{2+}-induced contractions during K^+-depolarization was fitted to a Hill equation with a least squares method. In strips not exposed to diltiazem, tension developed concentration-dependently within the range 100 nM and 3000 nM $[Ca^{2+}]_i$. The K_d value and Hill coefficient of the $[Ca^{2+}]_i$-tension relationship obtained in untreated strips was 356 ± 52 nM and 2.6 ± 0.4, respectively ($n = 6$). The $[Ca^{2+}]_i$-tension curve obtained in diltiazem-treated strips practically overlapped that obtained in untreated strips. In other words, treatment with diltiazem moved each point downwards along with the $[Ca^{2+}]_i$-tension curve of control contractions. The K_d value and Hill coefficient in treated strips were 307 ± 35 nM and 3.5 ± 0.8, respectively ($n = 6$) (not significantly different from K_d in the absence of diltiazem).

The $[Ca^{2+}]_i$-tension relationship of contractions induced by cumulative applications of histamine (from 10^{-7} to 10^{-4} M) is shown in Figure 8. The extent of tension development for a given change in $[Ca^{2+}]_i$ in K^+-depolarization- induced contractions was smaller than that for histamine-induced contractions, at $[Ca^{2+}]_i$ over 300 nM. Effects of diltiazem on the $[Ca^{2+}]_i$-tension relationship of 10^{-5} M histamine-induced contractions were examined at 8 min after the initiation of contractions (the second component). In the range 10^{-8} to 10^{-4} M, diltiazem caused a parallel displacement of the $[Ca^{2+}]_i$-tension relationship to the left of the curve produced by histamine alone.

Discussion

After incubation in fura-2/AM-containing media, strips of porcine coronary artery showed marked increases in fluorescence intensity. During 118 mM K^+-depolarization, there were no changes in 500 nm fluorescence intensities and ratio of fluorescence intensities at 340 nm and at 380 nm excitations, without fura-2-loading. However, after fura-2-loading, there was an increase in fluorescence intensity at 340 nm excitation,

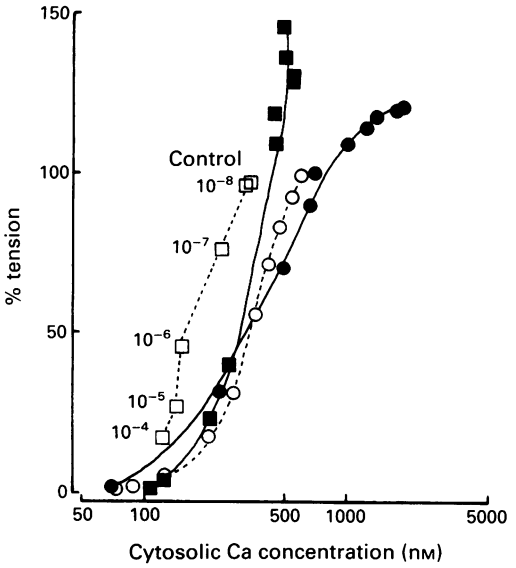

Figure 8 Effects of diltiazem of the $[Ca^{2+}]_i$-tension relationship. The $[Ca^{2+}]_i$-tension relationship was obtained from data in Figures 5 and 7. Absolute values of Ca^{2+} concentrations were calculated as described in the Methods section. (○, ●) Indicate values obtained with contractions induced by cumulative applications of extracellular Ca^{2+} during K^+-depolarization, with (○) or without (●) treatment with 10^{-6} M diltiazem, respectively ($n = 6$). (□) Indicate values obtained with 10^{-5} M histamine-induced contractions 8 min after application of histamine, in the strips treated with various concentrations of diltiazem ($n = 6$). (■) Indicate values obtained with contractions induced by cumulative applications of histamine. Numbers beside each open square indicate the concentration of diltiazem applied (in M).

a decrease in 380 nm excitation and an increase in fluorescence ratio. These results suggest that fura-2/AM was effectively incorporated into cells and converted intracellularly to fura-2 free acid. The changes in fluorescence reflect changes in Ca^{2+} concentration, mainly in the cytosol (Grynkiewicz *et al.*, 1985). Loading the strips with fura-2 did not alter the time courses and the maximum levels of tension development during 118 mM K^+-depolarization, thereby suggesting that tissue damage by possible acidification of the cells due to formaldehyde released on acetoxymethyl-ester hydrolysis did not occur (Tsien *et al.*, 1982; Rink & Pozzan, 1985). Thus, one can measure tension development and fura-2-fluorescence simultaneously, and carry out quantitative evaluations of the $[Ca^{2+}]_i$-tension relationship in intact tissues.

Histamine, a receptor stimulating compound, caused greater tension development for a given change in $[Ca^{2+}]_i$ than depolarization at $[Ca^{2+}]_i$ over 300 nM (Figure 8). This suggests that receptor-mediated stimulation increased Ca^{2+}-sensitivity of the contractile apparatus (Morgan & Morgan, 1984; Bruschi *et al.*, 1988; Himpens & Somlyo, 1988; Rembold & Murphy, 1988; Sato *et al.*, 1988). In addition, the $[Ca^{2+}]_i$-tension relationship of the Ca^{2+}-induced contraction during depolarization was similar to the pCa-tension relationship noted with skinned fibres of the porcine coronary artery (Itoh *et al.*, 1982) and of others (Saida & Nonomura, 1978; Kuriyama *et al.*, 1982).

In the present study, diltiazem concentration-dependently inhibited histamine-induced, extracellular Ca^{2+}-dependent increases in $[Ca^{2+}]_i$ (the second component) within the range 10^{-8} M to 10^{-5} M and only at higher concentrations (over 10^{-5} M) inhibited the histamine-induced Ca^{2+}-release from the intracellular store sites (the first component). As in the case of $[Ca^{2+}]_i$, diltiazem also inhibited the extracellular Ca^{2+}-dependent development of tension at lower concentrations ($\leq 10^{-5}$ M), and only at high concentrations ($> 10^{-5}$ M) inhibited the first component of contractions in normal PSS and in Ca^{2+}-free PSS. These results are consistent with our previous findings in rat aortic smooth muscle cells in primary culture. In rat cultured aortic smooth muscle cells, diltiazem inhibits histamine-mediated, extracellular Ca^{2+}-dependent increases in $[Ca^{2+}]_i$ with low IC_{50} values (for 10^{-5} M histamine, $IC_{50} = 0.18\,\mu$M); and only at high concentrations ($IC_{50} = 95.7\,\mu$M) did it inhibit the histamine-induced Ca^{2+}-release from intracellular store sites (Matsumoto *et al.*, 1989). We have also shown that diltiazem blocks [^{3}H]-mepyramine binding to membrane preparations from the porcine aorta at high concentrations ($K_i = 114\,\mu$M) (Matsumoto *et al.*, 1989). Ca^{2+}-antagonists inhibit the binding of several agonists to specific binding sites (Fairhurst *et al.*, 1980; Glossman & Hornung, 1980; Barnathan *et al.*, 1982; Karliner *et al.*, 1982; Nayler *et al.*, 1982; Motulsky *et al.*, 1983). Thus, inhibition of the receptor-mediated Ca^{2+}-release from intracellular store sites seen with very high concentrations of diltiazem (around 10^{-4} M) may be due to competitive binding to receptor-sites.

It has been found that histamine, through the H_1-receptor, depolarizes the membrane potential of the rabbit ear artery (Casteels & Suzuki, 1980) and guinea-pig main pulmonary artery (Suzuki & Kou, 1983). Nelson *et al.* (1988), with patch clamp study, showed that an agonist, even when administered outside of a pipette, enhanced the Ca^{2+} current through voltage-operated Ca^{2+} channels (VOCs). They suggested that an agonist could modulate the Ca^{2+} current in the case of depolarization by acting through second messengers. From evidence obtained in these electrophysiological studies, it seems likely that histamine can increase the Ca^{2+} current through VOCs by depolarizing the membrane potential or by activating other receptor-mediated pathways. In the present study, such events may explain why diltiazem decreased the histamine-induced and extracellular Ca^{2+}-dependent increases in $[Ca^{2+}]_i$ and contractions in the late phase. We did not obtain direct evidence that the late sustained phase of $[Ca^{2+}]_i$ increase is primarily due to a Ca^{2+} influx through VOCs or receptor-operated channels. As it has been found that therapeutic peak blood levels of diltiazem range from 10^{-7} M to 10^{-6} M (Koiwaya *et al.*, 1981), our results suggest that the clinical efficacy of diltiazem appears to be due to the inhibitory effects on Ca^{2+} influx through Ca^{2+} channels. In certain peripheral vasculatures, Ca^{2+}-antagonists are concentrated and reach a relatively high level (Kates *et al.*, 1981). The inhibition of receptor-mediated Ca^{2+}-release from intracellular store sites may be able to inhibit the contraction.

The present study showed that the Ca^{2+}-tension relationship during depolarization in diltiazem-treated strips overlaps with that seen in the untreated strips. This would suggest that diltiazem has no direct effects on the contractile elements. However, in the case of histamine-induced contractions, the Ca^{2+}-tension relationship of the second component obtained with diltiazem paralleled that of the Ca^{2+}-induced contractions obtained in the absence of diltiazem, the result being a leftward shift of the Ca^{2+}-tension curve. These observations suggest that the mechanisms by which histamine produces a greater extent of tension development for a given change in $[Ca^{2+}]_i$ than depolarization might be independent of $[Ca^{2+}]_i$ and not be affected by treatment with diltiazem. These findings also indicate that relaxation of the histamine-induced contractions by diltiazem is caused by a decrease in $[Ca^{2+}]_i$, with no direct effects on the contractile elements. The leftward shift of the Ca^{2+}-tension relationship does not indicate that diltiazem potentiates the Ca^{2+}-sensitivity of contractile elements. These observations are consistent with findings that diltiazem had no effect on myosin light chain kinase or on protein kinase C (Shächtele *et al.*, 1989) and that diltiazem did not affect the pCa-tension relationship in skinned muscles (Suzuki *et al.*, 1982). However, other workers have shown that diltiazem, at high concentrations, has direct effects on contractile elements (Boström *et al.*, 1981; Johnson *et al.*, 1982) and decreases the effectiveness of Ca^{2+}-tension relationship (Saida & van Breemen, 1983).

Diltiazem ($\geq 10^{-6}$ M) decreased the resting levels of $[Ca^{2+}]_i$ without causing relaxation in the normal PSS. This also occurs on exposure to Ca^{2+}-free PSS. Therefore, diltiazem-

sensitive Ca^{2+} channels are apparently active under resting conditions and play a role in maintaining the resting level of $[Ca^{2+}]_i$. These events may be caused by dissociation of the $[Ca^{2+}]_i$-tension relationship, or it may be due to difficulties in detecting decreases in the resting tension.

We simultaneously measured fura-2 fluorescence and contractions in the porcine coronary artery and examined the vasodilator mechanisms of diltiazem. It was observed that diltiazem ($\leq 10^{-5}$ M) specifically inhibited extracellular Ca^{2+}-dependent increases in $[Ca^{2+}]_i$, possibly Ca^{2+} influx through Ca^{2+} channels, and secondarily caused proportional decreases in tension development, with no effect on Ca^{2+}-sensitivity of the contractile elements. At high concentrations ($>10^{-5}$ M), diltiazem inhibited Ca^{2+}-release from intracellular store sites, possibly by inhibiting binding of the agonist at receptor sites.

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References

BARNATHAN, E.S., ADDONIZO, P. & SHATTIL, S.J. (1982). Interaction of verapamil with human platelet α-adrenergic receptors. *Am. J. Physiol.*, **242** (Heart Circ. Physiol., 11), H19–H23.

BOSTRÖM, S.L., LJUNG, B., MARDH, S., FORSEN, S. & THULIN, E. (1981). Interaction of the antihypertensive drug felodipine with calmodulin. *Nature*, **292**, 777–778.

BRUSCHI, G., BRUSCHI, M.E., REGOLISTI, G. & BORGHETTI, A. (1988). Myoplasmic Ca^{2+}-force relationship studied with fura-2 during stimulation of rat aortic smooth muscle. *Am. J. Physiol.*, **254**, (Heart Circ. Physiol., 23), H840–H854.

CASTEELS, R. & SUZUKI, H. (1980). The effects of histamine on the smooth muscle cells of the ear artery of the rabbit. *Pflügers Archiv.*, **387**, 17–25.

CAUVIN, C., LOUTZENHISER, R. & VAN BREEMEN, C. (1983). Mechanisms of calcium antagonist-induced vasodilation. *Ann. Rev. Pharmacol. Toxicol.*, **23**, 373–396.

FAIRHURST, A.S., WHITTAKER, M.L. & ELERT, F.J. (1980). Interaction of D600 (methoxy-verapamil) and local anesthetics with rat brain α-adrenergic and muscarinic receptors. *Biochem. Pharmacol.*, **29**, 155–162.

FLECKENSTEIN, A. (1977). Specific pharmacology of calcium in myocardium, cardiac pacemakers, and vascular smooth muscle. *Ann. Rev. Pharmacol. Toxicol.*, **17**, 149–166.

GLOSSMAN, H. & HORNUNG, R. (1980). Calcium- and potassium-channel blockers interact with α-adrenoceptors. *Mol. Cell Endocrinol.*, **19**, 243–251.

GREEN, F.J., FARMER, B.B., WISEMAN, G.L., JOSE, M.J.L. & WATANABE, A.M. (1985). Effect of membrane depolarization on binding of [^{3}H] nitrendipine to rat cardiac myocytes. *Circ. Res.*, **56**, 576–585.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

HIMPENS, B. & SOMLYO, A.P. (1988). Free-calcium and force transients during depolarization and pharmacomechanical coupling in guinea-pig smooth muscle. *J. Physiol.*, **395**, 507–530.

HIRANO, K., KANAIDE, H. & NAKAMURA, M. (1989). Effects of okadaic acid on cytosolic calcium concentrations and on contractions of the porcine coronary artery. *Br. J. Pharmacol.*, **98**, 1261–1266.

ITOH, T., KAJIWARA, M., KITAMURA, K. & KURIYAMA, H. (1982). Roles of stored calcium on the mechanical response evoked in smooth muscle cells of the porcine coronary artery. *J. Physiol.*, **322**, 107–125.

JOHNSON, J.D., VAGHY, P.L., CROUCH, T.H., POTTER, J.D. & SCHWARTZ, A. (1982). An hypothesis for the mechanism of action of some of the Ca^{2+} antagonist. Drug: calmodulin as a receptor. *Adv. Pharmacol. Ther.*, **3**, 121–138.

KAMM, K.E. & STULL, J.T. (1985). The function of myosin and myosin light chain phosphorylation in smooth muscle. *Ann. Rev. Pharmacol. Toxicol.*, **25**, 593–620.

KANAIDE, H., YOSHIMURA, R., MAKINO, R. & NAKAMURA, M. (1982). Regional myocardial function and metabolism during acute coronary artery occlusion. *Am. J. Physiol.*, **242** (Heart Circ. Physiol., 11), H980–H989.

KANAIDE, H., KOBAYASHI, S., NISHIMURA, J., HASEGAWA, M., SHOGAKIUCHI, Y., MATSUMOTO, Y. & NAKAMURA, M. (1988). Quin2 microfluorometry and effects of verapamil and diltiazem in calcium release from rat aorta smooth muscle cells in primary culture. *Circ. Res.*, **63**, 16–26.

KARLINER, J.S., MOTULSKY, H.J., DUNLAP, J., BROWN, J.H. & INSEL, P.A. (1982). Verapamil competitively inhibits α_1-adrenergic and muscarinic, but not β-adrenergic receptors in rat myocardium. *J. Cardiovascular Pharmacol.*, **4**, 515–520.

KATES, R.E., KEEFE, D., SCHWARTZ, J., HARAPAT, S., HARRISON, D., KIRSTEN, E. & JOHNSON, S.M. (1981). Deposition kinetics of verapamil and norverapamil (abstract). *Clin. Pharmacol. Ther.*, **29**, 257.

KOBAYASHI, S., KANAIDE, H. & NAKAMURA, M. (1985). Cytosolic-free calcium transients in cultured vascular smooth muscle cells: microfluorometric measures. *Science*, **229**, 553–556.

KODAMA, M., KANAIDE, H., ABE, S., HIRANO, K., KAI, H. & NAKAMURA, M. (1989). Endothelin-induced Ca-independent contraction of the porcine coronary artery. *Biochem. Biophys. Res. Commun.*, **160**, 1302–1308.

KOIWAYA, Y., ASHIHARA, T., NAKAMURA, M. & ETOH, A. (1981). Plasma concentration of diltiazem after oral administration in normal volunteers. *Clin. Ther.*, **3**, 436–440.

KURIYAMA, H., ITOH, Y., SUZUKI, H., KITAMURA, K. & ITOH, T. (1982). Factors modifying contraction-relaxation cycle in vascular smooth muscles. *Am. J. Physiol.*, **243** (Heart Circ. Physiol., 12), H641–H662.

MATSUMOTO, T., KANAIDE, H., NISHIMURA, J., KUGA, T., KOBAYASHI, S. & NAKAMURA, M. (1989). Histamine-induced calcium transients in vascular smooth muscle cells: effects of verapamil and diltiazem. *Am. J. Physiol.*, **257** (Heart Circ. Physiol., 26), H563–H570.

MORGAN, J.P. & MORGAN, K.G. (1984). Stimulation-specific patterns of intracellular calcium levels in smooth muscle of ferret portal vein. *J. Physiol.*, **351**, 155–167.

MOTULSKY, H.J., SNAVELY, M.D., HUGHES, R.J. & INSEL, P.A. (1983). Interaction of verapamil and other calcium channel blockers with α_1- and α_2-adrenergic receptors. *Circ. Res.*, **52**, 226–231.

NELSON, M.T., STANDEN, N.B., BRAYDEN, J.E. & WORLEY III, J.F. (1988). Noradrenaline contracts arteries by activating voltage-dependent calcium channels. *Nature*, **336**, 382–385.

NAYLER, W.G., THOMPSON, J.E. & JARROTT, B. (1982). The interaction of calcium antagonists (slow channel blockers) with myocardial alpha adrenoceptors. *J. Mol. Cell Cardiol.*, **14**, 185–188.

REMBOLD, C.M. & MURPHY, R.A. (1988). Myoplasmic $[Ca^{2+}]$ determines myosin phosphorylation in agonist-stimulated swine arterial smooth muscle. *Circ. Res.*, **63**, 593–603.

RINK, T.J. & POZZAN, T. (1985). Using quin2 in cell suspensions. *Cell Calcium*, **6**, 133–144.

SAIDA, K. & NONOMURA, Y. (1978). Characteristics of Ca^{2+}- and Mg^{2+}-induced tension development in chemically skinned smooth muscle fibers. *J. Gen. Physiol.*, **72**, 1–14.

SAIDA, K. & VAN BREEMEN, C. (1983). Mechanism of Ca^{2+} antagonist-induced vasodilation-intracellular actions. *Circ. Res.*, **52**, 137–142.

SATO, K., OZAKI, H. & KARAKI, H. (1988). Changes in cytosolic calcium level in vascular smooth muscle strip measured simultaneously with contraction using fluorescent calcium indicator fura2. *J. Pharmacol. Exp. Ther.*, **246**, 294–300.

SCATCHARD, G. (1949). The attractions of protein for small molecules and ion. *Ann. New York Acad. Sci.*, **51**, 660–672.

SCHÄCHTELE, C., WAGNER, B. & RUDOLPH, C. (1989). Effect of Ca^{2+} entry blockers on myosin light-chain kinase and protein kinase C. *Eur. J. Pharmacol.*, **163**, 151–155.

SEGEL, I.H. (1976). *Biochemical Calculation*, 2nd ed. New York: John Wiley & Sons, Inc.

SHIMOKAWA, H., TOMOIKE, H., NABEYAMA, S., YAMAMOTO, H., ARAKI, H., NAKAMURA, M., ISHII, Y. & TANAKA, K. (1983). Coronary artery spasm induced in atherosclerotic miniature swine. *Science*, **221**, 560–562.

SOMLYO, A.P. (1985). Excitation-contraction coupling and the ultrastructure of smooth muscle. *Circ. Res.*, **57**, 497–507.

SOMMERVILLE, L.E. & HARTSHORNE, D.J. (1986). Intracellular calcium and smooth muscle contraction. *Cell Calcium*, **7**, 353–364.

SUZUKI, H., ITOH, T. & KURIYAMA, H. (1982). Effects of diltiazem on smooth muscles and neuromuscular junction in the mesenteric artery. *Am. J. Physiol.*, **242** (Heart Circ. Physiol., 11), H325–H336.

SUZUKI, H. & KOU, K. (1983). Direct and indirect effects of histamine on the smooth muscle cells of the guinea-pig main pulmonary artery. *Pflügers Archiv.*, **339**, 46–53.

TSIEN, R.Y., POZZAN, T. & RINK, T.J. (1982). Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescence indicator. *J. Cell Biol.*, **94**, 325–334.

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Zacopride and BRL 24924 induce an increase in EEG-energy in rats

[1]Hendrik W.G.M. Boddeke & Hans O. Kalkman

Preclinical Research Sandoz Pharma Ltd, CH-4002, Basel, Switzerland

1 The substituted benzamides, zacopride and BRL 24924 induced dose-dependent increases of the total EEG-energy of rats when applied intracerebroventricularly (i.c.v.) with ED_{50} values of 8.0 ± 0.6 and $3.6 \pm 0.9\,\mu g$, respectively. Not only the energy of the low frequency hippocampal theta rhythm but also that of the other frequency bands was increased.

2 In contrast to i.c.v. application intraperitoneal administration of zacopride or BRL 24924 (1 and $10\,mg\,kg^{-1}$) did not lead to an increase in EEG-energy.

3 The increase in EEG-energy induced by zacopride ($10\,\mu g$, i.c.v.) was blocked by ICS 205-930 ($1\,\mu g$, i.c.v.). Neither the 5-HT_3 receptor agonist 2-methyl-5-hydroxytryptamine ($30\,\mu g$, i.c.v.) nor the selective 5-HT_3 receptor antagonist MDL 72222 ($30\,\mu g$, i.c.v.) had any effect upon rat EEG.

4 Scopolamine ($0.01\,\mu g$ and $0.1\,\mu g$, i.c.v.) dose-dependently antagonized the effect of zacopride ($10\,\mu g$, i.c.v.).

5 An agonist action of zacopride and BRL 24924 and inhibition of these effects by ICS 205-930 but not by MDL 72222 was recently described in isolated colliculi neurones from neonatal mice. The receptor involved was described as '5-HT_4'. The present results indicate that the central effects of zacopride and BRL 24924 may be due to activation of such a 5-HT receptor.

Introduction

The subdivision of 5-hydroxytryptamine (5-HT) receptors into 5-HT_1-like, 5-HT_2 and 5-HT_3 receptor types has been established by radioligand binding, biochemical and functional studies (Bradley *et al.*, 1986; Peroutka, 1988). Many responses to 5-HT can be accommodated into the scheme of 5-HT_1-like, 5-HT_2 and 5-HT_3; for instance, the 'M' (or 5-HT_M) receptor, as originally described by Gaddum & Picarelli (1957), is now considered to be a 5-HT_3 receptor (Bradley *et al.*, 1986; Craig & Clarke, 1989). Nevertheless, several responses mediated by 5-HT cannot be explained by activation of 5-HT_1-like or by 5-HT_2 or 5-HT_3 receptors.

Enteric neurones contain a 5-HT receptor that is different from 5-HT_1, 5-HT_2 or 5-HT_3 subtypes (Craig & Clarke, 1989; 1990). These as yet undefined receptors are stimulated by substituted benzamides like, zacopride, cisapride, BRL 24924 and metoclopramide as well as by the tryptamine derivatives α-methyltryptamine and 5-methoxytryptamine (Craig & Clarke, 1989). The effects of these agonists are antagonized competitively by ICS 205-930, albeit at concentrations that are higher than those required to block 5-HT_3 receptors (Craig & Clarke, 1990). The response mediated by this receptor can be antagonized by atropine (Buchheit *et al.*, 1985). Consequently, it may be assumed that this receptor induces release of acetylcholine.

Another non-classical 5-HT receptor has recently been described by Dumuis *et al.* (1988a). These so called '5-HT_4' receptors mediate stimulation of adenylate cyclase activity in mouse embryo superior colliculi neurones and in guinea-pig hippocampal membranes (Dumuis *et al.*, 1988b; 1989a,b). Compounds which activate 5-HT_4 receptors include substituted benzamides, e.g. zacopride, BRL 24924 and metoclopramide and the effects can be blocked by ICS 205-930 at high (micromolar) concentrations (Dumuis *et al.*, 1989a,b). The atypical 5-HT receptor in the ileum and the 5-HT_4 receptor in the brain show closely similar pharmacological characteristics and may be identical (Craig & Clarke, 1989; Clarke *et al.*, 1989).

Since muscarinic agonists increase theta rhythm and 5-HT_4 receptor activation causes acetylcholine release in guinea-pig ileum, it is possible that 5-HT_4 receptor stimulation in the brain may cause a release of acetylcholine and a subsequent increase in theta rhythm. Therefore, in the present study the effects of zacopride and BRL 24924 upon rat EEG were investigated.

Methods

Male Sprague Dawley rats weighing 250–300 g were used throughout. For i.c.v. administration the rats were anaesthetized with $50\,mg\,kg^{-1}$ pentobarbitone-sodium. A stainless steel guide (0.8 mm o.d., 0.5 mm i.d., 11 mm length) was placed stereotaxically with its mandrel into the right lateral ventricle (coordinates A: −1.3 mm, L: 1.7 mm, V: −3.5 mm) and its position was fixed with acrylic cement (DeTrey, Zurich) and three screws. The wound was then treated with sulpadiazine and sutured. The animals were allowed to recover for two days. On the day of the experiment, anaesthesia was induced and maintained by a mixture of 1.5% halothane in oxygen. Body temperature was kept at 38°C by means of a heating table. One femoral artery was cannulated for blood pressure monitoring. The rats were placed in a stereotaxic apparatus with the mouthbar positioned 5 mm above the earbar. Concentric bipolar electrodes (Clark: SNEX 100 × 5 mm) were attached to a micromanipulator and implanted in the hippocampus at the following coordinates (calculated from bregma) A: −0.4 mm, L 2.0 mm and V: −2.5 mm. EEG activity was recorded monopolarly. Following amplification with either Grass 7P551 or WPI DAM5AR amplifiers, the signal was monitored by a computer. Realtime on-line Fourier-analysis was performed on computer using an Ariel FFT chart.

For drug infusion the mandrel was removed and replaced by a needle connected by means of a polyethylene tube to an infusion pump. Single concentration-response curves for zacopride and BRL 24924 were made; in each animal 2 or 3 concentrations of zacopride were tested. In experiments where antagonists were used, the animals were pretreated with antagonist for 60 min. All drugs were dissolved in non-buffered 0.9% w/v NaCl solution (pH = 6.9–7.3) and single doses were infused i.c.v. (infusion rate $1\,\mu l\,min^{-1}$) or in some experiments injected i.p., in volumes of 1 μl or 1 ml, respectively. A 30 min baseline recording was obtained before application of test compounds.

[1] Author for correspondence.

Statistical significance was evaluated by Student's two tailed test. The significance level was considered at $P < 0.05$.

Drugs

The following drugs were used (source in parentheses): ICS 205-930 ([3α-tropanyl]-1H-indole-3-carboxylic acid ester hydrochloride, Sandoz); MDL 72222 (1αH,3α,5αH-tropan-3-yl-3,5-dichlorobenzoate methanesulphonate, Merrell-Dow); 2-methyl-5-hydroxytryptamine (Sandoz); zacopride (A.H. Robins); BRL 24924 ([(±)-(endo)]-4-amino-5-chloro-2-methoxy-N-(1-azabicyclo-[3.3.1]-non-4-yl)-benzamide hydrochloride, Beecham); scopolamine (Sigma).

Results

In control experiments EEG recordings of rats anaesthetized with halothane remained constant for at least 5 h. No effect upon EEG was observed after i.c.v. or i.p. administration of NaCl (0.9%). I.c.v. application of single doses of zacopride induced a dose-dependent ($ED_{50} = 8.0 \pm 0.6\,\mu g$, $n = 8$) increase in total (1.2–49 Hz) EEG-energy (Figure 1). A similar effect was seen with BRL 24924, which was, however, more potent ($ED_{50} = 3.6 \pm 0.9\,\mu g$, $n = 5$, Figure 2). Not only the energy of the low frequency band of the hippocampal theta rhythm was increased, but also the energy of the other frequency bands of the EEG spectrum (Figures 1 and 2). The effects of zacopride and BRL 24924 were long lasting and maximal durations of approximately 90 min were found after

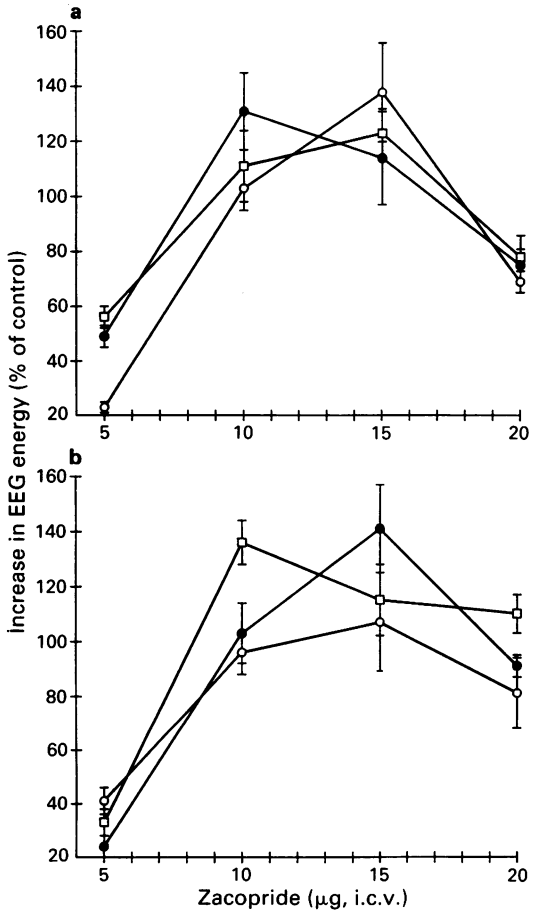

Figure 1 (a) The dose-response curves (% of the control value) of the increase in energy of the total (1.2–49 Hz) (○) and of the frequency bands: 1.2–4.9 (●) and 2.6–5.6 Hz (□) induced by zacopride. (b) The dose-response curves (% of the control value) of the increase in energy of the frequency bands 4.2–8.2 (○), 8–12 (●) and 12–24 Hz (□) of the EEG spectrum induced by zacopride. The symbols represent mean values with s.e.mean shown by vertical bars ($n = 4$–6).

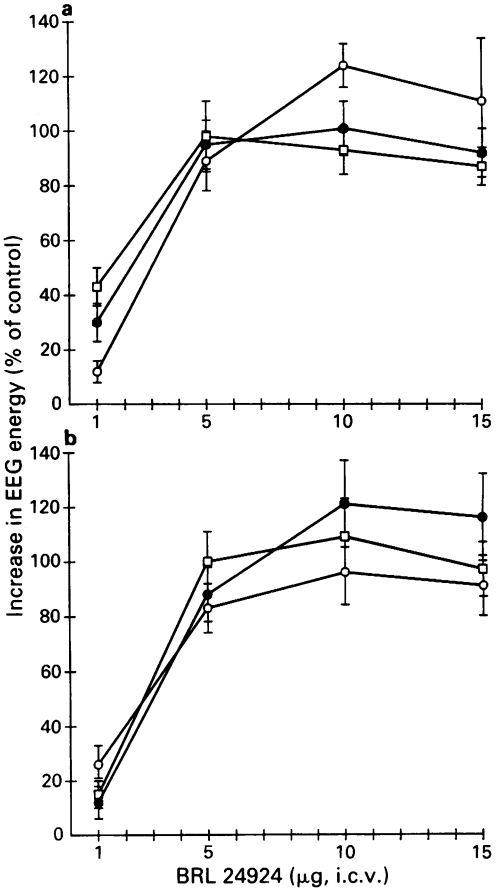

Figure 2 (a) The dose-response curves (% of the control value) of the increase in energy of the total (1.2–49 Hz) (○) and of the frequency bands: 1.2–4.9 (●) and 2.6–5.6 Hz (□) induced by BRL 24924. (b) The dose-response curves (% of the control value) of the increase in energy of the frequency bands 4.2–8.2 (○), 8–12 (●) and 12–24 Hz (□) of the EEG spectrum induced by BRL 24924. The symbols represent mean values with s.e.mean shown by vertical bars ($n = 4$–6).

i.c.v. application of a single dose of either drug. In contrast to i.c.v. administration, intraperitoneal application of zacopride or BRL 24924 (1 and 10 mg kg^{-1}) did not induce the excitatory EEG activity (data not illustrated).

Pretreatment with ICS 205-930 (1 μg, i.c.v.) did not affect the EEG but blocked completely the effect of zacopride (10 μg, i.c.v., Figure 3). ICS 205-930 (1 μg, i.c.v.) was able to inhibit the effect of zacopride (10 μg, i.c.v.) when administered 45 min

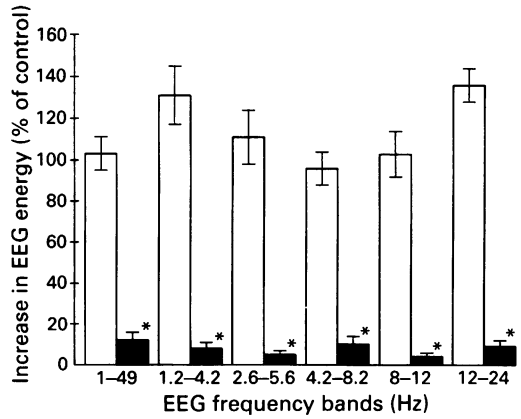

Figure 3 The effect of zacopride (10 μg, i.c.v.) without (open columns) and with (filled columns) ICS 205-930 (1 μg, i.c.v.) ($n = 8$). The data represent mean values with s.e.mean shown by vertical bars. The significance ($P < 0.05$) of the difference between the responses of zacopride with and without ICS 205-930 are indicated (*).

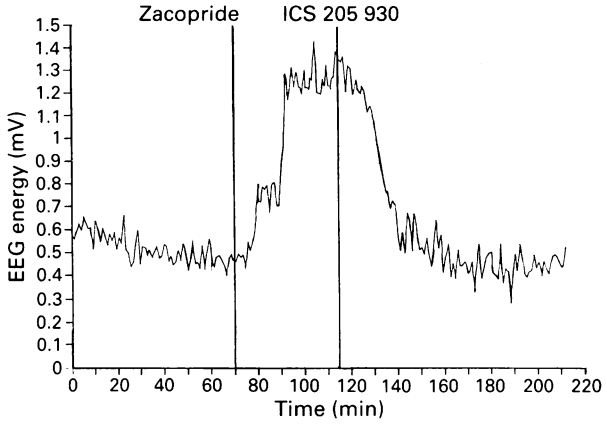

Figure 4 Recording of the total EEG spectrum (1.2–49 Hz), showing the effect of zacopride (10 μg, i.c.v.) followed by application of ICS 205-930 (1 μg, i.c.v.). The vertical axis represents the EEG energy (mV). The horizontal axis indicates the recording time (min).

after the application of zacopride (Figure 4). The 5-HT_3 receptor antagonist MDL 72222 (30 μg, i.c.v.) had no effect *per se* and did not significantly ($P < 0.05$) affect the response elicited by zacopride (10 μg i.c.v., Figure 5). In addition, i.c.v. application of the 5-HT_3 receptor agonist, 2-methyl-5-hydroxytryptamine (30 μg), showed no effect on rat EEG (Figure 6).

I.c.v. application of scopolamine even at a high dose of 0.1 μg did not affect the EEG in rats. However, after pretreatment with a low dose of scopolamine (0.01 μg) the response to zacopride (10 μg, i.c.v.) was partially blocked (Figure 7) and a higher dose of 0.1 μg scopolamine completely abolished the effect (data not illustrated). Doses of 0.1 and 0.01 μg (i.c.v.) scopolamine are sufficient to antagonize the excitatory EEG effects of cholinoceptor agonists (authors unpublished).

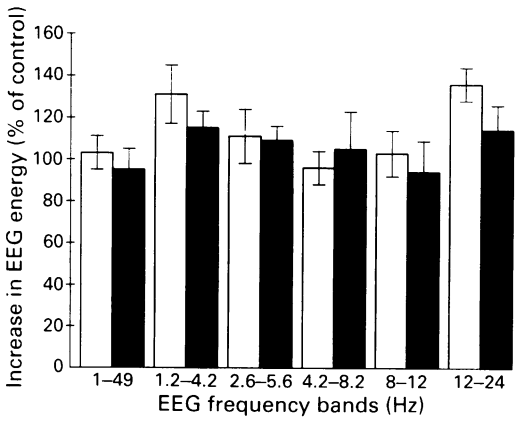

Figure 5 The effect of zacopride (10 μg, i.c.v.) without (open columns) and with (filled columns) MDL 72222 (30 μg, i.c.v.) pretreatment. The data represent mean values with s.e.mean shown by vertical bars ($n = 6$).

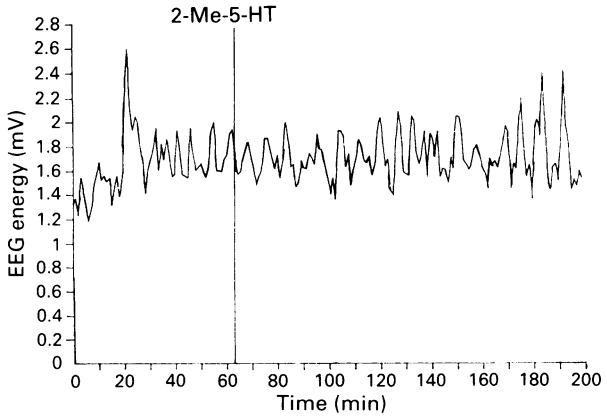

Figure 6 A recording of rat EEG (1–49 Hz) before and after application of 2-methyl-5-hydroxytryptamine (2-Me-5-HT, 30 μg, i.c.v.). The vertical axis represents the EEG energy (mV). The horizontal axis indicates the recording time (min).

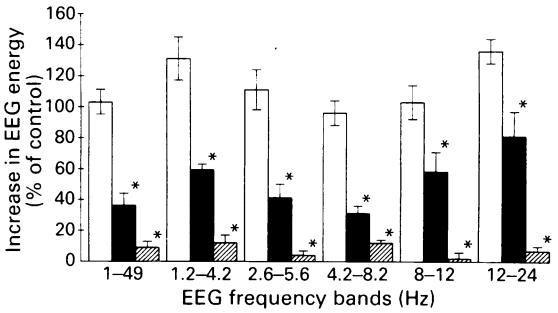

Figure 7 The effect of zacopride (10 μg, i.c.v.) in control (open columns) and in scopolamine (0.01 μg i.c.v., filled columns, 0.1 μg i.c.v., cross-hatched columns) pretreated rats. The data represent mean values with s.e.mean shown by vertical bars ($n = 6$). The significance ($P < 0.05$) of the difference between the responses to zacopride with and without scopolamine are indicated (*).

Discussion

Since the presence of 5-HT_4 receptors is established in central nervous tissue (Dumuis *et al.*, 1988a,b; 1989a,b), the question arises what functional consequences activation of central 5-HT_4 receptors might have. To our knowledge, no electrophysiological or behavioural studies using 5-HT_4 agonists have been described. Therefore, it seemed of interest to study the effects of published 5-HT_4 receptor agonists and antagonists upon rat EEG activity. In order to avoid the problem of penetration of the blood brain barrier, all compounds investigated were applied i.c.v., except for some experiments where we injected zacopride and BRL 24924 i.p. to find out whether these compounds are active when applied peripherally.

Both zacopride and BRL 24924 increased the energy of the EEG spectrum. Since both zacopride and BRL 24924 are 5-HT_3 receptor antagonists the possible involvement of 5-HT_3 receptors was investigated. The selective 5-HT_3 receptor antagonist MDL 72222, at a high dose of 30 μg (i.c.v.), had no effect on EEG. It remains a question whether the dose of MDL 72222 used is sufficient to block possible 5-HT_3-mediated responses. BRL 24924 and MDL 72222 show approximately equal affinity for 5-HT_3 receptors (Hoyer, 1989; Hoyer *et al.*, 1989). Accordingly, the dose used in the present study, which is at least 7-times higher than the ED_{50} value of BRL 24924, should be sufficient to block 5-HT_3 receptors. Also the 5-HT_3 receptor agonist, 2-methyl-5-hydroxytryptamine (30 μg, i.c.v.), had no effect itself on EEG. Whilst we cannot be certain that the dose of 2-methyl-5-hydroxytryptamine would be adequate to activate 5-HT_3 receptors, this seems likely since, if the dose were distributed evenly throughout the brain, a concentration of approximately 10^{-5} M would be reached and this is 10 times higher than the pK_D value for 5-HT_3 receptors (Hoyer *et al.*, 1989). MDL 72222 (30 μg, i.c.v.) also did not antagonize the excitatory activity of zacopride. Finally, the affinity of zacopride for 5-HT_3 receptors is higher than that of BRL 24924 (Hoyer 1989; Hoyer *et al.*, 1989) and this is opposite to the activities observed in the present experiments. Taken together, these data indicate that 5-HT_3 receptors are not involved in the excitatory activity of zacopride and BRL 24924.

On the other hand, the effect of zacopride was completely blocked by ICS 205-930 (1 μg, i.c.v.). The 5-HT_4 receptor-mediated stimulation of adenylate cyclase in isolated colliculi neurones, was inhibited by ICS 205-930, but not by MDL

72222 (Dumuis *et al.*, 1988a,b). In addition, ICS 205-930 has negligible affinity for muscarinic cholinoceptors (Richardson *et al.*, 1985). Therefore, the present data are consistent with the central effect of zacopride and BRL 24924 being mediated by 5-HT_4 receptors. This conclusion is supported by the fact that the ratio of relative potency is consistent with 5-HT_4 (BRL 24924 more potent than zacopride; Craig & Clarke, 1990; Dumuis *et al.*, 1989b) and is the opposite of that to be expected if 5-HT_3 receptors were involved (see above).

In guinea-pig ileum the activation of 5-HT_4-like receptors induces release of acetylcholine (Buchheit *et al.*, 1985). Therefore, we investigated whether the central effects were the direct result of 5-HT_4 receptor activation or the consequence of 5-HT_4-mediated release of acetylcholine. In previous experiments we observed that scopolamine blocks central cholinergic effects at concentrations of 0.01 and 0.1 μg when applied i.c.v. (authors unpublished). Scopolamine blocked in a dose-dependent manner the effect of zacopride. Scopolamine not only antagonized the increase in theta rhythm, but the increase of all frequency bands. This is an observation which is, at this stage, difficult to explain, since the usual response to directly acting muscarinic agonists, is a specific increase of the hippocampal theta rhythm (2–5 Hz) (Olpe *et al.*, 1987). A possible explanation for the non-specific character of the excitation by zacopride might be the concomitant activation of other neurotransmitter systems. This assumption, however, needs to be verified, and would be in contrast with the fact that the response of zacopride can be blocked completely with scopolamine.

An interesting phenomenon observed, was the complete lack of activity of zacopride (1 and 10 mg kg^{-1}) and BRL 24924 (1 and 10 mg kg^{-1}) after peripheral application. Since the effect of zacopride when applied i.c.v., occurs at relatively high doses and considering the relatively hydrophilic nature of the compound, a likely explanation is that after i.p. application insufficient amounts of compound reach the locus of action within the brain. Whereas previous work on 5-HT_4 receptors has been performed on guinea-pig and mouse tissue (for summary, see Clark *et al.*, 1989) in this study rats were used. This indicates that 5-HT_4 receptors are also present in rats.

In conclusion, zacopride and BRL 24924 induce increases in EEG energy. The pharmacology of this response is consistent with a 5-HT_4-mediated effect since it was abolished by ICS 205-930 which is known to antagonize 5-HT_4 receptors, but unaffected by MDL 72222, a selective 5-HT_3 receptor antagonist. As in the guinea-pig ileum, the response appears to be indirect and involves (at least in part) the release of acetylcholine. Since excitation by cholinoceptor agents is assumed to play a role in learning and memory it may be worthwhile to evaluate the effects of 5-HT_4 receptor agonists in tests of cognitive function.

References

BRADLEY, P.B., ENGEL, G., FENIUK, W., FOZARD, J.R., HUMPHREY, P.P.A., MIDDLEMISS, D.N., MYLECHARANE, E.J., RICHARDSON, B.P. & SAXENA, P.R. (1986). Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. *Neuropharmacology*, **25**, 563–576.

BUCHHEIT, K.H., ENGEL, G., MUTSCHLER, E. & RICHARDSON, B.P. (1985). Study of the contractile effect of 5-hydroxytryptamine (5-HT) in the isolated longitudinal muscle strip from guinea-pig ileum. Evidence for two distinct release mechanisms. *Naunyn Schmiedebergs Arch. Pharmacol.*, **319**, 36–41.

CLARKE, D.E., CRAIG, D.A. & FOZARD, J.R. (1989). The 5-HT_4 receptor: naughty but nice. *Trends Pharmacol. Sci.*, **10**, 385–386.

CRAIG, D.A. & CLARKE, D.E. (1989). 5-Hydroxytryptamine and cholinergic mechanisms in guinea-pig ileum. *Br. J. Pharmacol.*, **96**, 247P

CRAIG, D.A. & CLARKE, D.E. (1990). Pharmacological characterization of a neuronal receptor for 5-HT in guinea-pig ileum with properties similar to the 5-HT_4 receptor. *J. Pharmacol. Exp. Ther.*, **252**, 1378–1386.

DUMUIS, A., BOUHELAL, R., SEBBEN, M. & BOCKAERT, J. (1988a). A 5-HT receptor positively coupled with adenylate cyclase is antagonized by ICS 205-930 in the central nervous system. *Eur. J. Pharmacol.*, **146**, 187–188.

DUMUIS, A., BOUHELAL, R., SEBBEN, M., CORY, R. & BOCKAERT, J. (1988b). A non-classical 5-hydroxytryptamine receptor positively coupled with adenylate cyclase in the central nervous system. *Mol. Pharmacol.*, **34**, 880–887.

DUMUIS, A., SEBBEN, M. & BOCKAERT, J. (1989a). BRL 24924: a potent agonist at a non-classical 5-HT receptor positively coupled with adenylate cyclase in colliculi neurons. *Eur. J. Pharmacol.*, **162**, 381–384.

DUMUIS, A., SEBBEN, M. & BOCKAERT, J. (1989b). The gastrointestinal prokinetic benzamide derivatives are agonists at the non-classical 5-HT receptor (5-HT_4) positively coupled to adenylate cyclase in neurons. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **340**, 403–410.

GADDUM, J.H. & PICARELLI, Z.P. (1957). Two kinds of tyrptamine receptor. *Br. J. Pharmacol. Chemother.*, **12**, 323–328.

HOYER, D. (1989). 5-hydroxytryptamine receptors and effector coupling mechanisms in peripheral tissues. In *Peripheral Action of 5-HT*. ed. Fozard, J.R. pp. 72–99. Oxford: University Press.

HOYER, D., GOZLAN, H., BOLANOS, F., SCHLECHTER, L.E. & HAMON, M. (1989). Interaction of psychotropic drugs with central 5-HT_3 recognition sites: fact or artifact. *Eur. J. Pharmacol.*, **171**, 137–139.

OLPE, H.R., KLEBS, K., KUNG, E., CAMPICHE, P., GLATT, A., ORTMANN, R., D'AMATO, F., POZZA, M.F. & MONDADORI, C. (1987). Cholinomimetics induce theta rhythm and reduce hippocampal pyramidal cell excitability. *Eur. J. Pharmacol.*, **142**, 275–283.

PEROUTKA, S.J. (1988). 5-hydroxytryptamine receptor subtypes: molecular biochemical and physiological characterization. *Trends Neurol. Sci.*, **11**, 496–500.

RICHARDSON, B.P., ENGEL, G., DONATSCH, P. & STADLER, P.A. (1985). Identification of serotonin M-receptor subtypes and their specific blockade by a new class of drugs. *Nature*, **316**, 126–131.

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Functional evidence for heterogeneity of peripheral prejunctional α_2-adrenoceptors

Sonia Connaughton & [1]James R. Docherty

Department of Clinical Pharmacology, Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin 2, Ireland

1 We have examined the potencies of a series of α_2-adrenoceptor antagonists in functional studies of prejunctional α_2-adrenoceptors in rat atrium and vas deferens, and compared potencies with affinities for the α_{2A}-ligand binding site of human platelet and the α_{2B}- site of rat kidney.

2 Antagonist potency in rat atrium was expressed as an EC_{30} (concentration producing 30% increase in the stimulation-evoked overflow of tritium in tissues pre-incubated with [^{3}H]-noradrenaline). Antagonist potency in rat vas deferens was expressed as a pA_2 or K_B at antagonizing the inhibition by the α_2-adrenoceptor agonist xylazine of the isometric twitch to a single stimulus, or as an EC_{30}.

3 In ligand binding studies, K_i values were obtained for the displacement by α-adrenoceptor antagonists of [^{3}H]-yohimbine binding to human platelet or rat kidney membranes.

4 In functional studies, three antagonists (ARC 239, prazosin and chlorpromazine) distinguished between prejunctional α_2-adrenoceptors of rat atrium (EC_{30}) and rat vas deferens (pA_2) and showed 49, 12 and 7 times higher potency in rat atrium, respectively. ARC 239 was also 17 times more potent in rat atrium than rat vas deferens when EC_{30} values were compared.

5 The correlation of affinity for the α_{2A}- site of human platelet was better with prejunctional potency in rat vas deferens than rat atrium.

6 The correlation of affinity for the α_{2B}-site of rat kidney was better with prejunctional potency in rat atrium than rat vas deferens.

7 It is concluded that prejunctional α_2-adrenoceptors of rat vas deferens and rat atrium differ, and these receptors may resemble the α_{2A}- and α_{2B}-ligand binding sites, respectively.

Introduction

Heterogeneity of α_2-adrenoceptors has led to a subdivision into α_{2A}, α_{2B} and α_{2C} based on ligand binding studies (Cheung *et al.*, 1982; Bylund, 1985; Nahorski *et al.*, 1985; Murphy & Bylund, 1988; Bylund, 1988; Michel *et al.*, 1989a). Gene cloning techniques suggest that there are at least two genes coding for α_2-adrenoceptors (Regan *et al.*, 1988).

However, functional correlates of these ligand binding sites have proved difficult to find (see Docherty, 1989; Connaughton & Docherty, 1990). Prazosin, which has higher absolute potency and higher potency relative to yohimbine at the α_{2B}- than at that α_{2A}-binding site, has been shown to be only 5 times less potent than yohimbine in potentiating the stimulation-evoked release of noradrenaline in rat submandibular gland (Turner *et al.*, 1984) and rat cerebral cortex (Nasseri & Minneman, 1987), suggesting that these receptors may resemble the α_{2B}-binding site. Furthermore, Kapocsi *et al.* (1987) found that prazosin had higher potency at potentiating the stimulation-evoked release of noradrenaline from adrenergic nerves of rabbit pulmonary artery than from cholinergic nerves of guinea-pig ileum, suggesting that different subtypes of α_2-adrenoceptor are present on these nerve terminals.

However, most of the functional evidence has been obtained from studies in which prazosin has been compared to a reference antagonist. In this study, we have examined the potencies of a series of 10 α-adrenoceptor antagonists in functional studies of prejunctional α_2-adrenoceptors in rat atrium and rat vas deferens and compared potencies with affinities for the α_{2A}- site of human platelet (see Bylund, 1985) and the α_{2B}- site of rat kidney (Michel *et al.*, 1989a).

Methods

Male Wistar rats (250–350 g) were obtained from Trinity College, Dublin, Ireland and a variety of isolated tissues were used.

[1] Author for correspondence.

Rat vas deferens isometric contraction

Prostatic portions of rat vas deferens were placed between platinum electrodes in organ baths (50 ml) and bathed at 37°C in Krebs-Henseleit solution of the following composition (mmol l^{-1}): NaCl 119, $NaHCO_3$ 25, (+)-glucose 11.1, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.0. Two or four prostatic segments were obtained per rat and the tissues were attached to myograph transducers for recording of isometric contractions.

Responses to single pulse field stimulation (supramaximal voltage, 0.5 ms pulses) were obtained at intervals of 5 min. When consistent control responses had been obtained, antagonist or vehicle was administered and 15 min later the effects of the α_2-adrenoceptor agonists xylazine and oxymetazoline were assessed on nerve-stimulation evoked contractions. Agonists were added cumulatively in 0.5 log unit increments at intervals of 5 min. Agonist IC_{50} values (concentration producing 50% of maximum inhibition of the contraction to a single stimulus) were obtained from individual paired experiments in which one tissue received antagonist and the other received vehicle. Antagonist potency was expressed as the dissociation constant K_B from the equation $K_B = [B]/(DR - 1)$, where [B] is the concentration of antagonist and DR is the agonist dose-ratio produced by the antagonist, or as a pA_2 value. Antagonist pA_2 values were obtained from the x-intercept of the plot of log (agonist DR − 1) against log antagonist concentration, where the slope was not significantly different from negative unity (Arunlakshana & Schild, 1959).

Radioactive overflow experiments

Rat isolated atria or vas deferens were preincubated for 1 h in 1 ml medium containing [^{3}H]-noradrenaline (0.5 μmol l^{-1}, specific activity 39 Ci $mmol^{-1}$) before being superfused with [^{3}H]-noradrenaline-free Krebs-Henseleit solution of the same composition as stated above except that the ascorbic acid (0.28 mmol l^{-1}), tetrasodium EDTA (30 μmol l^{-1}), corticosterone (30 μmol l^{-1}) and propranolol (1 μmol l^{-1}) were

present. In addition, cocaine ($3\,\mu mol\,l^{-1}$) was present after pre-incubation with [^{3}H]-noradrenaline. Tissues were placed between platinum electrodes in organ baths, and superfused at a rate of $2\,ml\,min^{-1}$ at 37°C.

In all experiments, tissues were stimulated 4 or 5 times (S_0–S_4) for 3 min at a frequency of 2 Hz (atria) or 5 Hz (vas deferens) at intervals of 27 min, beginning after 99 min of superfusion. Effluent samples were collected in 6 ml aliquots beginning after 120 min of superfusion so that sampling began 6 min before S_1. Antagonist drugs or distilled water vehicle were added to the superfusion stream at a rate of $50\,\mu l\,min^{-1}$ in 2 or 3 cumulative concentrations beginning 12 min before S_2. At the end of the experiment tissues were made soluble in 1 ml of tissue solubiliser.

Human platelet membranes

Membrane preparation was carried out by a method adapted from that described by Buckley *et al.* (1986): 60 ml blood was drawn from an antecubital vein of healthy young adult subjects into lithium heparin tubes. The blood was centrifuged at $160\,g$ for 10 min and platelet-rich plasma removed. Ice-cold isotonic buffer (Tris-HCl $50\,mmol\,l^{-1}$, NaCl $100\,mmol\,l^{-1}$, EDTA $5\,mmol\,l^{-1}$, pH 7.4 at 4°C) was mixed with the remaining blood and the tubes were centrifuged again. The supernatant and platelet-rich plasma were combined and centrifuged at $27{,}000\,g$ for 10 min at 4°C. The pellets were resuspended in lysing buffer (Tris-HCl $5\,mmol\,l^{-1}$, EDTA $5\,mmol\,l^{-1}$, pH 7.4) at 4°C and homogenized. After the membranes had been in the lysing buffer for 10 min they were centrifuged at $27{,}000\,g$ for 10 min. The resultant pellet was used immediately or stored at −20°C for later use. Pellets were reconstituted in incubation buffer (Tris-HCl $50\,mmol\,l^{-1}$, $MgCl_2$ $8\,mmol\,l^{-1}$, EGTA $5\,mmol\,l^{-1}$, pH 7.4 at 37°C), homogenized and centrifuged at $27{,}000\,g$ for 10 min. Pellets were again reconstituted in 2.5 ml incubation buffer.

Rat kidney membranes

Membrane binding was carried out by a method adapted from those of Neylon & Summers (1985) and Michel *et al.* (1989b). Rat kidney cortex was homogenized in 10 volumes of ice-cold wash buffer (Tris HCl $50\,mmol\,l^{-1}$, EDTA $5\,mmol\,l^{-1}$, pH 7.4 at 4°C) for 30 s in an Ultra-Turrax homogenizer and centrifuged at $40{,}000\,g$ for 10 min at 4°C. The supernatant was discarded, the pellet was resuspended in fresh buffer and the centrifugation step was repeated. The pellet was resuspended in 10 volumes of incubation buffer (Tris HCl $50\,mmol\,l^{-1}$, EDTA $5\,mmol\,l^{-1}$, pH 7.4 at 25°C). Homogenates were filtered through two layers of 43 T-mesh to remove connective tissue.

Radioligand binding studies

In saturation experiments, aliquots of membrane suspension were incubated with various concentrations of [^{3}H]-yohimbine at 37°C (0.3–$20\,nmol\,l^{-1}$; human platelet) or 25°C (0.5–$30\,nmol\,l^{-1}$; rat kidney) for 30 min. In competition studies, [^{3}H]-yohimbine ($5\,nmol\,l^{-1}$) was incubated with displacing ligands in concentrations from $0.1\,nmol\,l^{-1}$ to $100\,\mu mol\,l^{-1}$ in 0.5 log unit increments for 30 min. Non-specific binding was determined in the presence of phentolamine $10\,\mu mol\,l^{-1}$. Assays were terminated by washing with ice-cold incubation buffer, followed by rapid vacuum filtration through Whatman GF/C filters, using a Brandel Cell Harvester. Radioactivity retained on filters was determined by liquid scintillation spectroscopy.

The inhibition constant (K_i) for displacement of radiolabelled ligand was determined from the formula: $K_i = IC_{50}/(1 + [^3H/K_D])$ where IC_{50} is the concentration of competing ligand that inhibits radioligand binding by 50%, K_D is the dissociation constant for the radioligand (platelet: $5.75 \pm 0.78\,nmol\,l^{-1}$, $n = 6$; kidney: $8.80 \pm 0.62\,nmol\,l^{-1}$, $n = 7$), and ^{3}H is the concentration of tritiated yohimbine employed ($5\,nmol\,l^{-1}$).

Statistical evaluation

Results are expressed as mean ± s.e.mean or mean and 95% confidence limits. Effects of antagonist on agonist IC_{50}, EC_{50} or maximum tension, or on the stimulation-evoked overflow of tritium were compared with the effects of vehicle by Student's *t* test for unpaired data, or Student's *t* test for paired data where appropriate, and analysis of variance. Slopes and elevation of Schild regressions were compared by covariance analysis (see Snedecor & Cochrane, 1980).

Drugs

The following drugs were used: ARC 239 (2-(2,4-(O-methoxyphenyl)-piperazin-1-yl)-ethyl-4,4 dimethyl-1,3-(2H,4H)-isoquinolindine chloride (gift: Karl Thomae, Biberach, F.R.G.), BDF 8933 (4-fluoro-2-(imidazolin-2-ylamino)-isindoline maleate; gift: Beiersdorf, Hamburg, F.R.G.); chlorpromazine hydrochloride (Sigma, Poole, U.K.); cocaine hydrochloride (Sigma); corticosterone (Sigma); idazoxan (Research Biochemicals, Natick, MA, U.S.A.); oxymetazoline hydrochloride (Sigma); phentolamine mesylate (gift: CIBA, Horsham, U.K.); prazosin hydrochloride (gift: Pfizer, Sandwich, U.K.); (±)-propranolol hydrochloride (Sigma); SKF 104078 (6-chloro-9-(3-methyl-2-butenyl) oxyl-3-methyl-1H-2,3,4,5-tetrahydro-3-benzazepine maleate (gift: Smith Kline & French, King of Prussia, PA, U.S.A.); urapidil (gift: Byk Gulden, Konstanz, F.R.G.); WB 4101 (2-2(,6-dimethoxyphenoxyethyl) aminoethyl-1,4-benzodioxane hydrochloride (Research Biochemicals); xylazine hydrochloride (gift: Bayer, Ireland); yohimbine hydrochloride (Sigma).

Drug stocks were dissolved in distilled water and dilutions made up in distilled water, with the exception of corticosterone which was dissolved in 100% ethanol.

Results

Vas deferens isometric contraction

In prostatic portions of the rat vas deferens, single pulse electrical stimulation produced an isometric contraction of 1.36 ± 0.12 g ($n = 31$). Chlorpromazine ($30\,\mu mol\,l^{-1}$) significantly reduced the stimulation-evoked isometric contraction to $63.9 \pm 16.8\%$ ($n = 5$) of control and SKF 104078 ($10\,\mu mol\,l^{-1}$) and ARC 239 ($30\,\mu mol\,l^{-1}$) significantly increased the response to $136.2 \pm 7.7\%$ ($n = 8$) and $133.6 \pm 9.2\%$ ($n = 4$) of control, respectively. Other antagonists in the concentrations employed did not significantly affect the response to a single stimulus. In vehicle experiments, xylazine (1–$1000\,nmol\,l^{-1}$) produced concentration-dependent inhibition of the stimulation evoked isometric contractions, with a maximum inhibition of $93.1 \pm 1.1\%$ ($n = 31$) and an IC_{50} of $74.1\,nmol\,l^{-1}$ (95% confidence limits of 64.5–$85.1\,nmol\,l^{-1}$). It was not possible to construct a Schild plot for chlorpromazine due to its low potency and since higher concentrations reduced stimulation-evoked contractions (see above), but a K_B of 5.70 ± 0.33 (−log M, $n = 3$) was obtained with chlorpromazine ($3\,\mu mol\,l^{-1}$) and a K_B of 5.59 ± 0.35 ($n = 3$) was obtained with $10\,\mu mol\,l^{-1}$, suggesting a competitive interaction. Slopes of Schild plots were not significantly different from negative unity for all other antagonists except prazosin and ARC 239. For prazosin, ARC 239 and chlorpromazine, potency was expressed as $-\log K_B$ values from the effects of the two lowest concentrations to produce shifts in agonist potency. The pA_2 or K_B values obtained are shown in Tables 1 and 2.

Table 1 pA_2 values obtained for α-adrenoceptor antagonists from Schild plots of slope not significantly different from negative unity

	pA_2	Slope
WB 4101	6.82 (5.95–8.07) $n = 11$	-0.88 ± 0.20
BDF 8933	8.92 (7.94–12.59) $n = 12$	-1.15 ± 0.25
Yohimbine	7.50 (6.82–8.97) $n = 10$	-1.06 ± 0.25
SKF 104078	6.45 (5.69–8.56) $n = 9$	-0.98 ± 0.26
Urapidil	5.29 (4.86–5.96) $n = 8$	-1.08 ± 0.16
Phentolamine	7.77 (7.35–8.43) $n = 8$	-1.02 ± 0.14
Idazoxan	7.82 (7.28–8.58) $n = 14$	-1.13 ± 0.17

Values are pA_2 and 95% confidence limits and slope ± s.e.mean, with n the number of points.

The α_2-adrenoceptor agonist oxymetazoline (1–1000 nmol l^{-1}) produced concentration-dependent inhibition of the stimulation-evoked isometric contraction with a maximum inhibition of 75.5 ± 11.5% ($n = 5$) and an IC_{50} of 85.1 nmol l^{-1} (11.2–64.5 nmol l^{-1}). Oxymetazoline, in concentrations of 3 μmol l^{-1} and above produced spontaneous contractions of the tissue and potentiated the response to a single pulse. In interaction experiments, the slope of the Schild plot for the antagonist ARC 239 against oxymetazoline was significantly different from negative unity, so that a K_B value of 5.67 ± 0.45 ($n = 6$) was obtained from the effects of the 2 lowest concentrations to produce shifts in agonist potency.

Radioactive overflow experiments

Isolated atria Field stimulation of rat isolated atria at a frequency of 2 Hz for 3 min produced an evoked overflow of tritium at S_1 of 1.41 ± 0.10% of tissue tritium ($n = 30$). Basal outflow of tritium was 0.16 ± 0.01% of tissue tritium per min ($n = 30$) before S_1. Yohimbine (10–100 nmol l^{-1}), SK&F 104078 (1 μmol l^{-1}), phentolamine (0.03–0.3 μmol l^{-1}), prazosin (0.1–0.3 μmol l^{-1}), urapidil (10 μmol l^{-1}), ARC 239 (0.01–1 μmol l^{-1}), chlorpromazine (0.1–1 μmol l^{-1}), WB 4101 (0.1–1 μmol l^{-1}), BDF 8933 (0.001–0.1 μmol l^{-1}) and idazoxan (0.01–1 μmol l^{-1}) significantly increased the stimulation-evoked overflow of tritium without increasing basal outflow. Potency of antagonist was expressed as an EC_{30} value (concentration producing a 30% increase in stimulation-evoked overflow of tritium) and values are shown in Table 2.

Table 2 K_i values obtained with α-adrenoceptor antagonists for the displacement of [^{3}H]-yohimbine binding in human platelet (α_{2A}) and rat kidney (α_{2B}) membranes, and their prejunctional potencies in rat vas deferens (pA_2) and rat atrium (EC_{30})

Antagonist	Platelet K_i	Kidney K_i	Vas deferens pA_2	Rat atrium EC_{30}
Chlorpromazine	6.48	6.29	$5.65 \pm 0.18^+$	6.53 ± 0.36
WB 4101	7.0	6.94	6.82	7.70 ± 0.62
BDF 8933	8.72	8.91	8.92	9.18 ± 0.24
Prazosin	5.62	7.12	$5.88 \pm 0.20^+$	6.97 ± 0.26
ARC 239	5.45	6.76	$5.71 \pm 0.24^+$	7.40 ± 0.63
Yohimbine	8.04	7.93	7.50	7.89 ± 0.14
SKF 104078	6.52	6.25	6.45	6.68 ± 0.16
Urapidil	5.52	5.72	5.29	5.80 ± 0.52
Phentolamine	7.61	7.24	7.77	7.60 ± 0.23
Idazoxan + K_B	7.30	7.49	7.82	7.97 ± 0.12

Values are mean and 95% confidence limits (−log M) from at least 3 experiments. Full data for vas deferens are shown in Table 1, and full data for platelet and kidney are shown in Table 4.

Table 3 EC_{30} values obtained for α-adrenoceptor antagonists in rat atrium and rat vas deferens

Antagonist	Rat atrium EC_{30}	Rat vas deferens EC_{30}
Chlorpromazine	6.53 ± 0.36	<6.0
BDF 8933	9.18 ± 0.24	9.26 ± 0.32
Prazosin	6.97 ± 0.26	<6.5
ARC 239	7.40 ± 0.63	6.17 ± 0.71*
Yohimbine	7.89 ± 0.14	7.60 ± 0.04

Values are mean and 95% confidence limits from at least 3 experiments. Asterisks denote potency in rat vas deferens significantly different from potency in rat atrium (Student's t test, * $P < 0.05$).

Isolated vas deferens Field stimulation of rat isolated vas deferens at a frequency of 5 Hz for 3 min produced an evoked overflow of tritium at S_1 of 1.44 ± 0.11% of tissue tritium ($n = 20$). Basal outflow of tritium was 0.13 ± 0.01% of tissue tritium per min ($n = 20$) before S_1. Yohimbine (0.01–1 μmol l^{-1}), BDF 8933 (0.001–0.1 μmol l^{-1}) and ARC 239 (1 μmol l^{-1}) significantly increased stimulation-evoked overflow of tritium without increasing basal outflow, and EC_{30} values are shown in Table 1. Prazosin (1 μmol l^{-1}) and chlorpromazine (10 μmol l^{-1}) significantly increased the basal outflow of tritium and an EC_{30} could not be calculated. The potencies of yohimbine and BDF 8933 in rat vas deferens were similar to their potencies in rat atrium, but ARC 239 was significantly less potent in rat vas deferens than in rat atrium (Table 3).

Human platelet and rat kidney membranes

K_i values and Hill slopes for the displacement by α-adrenoceptor antagonists of [^{3}H]-yohimbine binding to human platelet and rat kidney membranes are shown in Tables 2 and 4. For all displacing ligands, Hill slopes were close to unity, so that it was assumed that a single population of binding sites was present in both tissues.

Correlation between tissues

Figure 1 shows the correlation between K_i in human platelet membranes and prejunctional pA_2, K_B or EC_{30} obtained in rat vas deferens and rat atrium for 10 antagonists. The correlation of the α_{2A}-site of human platelet was better with antagonist pA_2 or K_B in rat vas deferens (slope = 1.00 ± 0.13, $r = 0.94$, $P < 0.001$) than with antagonist EC_{30} in rat atrium (slope = 0.68 ± 0.17, $r = 0.81$, $P < 0.01$).

Figure 2 shows the correlation between K_i in rat kidney membranes and prejunctional pA_2, K_B or EC_{30} obtained in rat vas deferens and rat atrium for 10 antagonists. The correlation of the α_{2B}- site of rat kidney was better with antagonist EC_{30} in rat atrium (slope = 0.97 ± 0.11, $r = 0.95$, $P < 0.001$) than with antagonist pA_2 or K_B in rat vas deferens (slope = 1.14 ± 0.22, $r = 0.88$, $P < 0.001$).

The correlation between human platelet K_i and rat kidney K_i gave a slope = 0.99 ± 0.25, $r = 0.81$, $P < 0.01$. The correlation between antagonist EC_{30} in rat atrium and antagonist pA_2 or K_B in rat vas deferens gave a slope = 1.14 ± 0.20, $r = 0.90$, $P < 0.001$.

Table 4 K_i values (−log M) and Hill slopes obtained with α-adrenoceptor antagonists for the displacement of [^{3}H]-yohimbine binding in human platelet (α_{2A}) and rat kidney (α_{2B}) membranes

Antagonist	*Platelet* K_i	*Kidney* K_i
Chlorpromazine	6.48 ± 0.29	6.29 ± 0.08
	0.94 ± 0.03	1.10 ± 0.09
WB 4101	7.08 ± 0.84	6.94 ± 0.09
	1.00 ± 0.03	1.18 ± 0.04
BDF 8933	8.72 ± 0.25	8.91 ± 0.60
	1.05 ± 0.05	1.04 ± 0.05
Prazosin	5.62 ± 0.40	7.12 ± 0.17
	0.99 ± 0.04	1.11 ± 0.05
ARC 239	5.45 ± 0.04	6.76 ± 0.13
	1.04 ± 0.05	1.18 ± 0.04
Yohimbine	8.04 ± 0.25	7.93 ± 0.07
	0.98 ± 0.04	1.07 ± 0.06
SKF 104078	6.52 ± 0.54	6.25 ± 0.06
	0.83 ± 0.03	1.10 ± 0.05
Urapidil	5.52 ± 0.76	5.72 ± 0.32
	1.04 ± 0.08	1.10 ± 0.04
Phentolamine	7.61 ± 0.28	7.24 ± 0.50
	0.93 ± 0.06	1.04 ± 0.03
Idazoxan	7.30 ± 0.37	7.49 ± 0.23
	0.98 ± 0.11	0.99 ± 0.04

Values are mean and 95% confidence limits (K_i values) or mean ± s.e.mean (Hill slopes) from at least 3 experiments.

Discussion

In this study we present evidence for differences between prejunctional α_2-adrenoceptors of rat atrium and vas deferens and correlate these subtypes of receptor with ligand binding sites. Before discussing these two points it is first necessary to discuss methodologies employed.

The isometric twitch evoked by single electrical pulse stimulation in prostatic portions of rat vas deferens is non-adrenergic, non-cholinergic and probably involves adenosine 5'-triphosphate (ATP) (Blakely *et al.*, 1981; Brown *et al.*, 1983). This twitch response is unaffected postjunctionally by most α_1- or α_2-adrenoceptor antagonists (see present results) and is relatively resistant to pharmacological intervention, except by calcium entry blockers which abolish the response (see Blakely *et al.*, 1981). The twitch response is therefore suitable for the examination of the prejunctional interaction between α_2-antagonists and agonists. However, α_1-adrenoceptor agonist drugs act postjunctionally to produce intermittent contractions of the vas deferens and to potentiate the twitch response to a single stimulus. Hence, it is important to choose an α_2-adrenoceptor agonist which has little or no effect postjunctionally in this tissue, and in our studies with rat vas deferens xylazine has proven to be the most suitable agonist. In contrast, oxymetazoline produces contractions of the tissue (see Results) and in previous studies clonidine was found to produce contractions especially in tissues from aged rats (Hyland & Docherty, 1985). Seven of the ten antagonists employed in this study behaved competitively against xylazine, as judged by slopes of Schild plots, 1 drug (chlorpromazine) could only be used in 2 concentrations, and the 2 drugs which did not behave competitively (prazosin and ARC 239) admittedly showed low potency. These latter 3 drugs were those which distinguished between vas and atrium (see below).

In rat isolated atria, a convenient measure of drug action is the ability to potentiate simulation-evoked release of tritium in tissues pre-incubated with [^{3}H]-noradrenaline. We have assumed that the EC_{30} values obtained do reflect potency at α_2-adrenoceptors, although other actions such as stimulation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) production can increase stimulation-evoked release (see Musgrave *et al.*, 1987). However, these effects occurred at concentrations which correlated with potency or affinity in one or more other tissues. Secondly, since differences were found for some agents between pA_2 in rat vas deferens and EC_{30} in rat atrium, EC_{30} values from overflow studies were carried out in

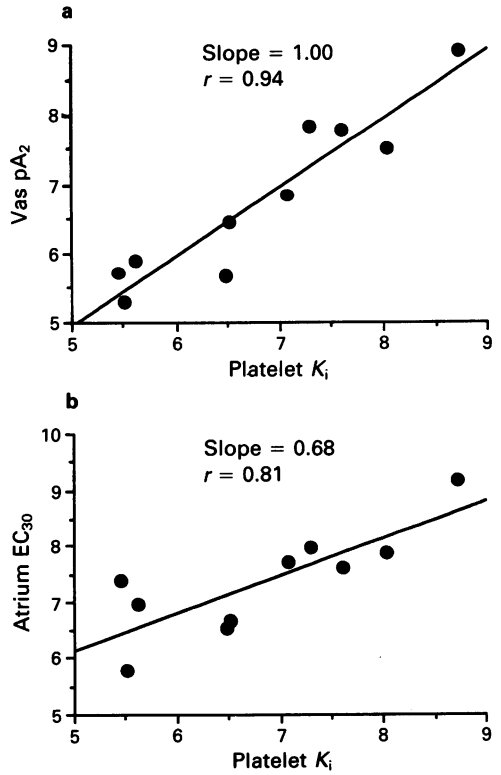

Figure 1 Correlation between K_i (−log M) obtained at α_{2A}- binding sites in human platelet membranes, and the antagonist pA_2, K_B or EC_{30} obtained at prejunctional α_2-adrenoceptors in rat vas deferens (a) or rat atrium (b).

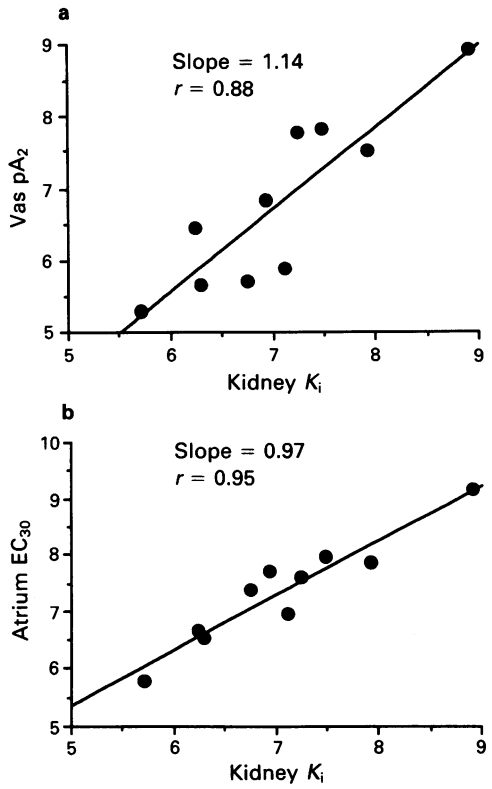

Figure 2 Correlation between K_i (−log M) obtained at α_{2B}- binding sites in rat kidney membranes, and the antagonists pA_2, K_B or EC_{30} obtained at prejunctional α_2-adrenoceptors in rat vas deferens (a) or rat atrium (b).

rat vas deferens to confirm that these differences were not due to methodological differences (see below).

The evidence for differences between the prejunctional α_2-adrenoceptors of rat atrium and vas deferens can be discussed under two headings: examination of the actions of individual antagonists and correlation analysis. In functional studies, three antagonists distinguished between the prejunctional α_2-adrenoceptors of rat atrium and vas deferens. ARC 239, prazosin and chlorpromazine were 49, 12 and 7 times, respectively more potent in rat atrium in terms of EC_{30} than in rat vas deferens in terms of pA_2. This difference was maintained when responses in both rat atrium and rat vas deferens were expressed as EC_{30} values, with ARC 239, prazosin and chlorpromazine 17, >3 and >3 times more potent, respectively in rat atrium, whereas yohimbine and BDF 8933 did not distinguish between atrium and vas deferens in terms of EC_{30} values. ARC 239 and prazosin had 20 and 31 times higher affinity at α_{2B}- than α_{2A}-sites in our binding studies, although chlorpromazine did not distinguish between these two tissues. Other authors have demonstrated that ARC 239, prazosin and chlorpromazine have 55, 50 and 24 times higher affinity for α_{2B}- than α_{2A}-receptors (Bylund *et al.*, 1988), and that prazosin and chlorpromazine have 78 and 16 times higher affinity for α_{2B}- than α_{2A}-receptors (Michel *et al.*, 1989a). Hence, the differences between the prejunctional α_2-adrenoceptors of rat vas deferens and atrium are similar to the differences between the α_{2A}- and α_{2B}-ligand binding sites. Other authors have found that prazosin has relatively high potency at the prejunctional α_2-adrenoceptors of rat submandibular gland (Turner *et al.*, 1984), rat cerebral cortex (Nasseri & Minneman, 1987) and rabbit pulmonary artery (Kapocsi *et al.*, 1987), suggesting that the prejunctional α_2-adrenoceptors in these tissues may resemble the α_{2B}-ligand binding site. The low potency of prazosin prejunctionally at the cholinergic nerves of guinea-pig ileum (Kapocsi *et al.*, 1987) suggests that this receptor may resemble the α_{2A}-ligand binding site.

In terms of correlation analysis, it should be remembered that most of the antagonists examined in this study did not distinguish between subtypes of α_2-adrenoceptor, so that a correlation even between the α_{2A}-binding site of human platelet and the α_{2B}-binding site of rat kidney had an r value of 0.81. However, the correlation between affinity for the α_{2A}-binding site of human platelet and antagonist pA_2 in rat vas deferens was better both in terms of r value (0.94) and slope (1.00) than with antagonist EC_{30} in rat atrium ($r = 0.81$, slope = 0.68). This suggests that the prejunctional α_2-adrenoceptor of rat vas deferens resembles the α_{2A}-binding site of human platelet, but that the prejunctional α_2-adrenoceptor of rat atrium is clearly dissimilar to the α_{2A}-binding site. The correlation between affinity for the α_{2B}-binding site of rat kidney and the antagonist EC_{30} in rat atrium was better both in terms of r value (0.95) and slope (0.97) than with antagonist pA_2 in rat vas deferens ($r = 0.88$, slope = 1.14). This suggests that the prejunctional α_2-adrenoceptor of rat atrium resembles the α_{2B}-binding site of rat kidney, but that the prejunctional α_2-adrenoceptor of rat vas deferens is clearly dissimilar to the α_{2B}-binding site.

A third subtype of α_2-ligand binding site, α_{2C}, has, like α_{2B}, high absolute affinity for ARC 239 and prazosin but differs from the α_{2B}-receptor in the affinities of these antagonists relative to yohimbine (Murphy & Bylund, 1988). In binding studies, ARC 239 and prazosin had 1.5 and 5 times (Bylund *et al.*, 1988) lower affinity than yohimbine at α_{2B}-receptors, whereas they had 43 and 41 times (Murphy & Bylund, 1988) lower affinity at α_{2C}-receptors, respectively. In our functional studies of rat atrium, ARC 239 and prazosin were 3 and 8 times less potent than yohimbine, which may suggest that this receptor is the α_{2B} rather than α_{2C} subtype, although further characterization is necessary. In rat vas deferens, ARC 239 and prazosin were 61 and 42 times less potent than yohimbine respectively (present results) as compared with 389 and 263 (present results) or 87 and 269 (Bylund *et al.*, 1988) times lower affinity, respectively at α_{2A}-binding sites.

Although it is difficult to make correlations between functional data from this study and published ligand binding data from other laboratories, due to the limited number of antagonists common to each study, there were good correlations between antagonist pA_2 in rat vas deferens and published K_1 values at α_{2A}-sites with r values of 0.95 (versus Bylund *et al.*, 1988) and 0.80 (versus Michel *et al.*, 1989a) ($n = 6$ antagonists in each case). For antagonist EC_{30} values in rat atrium the correlation was better for the α_{2C} than α_{2B}-site of Bylund *et al.* (1988) (r values of 0.88 and 0.65, respectively), and for the unclassified α_2-subtype of rat submaxillary gland than for the α_{2B}-site of Michel *et al.* (1989a) (r values of 0.92 and 0.58, respectively) (in all cases $n = 6$).

In this study we have made the assumption that the prejunctional α_2-adrenoceptors of rat atrium and vas deferens represent different but homogeneous populations of receptors. Oxymetazoline has been shown to be an α_{2A}-selective agent (Bylund, 1988), but the interaction of oxymetazoline with ARC 239 in rat vas deferens, like the interaction of xylazine with ARC 239, was non-competitive. The non-competitive interaction between ARC 239 and both agonists in rat vas deferens may reflect the fact that, in the relatively high concentrations employed, ARC 239 has other effects on the vas deferens including potentiation of the isometric response to a single stimulus (see Results). All that can be said with certainty is that the predominant receptor subtypes in these tissues are different, so we are able to demonstrate differences between tissues.

In conclusion, the prejunctional α_2-adrenoceptor of rat atrium differs from that of the rat vas deferens. The prejunctional α_2-adrenoceptor of rat vas deferens strongly resembles the α_{2A}-ligand binding site of human platelet. The prejunctional α_2-adrenoceptor of rat atrium resembles the α_{2B}-ligand binding sites of rat kidney, but may also resemble the α_{2C}-ligand binding site (Bylund, 1988).

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References

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BLAKELY, A.G.H., BROWN, D.A., CUNNANE, T.C., FRENCH, A.M., McGRATH, J.C. & SCOTT, N.C. (1981). Effects of nifedipine on electrical and mechanical responses of rat and guinea-pig vas deferens. *Nature*, **294**, 759–761.

BROWN, D.A., DOCHERTY, J.R., FRENCH, A.M., MacDONALD, A., McGRATH, J.C. & SCOTT, N.C. (1983). Separation of adrenergic and non-adrenergic contractions to field stimulation in the rat vas deferens. *Br. J. Pharmacol.*, **79**, 379–393.

BUCKLEY, C., CURTIN, D., WALSH, T. & O'MALLEY, K. (1986). Ageing and platelet α_2-adrenoceptors. *Br. J. Clin. Pharmacol.*, **21**, 721–722.

BYLUND, D.B. (1985). Heterogeneity of alpha-2 adrenergic receptors. *Pharmacol. Biochem. Behav.*, **22**, 835–843.

BYLUND, D.B. (1988). Subtypes of α_2-adrenoceptors: Pharmacological and molecular biological evidence converge. *Trends Pharmacol. Sci.*, **9**, 356–361.

BYLUND, D.B., RAY-PRENGER, C. & MURPHY, T.J. (1988). Alpha-2A and alpha-2B adrenergic receptor subtypes: antagonist binding in tissues and cell lines containing only one subtype. *J. Pharmacol. Exp. Ther.*, **245**, 600–607.

CHEUNG, Y.-D., BARNETT, D.B. & NAHORSKI, S.R. (1982). ^{3}H-Rauwolscine and ^{3}H-yohimbine binding to rat cerebral and human platelet membranes: evidence for possible heterogeneity of alpha$_2$ adrenoceptors. *Eur. J. Pharmacol.*, **84**, 79–85.

CONNAUGHTON, S. & DOCHERTY, J.R. (1990). No evidence for diffences between pre- and postjunctional α_2-adrenoceptors in the periphery. *Br. J. Pharmacol.*, **99**, 97–102.

DOCHERTY, J.R. (1989). The pharmacology of α_1 and α_2 adrenoceptors: evidence for and against a further subdivision. *Pharmacol. Ther.*, **44**, 241–284.

HYLAND, L. & DOCHERTY, J.R. (1985). Further examination of the effects of ageing on the adrenoceptor responsiveness of the rat vas deferens. *Eur. J. Pharmacol.*, **111**, 241–246.

KAPOCSI, J., SOMOGYI, G.T., LUDVIG, N., SERFOZO, P., HARSING, L.G., WOODS, R.J. & VIZI, E.S. (1987). Neurochemical evidence for two types of presynaptic alpha$_2$-adrenoceptors. *Neurochem. Res.*, **12**, 141–147.

MICHEL, A.D., LOURY, D.N. & WHITING, R.L. (1989a). Differences between the α_2-adrenoceptor in rat submaxillary gland and the α_{2A}- and α_{2B}-adrenoceptor subtypes. *Br. J. Pharmacol.*, **98**, 890–897.

MICHEL, M.C., BRODDE, O.-E., SCHNEPEL, B., BEHRENDT, J., TSCHADA, R., MOTULSKY, H.J. & INSEL, P.A. (1989b). [^{3}H] idazoxan and some other α_2-adrenergic drugs also bind with high affinity to a nonadrenergic site. *Molec. Pharmacol.*, **35**, 324–330.

MURPHY, T.J. & BYLUND, D.B. (1988). Characterization of alpha-2 adrenergic receptors in the OK Cell, an opossum kidney cell line. *J. Pharmacol. Exp. Ther.*, **244**, 571–578.

MUSGRAVE, I., MARLEY, P. & MAJEWSKI, H. (1987). Pertussis toxin does not attenuate α_2-adrenoceptor mediated inhibition of noradrenaline release in mouse atria. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **336**, 280–286.

NAHORSKI, S.R., BARNETT, D.B. & CHEUNG, Y.-D. (1985). α-Adrenoceptor-effector coupling: affinity states or heterogeneity of the α_2-adrenoceptor? *Clin. Sci.*, **68**, (Suppl. 10), 39s-42s.

NASSERI, A. & MINNEMAN, K.P. (1987). Relationship between α_2-adrenergic receptor binding sites and the functional receptors inhibiting norepinephrine release in rat cerebral cortex. *Mol. Pharmacol.*, **32**, 655–662.

NEYLON, C.B. & SUMMERS, R.J. (1985). [^{3}H]-rauwolscine binding to α_2-adrenoceptors in the mammalian kidney: apparent receptor heterogeneity between species. *Br. J. Pharmacol.*, **85**, 349–359.

REGAN, J.W., KOBILKA, T.S., YANG-FENG, T.L., CARON, M.G., LEFKOWITZ, R.J. & KOBILKA, B.K. (1988). Cloning and expression of a human kidney cDNA for an α-adrenergic receptor subtype. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 6301–6305.

SNEDECOR, G.W. & COCHRAN, W.G. (1980). *Statistical Methods*, 7th ed. Ames, Iowa: Iowa State University Press.

TURNER, J.T., PIERCE, D.L. & BYLUND, D.B. (1984). Alpha-2 adrenergic regulation of norepinephrine release in the rat submandibular gland as measured by HPLC-EC. *Life Sci.*, **35**, 1385–1394.

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High- and low-affinity binding sites for [^{3}H]-α,β-methylene ATP in rat urinary bladder membranes

Xuenong Bo & [1]Geoffrey Burnstock

Department of Anatomy and Developmental Biology and Centre for Neuroscience, University College London, Gower Street, London WC1E 6BT

1 The characteristics of [^{3}H]-α,β-methylene adenosine 5'-triphosphate ([^{3}H]-α,β-MeATP) binding to membrane preparations of rat urinary bladder detrusor were studied.

2 The rat bladder membrane preparation was obtained by multiple centrifugation. [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding to this preparation demonstrated that the muscarinic receptor density was 4.32 times higher than that in the homogenate. [^{3}H]-α,β-MeATP binding was increased 3.88 times.

3 Saturation analysis revealed that the rat bladder membrane contained a high density of [^{3}H]-α,β-MeATP binding sites, which could be divided into a high-affinity component (K_d = 8.1–8.9 nM) and a low-affinity component (K_d = 67.0–119.8 nM).

4 Magnesium ions inhibited the maximum binding in a concentration-dependent manner. The maximum high-affinity binding was reduced from 10.32 pmol mg^{-1} protein in magnesium-free buffer to 4.62 pmol mg^{-1} protein with 25 mM $MgCl_2$, while the maximum low-affinity binding was reduced from 58.84 pmol mg^{-1} protein to 14.24 pmol mg^{-1} protein. K_d values were not greatly affected.

5 The binding was a rapid reversible process. The association rate constants were 7.64 × 10^7 M^{-1} min^{-1} for high-affinity binding, and 7.31 × 10^6 M^{-1} min^{-1} for low-affinity binding. The dissociation rate constants were 0.2896 min^{-1} for high-affinity binding, and 0.6348 min^{-1} for the low-affinity binding.

6 Displacement experiments with unlabelled purinoceptor ligands confirmed that [^{3}H]-α,β-MeATP mainly binds to P_{2X}-purinoceptors. The potency order was: α,β-methylene ATP > β,γ-methylene ATP > suramin > ATP > ADP > 2-methylthio ATP ≫ adenosine.

7 The results indicate that [^{3}H]-α,β-MeATP is a radioligand for the P_{2X}-purinoceptor, which satisfies the basic criteria for use in radioligand binding assay.

Introduction

Amongst the growing literature on purinergic nerve transmission, there are many studies of mammalian urinary bladder. Excitatory purinergic responses have been reported in bladders of rabbit (Dean & Downie, 1978), guinea-pig (Burnstock *et al.*, 1978), rat (Burnstock *et al.*, 1972), mouse (Acevedo & Contreras, 1985), ferret and marmoset (Moss & Burnstock, 1985), and cat (Theobald, 1983). In human isolated bladder strips adenosine 5'-triphosphate (ATP) can induce concentration-dependent contractions (Husted *et al.*, 1983; Hoyle *et al.*, 1989). The atropine-resistant residuals of the electrical field stimulation-elicited contractions in the human bladder strips can be abolished following desensitization with α,β-methylene ATP (α,β-MeATP) (Hoyle *et al.*, 1989). The purinoceptors which mediate the exitatory mechanical responses of mammalian bladders to field stimulation, ATP and its analogues were classified as of the P_{2X}-subtype (Burnstock & Kennedy, 1985; Hourani *et al.*, 1985).

Radioligand binding assay and autoradiography of P_1-purinoceptors (A_1 and A_2 adenosine receptors) have been carried out for many years (Schwabe, 1985), and a series of radioligands are commercially available. In 1983 Levin *et al.* used [^{3}H]-ATP to label its binding sites in rabbit urinary bladder and reached the conclusion that the binding sites are not related to ATPase activity (Levin *et al.*, 1983). However, the rapid degradation of [^{3}H]-ATP limits its use in the characterization of P_2-purinoceptors. Specific binding sites for adenosine 5'-[α-[^{35}S]thio]triphosphate (ATPα[^{35}S]) have been observed on rat hepatocytes and purified liver plasma membranes (Keppens & De Wulf, 1986) and human liver plasma membranes (Keppens *et al.*, 1989). Competition experiments suggest that the binding sites are P_{2Y}-purinoceptors. The main disadvantage of this radioligand is the existence of the nonsaturable low-affinity binding site. Adenosine-5'-O-2-thio [^{35}S]-diphosphate ([^{35}S]-ADPβS) was reported as a radioligand for P_{2Y}-purinoceptors on turkey erythrocyte membrane (Cooper *et al.*, 1989). However, as was pointed out in that paper, [^{35}S]-ADPβS also has intracellular binding sites, which are unlikely to be P_{2Y}-purinoceptors. Therefore, this radioligand may not be suitable for the autoradiographic localization of P_{2Y}-purinoceptors. Recently we showed that [^{3}H]-α,β-MeATP is a radioligand for P_2-purinoceptors (preferentially for P_{2X}-purinoceptors) (Bo & Burnstock, 1989). In this paper we present a detailed study of the binding of [^{3}H]-α,β-MeATP to the membrane preparation of rat urinary bladder. The methods used in the first paper have been modified and extended.

Methods

Preparation of rat urinary bladder membranes

Male Wistar rats (200–250 g) were killed by cervical dislocation. Urinary bladders were removed immediately and the adipose tissue and serosa were trimmed off. The bladder was minced and homogenized in 3 ml 50 mM Tris-HCl buffer (pH 7.40, 20°C). The homogenate was centrifuged at 170 *g* for 4 min. The supernatant was drawn off and saved. The pellet was resuspended in 3 ml buffer, rehomogenized and recentrifuged as above. The supernatant was collected. The procedure was repeated four times. The supernatant was then centrifuged at 165,000 *g* for 1 h in a MSE Europa M-50 Ultracentrifuge. The supernatant was discarded and the pellet was suspended in 2 ml buffer for further dilution according to need. The harvest of crude membrane protein was 0.5 to 0.75 mg per bladder (70 to 76 mg wet weight). Protein concentration was determined by the method of Lowry *et al.* (1951).

[1] Author for correspondence.

Estimation of membrane purity by [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding compared with [^{3}H]-α,β-MeATP binding

Two rat bladders were homogenized in 50 mM Tris-HCl buffer (containing 25 mM $MgCl_2$, pH 7.40). Two-thirds of the homogenate was centrifuged four times at 170*g* and the collected supernatant was then centrifuged at 165,000*g* for 1 h. The homogenate, supernatant and the pellet suspension from the ultracentrifugation were used for receptor binding. [^{3}H]-QNB (50 μl, final concentration ranging from 0.025 to 1.5 nM) was mixed with 50 μl Tris-HCl buffer and 400 μl homogenate, supernatant, or pellet suspension. To determine the non-specific binding, 50 μl atropine (10 μM) was added instead of 50 μl buffer. The incubation was carried out at 30°C for 30 min. The reaction was terminated by quick filtration through double layers of Whatman GF/C glass fibre filters. The filters were washed three times with 12 ml ice-cold buffer, soaked in 8 ml scintillation cocktail (OptiPhase MP, LKB) for 6 h, then the trapped radioactivity was measured in a Beckman LS 7500 scintillation counter with an efficiency of 46% to 50%.

In order to compare the binding of [^{3}H]-QNB and [^{3}H]-α,β-MeATP, parallel experiments were carried out on the binding of [^{3}H]-α,β-MeATP to the bladder homogenate, the supernatant and pellet suspension obtained from ultracentrifugation. The basic protocol is as described below with the exception that this experiment was done in the Tris-HCl buffer with 25 mM $MgCl_2$.

Saturation analysis and the effects of magnesium ions on [^{3}H]-α,β-MeATP binding

In the preliminary experiment we found that the magnesium ions in the incubation solution inhibited the [^{3}H]-α,β-MeATP binding. Therefore, parallel binding experiments were done in four kinds of incubation solutions containing either zero, 2 mM, 10 mM, or 25 mM $MgCl_2$. For one experiment the membrane preparations (in magnesium-free Tris-HCl buffer) from four rat bladders were mixed, and then divided into four 2 ml portions. Each portion was diluted to 13 ml with one of the four Tris-HCl buffer solutions containing zero, 2.95 mM, 14.8 mM, or 36.9 mM $MgCl_2$. Protein concentration was around 50 μg ml^{-1}. Stock solutions of [^{3}H]-α,β-MeATP and β,γ-methylene ATP (β,γ-MeATP) were prepared with magnesium-free Tris-HCl buffer. The tubes for total binding contained 50 μl [^{3}H]-α,β-MeATP (final concentration from 1.25 to 160 nM), 50 μl magnesium-free buffer and 400 μl membrane preparation. In the tubes for non-specific binding, 50 μl β,γ-MeATP (final concentration 100 μM) was added instead of 50 μl buffer. The incubation was carried out at 30°C for 15 min. The reaction was terminated by vacuum filtration through double layers of Whatman GF/C glass fibre filters. The filters were washed four times with 20 ml ice-cold buffer (with corresponding $MgCl_2$ concentration). The filters were put at the bottom of 20 ml scintillation vials and 8 ml of scintillation cocktail was added (OptiPhase MP, LKB). The samples were put aside for 6 h before measurements were taken. Data from 10 experiments were collected. Each experiment was carried out in duplicate.

Kinetics of [^{3}H]-α,β-MeATP binding

The plasma membrane was prepared in Tris-HCl buffer with 25 mM $MgCl_2$. For the measurement of the association rate, each tube contained 50 μl [^{3}H]-α,β-MeATP (final concentration 10 nM, in buffer with 25 mM $MgCl_2$), 50 μl buffer and 400 μl membrane preparation. The incubation was carried out at 30°C. The reaction was terminated at different times (from 5 s to 10 min) by rapid filtration under vacuum, followed by washing with 5 ml ice-cold buffer four times. For the measurement of dissociation rate the samples were prepared as above. They were incubated at 30°C for 15 min, and then 50 μl β,γ-MeATP (100 μM) was added to the mixture. The reaction was terminated at different times (from 15 s to 30 min).

The effects of purinoceptor ligands on the [^{3}H]-α,β-MeATP binding

Several compounds that have been shown by pharmacological experiments to be ligands for purinoceptors were chosen to displace the [^{3}H]-α,β-MeATP binding. [^{3}H]-α,β-MeATP (50 μl, final concentration 10 nM, in Tris-HCl buffer with 25 mM $MgCl_2$) was incubated with 400 μl membrane preparation in the presence of 50 μl non-labelled ligand. The concentration ranges of the non-labelled ligands were as follows: α,β-MeATP, 10^{-9}–10^{-4} M; β,γ-MeATP, 10^{-9}–10^{-4} M; suramin, 10^{-9}–10^{-4} M; 2-methylthio ATP (2-MeSATP), 10^{-9}–10^{-4} M; ATP, 10^{-9}–10^{-3} M; ADP, 10^{-9}–10^{-3} M; and adenosine, 10^{-9}–10^{-3} M. The incubation was carried out at 30°C for 15 min.

Other factors that could influence binding

The effect of pH on the [^{3}H]-α,β-MeATP binding was tested. [^{3}H]-α,β-MeATP (50 μl, 10 nM) was incubated with 400 μl membrane preparation and 50 μl buffer at 30°C for 15 min. The stock solution of [^{3}H]-α,β-MeATP and the membrane were prepared in four kinds of 50 mM Tris-HCl buffer (25 mM $MgCl_2$) with pH values of 6.0, 7.0, 7.5, 8.0, 9.0.

Some radioligands can bind to filters and can be partially displaced by a non-labelled ligand, which will produce false specific binding. The binding of [^{3}H]-α,β-MeATP to Whatman GF/C glass fibre filters was estimated. [^{3}H]-α,β-MeATP solutions (1.25–160 nM) with or without 100 μM β,γ-MeATP were filtered through double layers of GF/C filters, followed by washing with 5 ml ice-cold buffer four times.

Drugs

[^{3}H]-α,β-MeATP was synthesized by Amersham International plc. α,β-MeATP was tritiated by catalytic exchange. Specific activity was 27 Ci mmol^{-1} with chemical purity of 98–99%. [^{3}H]-QNB with specific activity of 44 Ci mmol^{-1} was purchased from the same company. α,β-MeATP, β,γ-MeATP, ATP, ADP, and adenosine were from Sigma Chemical Company Ltd. 2-MeSATP was from Research Biochemical Inc., U.S.A. Atropine sulphate was from Antigen Ltd. Suramin was a gift from ICI Pharmaceuticals, Alderly Park, Cheshire.

Results

The saturation analysis of [^{3}H]-QNB binding to rat bladder homogenate, the supernatant and pellet suspension from ultracentrifugation showed that the maximum specific binding was 112 ± 17 fmol mg^{-1} protein in the homogenate (K_d = 0.063 nM), 484 ± 37 fmol mg^{-1} protein in the pellet suspension (K_d = 0.054 nM). Only a trace amount of specific binding was observed in the supernatant. Thus, the muscarinic receptor density was increased 4.32 times by this membrane separation method. The maximum high-affinity specific binding of [^{3}H]-α,β-MeATP was 1235 ± 165 fmol mg^{-1} protein in the homogenate (K_d = 10.2 nM) and 4793 ± 385 fmol mg^{-1} protein in the pellet suspension (K_d = 8.5 nM). The density was therefore increased 3.88 times, which was a little lower than that for [^{3}H]-QNB binding. The reason for this may be that the supernatant also contained a small amount of specific [^{3}H]-α,β-MeATP binding (324 ± 57 fmol mg^{-1} protein).

The specific binding of [^{3}H]-α,β-MeATP to the rat bladder membrane preparation was saturable in all four concentrations of magnesium ion buffer tested. A typical binding curve (in the buffer with 25 mM $MgCl_2$) is shown in Figure 1. Scatchard analysis revealed the existence of two binding sites (Figure 2). The K_d values for the high-affinity binding varied between 8.1 and 8.9 nM, while for the low-affinity binding the

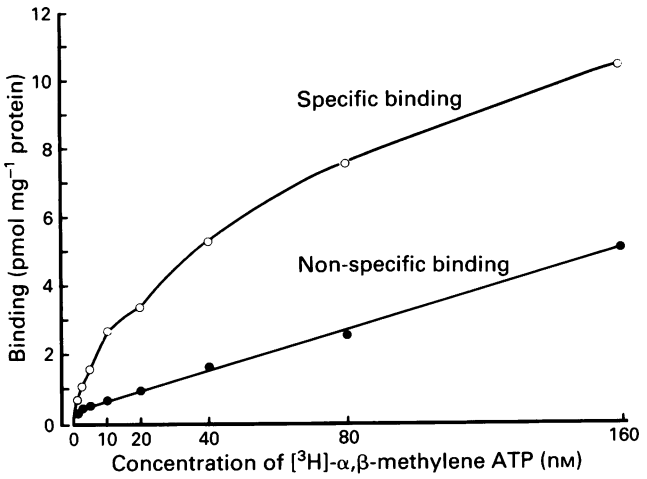

Figure 1 The saturation curve of [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP) binding to rat urinary bladder membrane preparation in 50 mM Tris-HCl buffer (pH 7.4) with 25 mM $MgCl_2$. Incubation was carried out at 30°C for 15 min. Non-specific binding (●) was determined by displacing the binding with 10 μM β,γ-MeATP. Each point represents the mean of 10 duplicated experiments.

range was 67.0 to 119.8 nM (Table 1). Magnesium ions had a significant inhibitory effect on the binding. The maximum high-affinity binding was 2.2 times higher in magnesium-free buffer than that in buffer with 25 mM $MgCl_2$, while the maximum low-affinity binding was 4.13 times higher in magnesium-free buffer than that in buffer with 25 mM $MgCl_2$. The K_d values for the high-affinity binding were slightly reduced with the increase of magnesium ion concentration in the buffer, but the K_d values for the low-affinity binding were reduced from 119.8 nM in the buffer with 2 mM $MgCl_2$ to 67 nM in the buffer with 25 mM $MgCl_2$. The non-specific binding was about 10% of the total binding at the lowest [^{3}H]-α,β-MeATP concentration and 50% at the highest [^{3}H]-α,β-MeATP concentration. Magnesium ions slightly increased the non-specific binding at lower [^{3}H]-α,β-MeATP concentrations (data not shown). The slopes of Hill plots showed that Hill coefficients (n_H) for all high- and low-affinity binding were around one (Table 1).

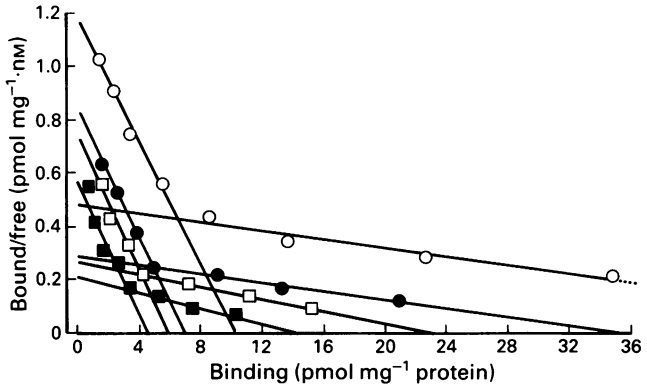

Figure 2 Scatchard plots of specific [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP) binding to rat urinary bladder membrane preparation in Tris-HCl buffer (pH 7.4) with zero (○), 2 mM (●), 10 mM (□), or 25 mM (■) $MgCl_2$. Each point represents the mean of 10 duplicated experiments. The B_{max} and K_d values are shown in Table 1.

At 30°C the binding of [^{3}H]-α,β-MeATP to the bladder plasma membrane preparation was a rapid process, which reached equilibrium in 10 min (Figure 3). Kinetic analysis showed that the process was composed of two components: a high-affinity and a low-affinity part (Figure 3, inset). The association rate constant of the high-affinity component was $7.64 \times 10^7\ M^{-1}\ min^{-1}$, and that of the low-affinity component was $7.31 \times 10^6\ M^{-1}\ min^{-1}$.

The binding of [^{3}H]-MeATP to bladder membrane was a reversible reaction. After the binding of 10 nM [^{3}H]-α,β-MeATP had reached equilibrium, the addition of a high concentration of β,γ-MeATP (100 μM) quickly displaced the bound [^{3}H]-α,β-MeATP (Figure 4). The dissociation process was also composed of two components: the dissociation rate constant was 0.2896 min^{-1} for the high-affinity binding, and 0.6348 min^{-1} for the low-affinity binding (Figure 4, inset). The half-life of the [^{3}H]-α,β-MeATP-receptor complex was 2.31 min for the high-affinity binding sites and 1.09 min for the low-affinity binding sites. Thus the dissociation process was also very rapid at 30°C. The K_d values calculated from the kinetic experiment were 3.8 and 86.8 nM, which were in fair agreement with the K_d values obtained from the saturation experiment.

All the unlabelled ligands tested showed inhibitory effects on the binding of [^{3}H]-α,β-MeATP to different degrees (Figure 5). The competition of the binding by the agonists and antagonists was in a concentration-dependent manner. According to the IC_{50} values (Table 2) α,β-MeATP was the most potent agent to displace the binding with an IC_{50} value of 91 nM, which was more than 20 fold higher than the competitive actions of β,γ-MeATP and suramin. However, β,γ-

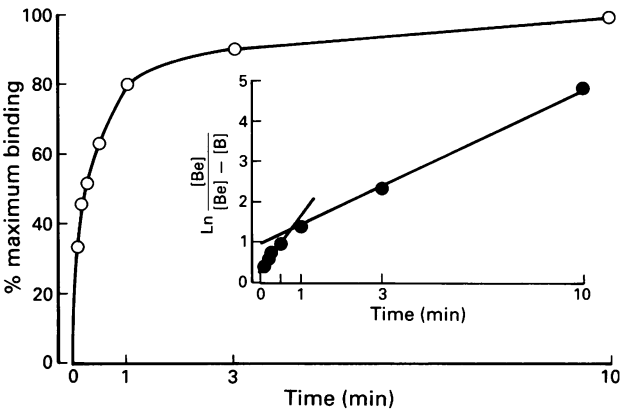

Figure 3 Association curve of [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP, 10 nM) binding to rat urinary bladder membrane preparation at 30°C. Inset shows the time course after the original data are transformed with pseudo-first-order rate equation for the calculation of association rate constants. Data from seven experiments are collected. [Be]: concentration of receptor-radioligand complex at equilibrium, [B]: concentration of receptor-radioligand complex at the time the reaction was terminated.

Table 1 The maximum binding (B_{max}), K_d values and Hill coefficients (n_H) of [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP) binding to rat urinary bladder membrane preparation at various magnesium ion concentrations (n = 7 to 10)

	High affinity			*Low affinity*		
	B_{max} (pmol mg^{-1} protein)	K_d (nM)	n_H	B_{max} (pmol mg^{-1} protein)	K_d (nM)	n_H
Mg^{2+}-free	10.32	8.9	0.99	58.84	112.7	1.00
2 mM $MgCl_2$	7.01	8.5	1.00	34.85	119.8	1.00
10 mM $MgCl_2$	5.97	8.3	1.00	23.68	91.7	1.01
25 mM $MgCl_2$	4.62	8.1	1.07	14.24	67.0	1.02

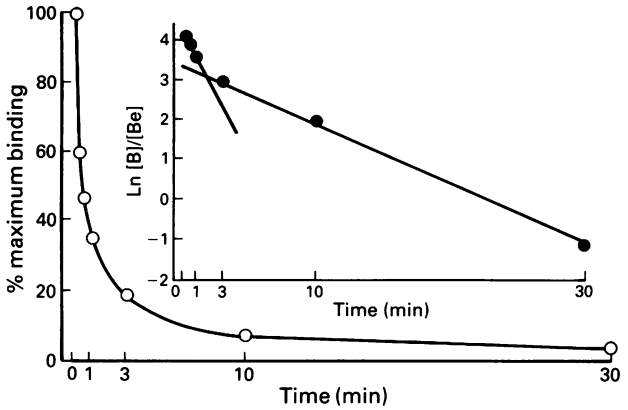

Figure 4 Dissociation curve of [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP, 10 nM) binding to rat urinary bladder membrane preparation at 30°C. β,γ-MeATP (10 μM) was used to displace the bound [^{3}H]-α,β-MeATP after the binding reached equilibrium. Inset shows the time course after the original data are transformed with the equation for dissociation rate constant. Data from seven experiments are collected. [Be]: concentration of receptor-radioligand complex at equilibrium, [B]: concentration of receptor-radioligand complex at the time the reaction was terminated.

MeATP and suramin were 16 fold more potent than ATP. The IC_{50} values of ATP, ADP and 2-MeSATP were in the same order, but from the concentration-effect curve it was estimated that even at higher concentrations 2-MeSATP could only partially displace the specific binding, while the actions of ATP and ADP were complete. Adenosine was the least potent agent among the ligands tested, even at a concentration of 1 mM, it could displace only 13% of the specific binding.

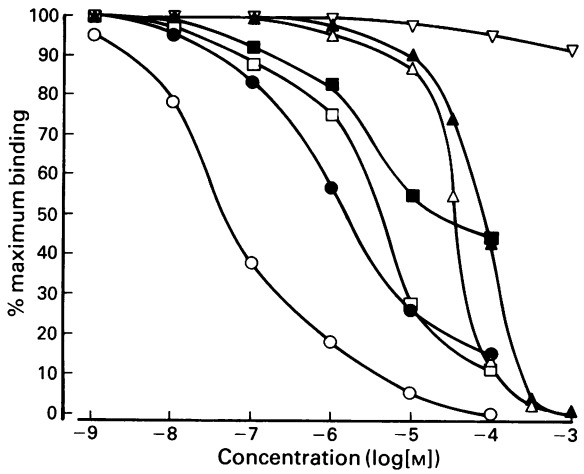

Figure 5 Displacement of [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP, 10 nM) binding to rat urinary bladder membrane preparation by unlabelled ligands (in Tris-HCl buffer with 25 mM $MgCl_2$): (○), α,β-MeATP; (●) β,γ-MeATP; (□) suramin; (■) 2-methylthio ATP (2-MeSATP); (△) ATP; (▲) ADP; (▽) adenosine. Each point represents the mean from nine experiments.

Table 2 The IC_{50} values of purinoceptor active compounds to displace the binding of [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP, 10 nM) to rat bladder membrane preparation ($n = 9$)

Ligands	*IC_{50} (μM)*
α,β-MeATP	0.091
β,γ-MeATP	1.96
Suramin	2.20
ATP	32.6
ADP	55.0
2-MeSATP	59.6
Adenosine	⩾1000 (13%)*

* In parentheses is the percentage of the displaced binding at the maximum tested concentration of adenosine.
2-MeSATP = 2-methylthio ATP.

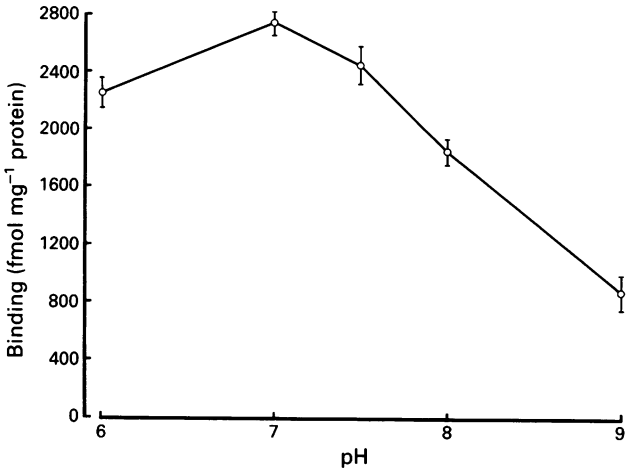

Figure 6 The effect of pH values of the incubation solution (50 mM Tris-HCl buffer with 25 mM $MgCl_2$) on the [^{3}H]-α,β-MeATP (10 nM) binding to rat urinary bladder membrane preparation. Data from six experiments are collected.

The effect of pH value on the [^{3}H]-α,β-MeATP binding is shown in Figure 6. The optimal pH value for the binding was around 7.0. It can be seen that an alkaline environment more profoundly inhibited the binding.

The binding of [^{3}H]-α,β-MeATP to Whatman GF/C glass fibre filters was very low, ranging from 0.14% (160 nM) to 0.22% (5 nM) of the total added radioactivity. No significant difference was observed between the binding with or without 100 μM β,γ-MeATP. Thus the interference of glass fibre filters on the [^{3}H]-α,β-MeATP binding can be excluded.

Discussion

Recently we reported in brief the discovery that [^{3}H]-α,β-MeATP can be used as a radioligand to label P_{2X}-purinoceptors (Bo & Burnstock, 1989). Because of the multiple functions of ATP in cell metabolism, we tried to develop a purified plasma membrane preparation to avoid the possibility of binding to sites on the intracellular organelles. It has been known that the separation of smooth muscle plasma membrane is more difficult than other kinds of tissues such as brain and liver (Batra, 1980). As to the urinary bladder, one successful separation of rabbit bladder membrane has been described (Batra, 1986). We modified the dextran-polyethylene glycol two-phase method (Brunette & Till, 1971) to purify the membrane of rat baldder. This method is very quick and does not need ultracentrifugation. The use of this kind of preparation had advantages over homogenate preparations for [^{3}H]-α,β-MeATP binding. However, the method is not as efficient in the separation of smooth muscle membranes as for L-cell membrane described in the original paper. In these experiments we used multiple centrifugation and harvested a purified plasma membrane. The [^{3}H]-QNB binding demonstrated that the muscarinic receptor density was increased by 4.32 times after the purification process, while the [^{3}H]-α,β-MeATP binding showed a similar increase. The parallel increase of binding sites of the two radioligands indicates that [^{3}H]-α,β-MeATP mainly binds to the proteins in the membrane and that the method is suitable to separate rat bladder membranes for use in radioligand binding assays.

In our first paper, saturation analysis showed only one [^{3}H]-α,β-MeATP binding site, with maximum binding of 3.52 pmol mg^{-1} protein and a K_d value of 58.2 nM in 50 mM Tris-HCl buffer with 25 mM $MgCl_2$. In fact, both the maximum binding and the K_d value were combined results of

two components. In the present experiments a purer membrane preparation was used and the concentration range of the radioligand was extended, so that the high- and low-affinity binding sites could be distinguished. The ratios of K_d values of the low- and high-affinity binding were around 8 to 14 fold. This phenomenon was common when agonists were used as the radioligands. In the radioligand binding assay of P_1-purinoceptors, [^{3}H]-(−)N^6-phenylisopropyladenosine ([^{3}H]-PIA) showed two binding sites in rat fat cell membrane, while [^{3}H]-^{5}N-ethylcarboxamidoadenosine ([^{3}H]-NECA) had two binding sites in human platelet membrane (Schwabe, 1985). In the experiments with [^{3}H]-ATP binding to rabbit bladder homogenate, two binding sites were also observed with K_d values of 20 nM and 45 nM for high- and low-affinity binding sites, respectively, which were not very far from our results.

Magnesium ions had profound effects on [^{3}H]-α,β-MeATP binding. With increase in magnesium ion concentration, both the high- and the low-affinity maximum binding were decreased. However, magnesium ions had only a slight effect on the K_d values for high-affinity binding, while the K_d values for the low-affinity binding showed larger increases at high magnesium concentration. The regulatory effects of magnesium ions and several other divalent ions such as manganese and calcium on the binding of P_1-purinoceptors have been known for many years (Goodman *et al.*, 1982; Hüttemann *et al.*, 1984). In the binding experiments at A_1-adenosine receptors, magnesium ions decreased the binding of [^{3}H]-N^6-cyclohexyladenosine ([^{3}H]-CHA) to rat fat cell membrane in the absence of guanine nucleotide; the binding was increased by magnesium ions in the presence of guanine nucleotide (Cooper *et al.*, 1984). Recently the study on P_{2Y}-purinoceptor binding with [^{35}S]-ADPβS indicated that without magnesium the high-affinity binding was almost completely lost (Cooper *et al.*, 1989). The binding of [^{3}H]-ATP to rabbit bladder homogenate also showed that a large proportion of the binding sites was magnesium-dependent (Levin *et al.*, 1983). The results from our experiment demonstrate that magnesium can modulate the P_{2X}-purinoceptor molecules and make the receptors lose their ability to bind to their ligands. The effect of guanine nucleotide on the [^{3}H]-α,β-MeATP binding has not yet been tested. Further investigation may reveal the relationship between P_{2X}-purinoceptor and G-protein, and may give more insight into the molecular mechanism of P_{2X}-purinoceptor action.

The existence of two binding sites for [^{3}H]-α,β-MeATP in rat bladder membrane was confirmed by the kinetic experiment. After the transformation of the original data with special equations, both the association and dissociation processes still demonstrated a curvilinear time-course, which could be separated into two components by curve-fitting. The K_d values derived from the kinetic experiment were similar to those from the saturation experiment, which implies that the binding may be a simple biomolecular reaction.

Both the association and dissociation processes were very rapid, but the [^{3}H]-α,β-MeATP-receptor complex lasted long enough to undergo vacuum filtration and the subsequent washing. The whole operation of filtration separation only took 12 s, so it can be estimated that the loss of the binding during separation was trivial.

The displacement experiment proved the specificity of [^{3}H]-α,β-MeATP binding to P_{2X}-purinoceptors. The potency order of the unlabelled ligands in displacing the [^{3}H]-α,β-MeATP binding is: α,β-MeATP > β,γ-MeATP > suramin > ATP > ADP > 2-MeSATP $\gg$ adenosine, which is in good agreement with the potency order of these ligands in their pharmacological actions on P_{2X}-purinoceptors (Burnstock & Kennedy, 1985). P_{2Y}-purinoceptor binding showed a different potency order: 2-MeSATP > ADPβS > ATP > ADP > App(NH)p > α,β-MeATP > β,γ-MeATP (Cooper *et al.*, 1989). This is also in accordance with the criteria for the classification of P_{2Y}-purinoceptors. It should be mentioned that the present displacement experiments were carried out at a radioligand concentration of 10 nM, which approximated to the K_d values for the high-affinity binding, thus the obtained potency order of the cold ligands probably represents their affinities to the high-affinity [^{3}H]-α,β-MeATP binding sites.

Suramin is a trypanocidal drug which was introduced into therapy in 1920. Recently it was reported that suramin could antagonize the response of mouse vas deferens to α,β-MeATP, and it was suggested as a specific antagonist for P_2-purinoceptors (Dunn & Blakeley, 1988). Our results confirm its affinity for P_{2X}-purinoceptors. However, pharmacological results from our laboratory show that suramin is also an antagonist of P_{2Y}-purinoceptors with an equal affinity (Hoyle *et al.*, 1990) so the action of suramin may not be specific.

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References

ACEVEDO, C.G. & CONTRERAS, R. (1985). Possible involvement of adenine nucleotides in the neurotransmission of the mouse urinary bladder. *Comp. Biochem. Physiol.*, **82C**, 357–361.

BATRA, S. (1980). Discussion summary: cell fractionation. In *Vascular Neuroeffector Mechanisms*. ed. Bevan, J.A., Godfraind, T., Maxwell, R.A. & Vanhoutte, P.M. pp. 9–12. New York: Raven Press.

BATRA, S. (1986). Use of ^{3}H-QNB in the isolation of plasma membrane from smooth muscle of the urinary bladder: effect of oxalate in calcium uptake by the membrane fractions. *Experientia*, **42**, 608–611.

BO, X. & BURNSTOCK, G. (1989). [^{3}H]-α,β-methylene ATP, a radioligand labelling P_2-purinoceptors. *J. Auton. Nerv. Syst.*, **28**, 85–88.

BRUNETTE, D.M. & TILL, J.E. (1971). A rapid method for the isolation of L-cell surface membranes using an aqueous two-phase polymer system. *J. Membrane Biol.*, **5**, 215–224.

BURNSTOCK, G., DUMSDAY, B. & SMYTHE, A. (1972). Atropine resistant excitation of the urinary bladder: the possibility of transmission via nerves releasing a purine nucleotide. *Br. J. Pharmacol.*, **44**, 451–461.

BURNSTOCK, G., COCKS, T., CROWE, R. & KASAKOV, L. (1978). Purinergic innervation of the guinea-pig urinary bladder. *Br. J. Pharmacol.*, **63**, 125–138.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.*, **5**, 433–440.

COOPER, C.L., MORRIS, A.J. & HARDEN, T.K. (1989). Guanine nucleotide-sensitive interaction of a radiolabeled agonist with a phospholipase C-linked P_{2Y}-purinergic receptor. *J. Biol. Chem.*, **264**, 6202–6206.

COOPER, D.M.F., YEUNG, S-M.H., PEREZ-REYES, E. & FOSSOM, L.H. (1984). A central role for magnesium in the regulation of inhibitory adenosine receptors. In *Neurotransmitter Receptors, Mechanisms of Action and Regulation*. ed. Kito, K., Segawa, T., Kuriyama, K., Yamamura, H.I. & Olsen, R.W. pp. 17–30. New York: Plenum Press.

DUNN, P.M. & BLAKELEY, A.G.H. (1988). Suramin: a reversible P_2-purinoceptor antagonist in the mouse vas deferens. *Br. J. Pharmacol.*, **93**, 243–245.

DEAN, D.M. & DOWNIE, J.W. (1978). Contribution of adrenergic and "purinergic" neurotransmission to contraction in rabbit detrusor. *J. Pharmacol. Exp. Ther.*, **207**, 431–445.

GOODMAN, R.R., COOPER, M.J., GAVISH, M. & SNYDER, S. (1982). Guanine nucleotide and cation regulation of the binding of [^{3}H]-cyclohexyladenosine and [^{3}H]-diethylphenylxanthine to adenosine A_1 receptors in brain membranes. *Mol. Pharmacol.*, **21**, 329–335.

HOURANI, S.M.O., WELFORD, L.A. & CUSACK, N.J. (1985). L-AMP-PCP, an ATP receptor agonist in guinea-pig bladder is inactive on taenia coli. *Eur. J. Pharmacol.*, **108**, 197–200.

HOYLE, C.H.V., CHAPPLE, C. & BURNSTOCK, G. (1989). Isolated human bladder: evidence for an adenine dinucleotide acting on P_{2X}-purinoceptors and for purinergic transmission. *Eur. J. Pharmacol.*, **174**, 115–118.

HOYLE, C.H.V., KNIGHT, G.E. & BURNSTOCK, G. (1990). Suramin antagonizes responses to P_2-purinoceptor agonists and purinergic nerve stimulation in the guinea-pig urinary bladder and taenia coli. *Br. J. Pharmacol.*, **99**, 617–621.

HUSTED, S., SJÖGREN, C. & ANDERSSON, K-E. (1983). Direct effects of adenosine and adenine nucleotides on isolated human urinary bladder and their influence on electrically induced contractions. *J. Urol.*, **130**, 392–398.

HÜTTEMANN, E., UKENA, D., LENSCHOW, V. & SCHWABE, U. (1984). R_a adenosine receptors in human platelets: characterization by 5'-N-ethylcarboxamido [^{3}H]adenosine binding in relation to adenylate cyclase activity. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **325**, 226–233.

KEPPENS, S. & DE WULF, H. (1986). Characterization of the liver P_2-purinoceptor involved in the activation of glycogen phosphorylase. *Biochem. J.*, **240**, 367–371.

KEPPENS, S., VANDEKERCKHOVE, A. & DE WULF, H. (1989). Characterization of purinoceptors present on human liver plasma membranes. *FEBS Lett.*, **248**, 137–140.

LEVIN, R.M., JACOBY, R. & WEIN, A.J. (1983). High-affinity, divalent ion-specific ^{3}H-ATP to homogenate derived from rabbit urinary bladder. *Mol. Pharmacol.*, **23**, 1–7.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

MOSS, H.E. & BURNSTOCK, G. (1985). A comparative study of electrical field stimulation of the guinea-pig, ferret and marmoset urinary bladder. *Eur. J. Pharmacol.*, **114**, 311–316.

SCHWABE, U. (1985). Use of radioligands in the identification, classification, and study of adenosine receptors. In *Methods Used in Adenosine Research*. ed. Paton, D.M. pp. 239–278. New York and London: Plenum Press.

THEOBALD, R.T. Jr. (1983). The effect of arylazido aminopropionyl ATP on atropine resistant contractions of the cat urinary bladder. *Life Sci.*, **32**, 2479–2484.

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Effects of galanin, its analogues and fragments on rat isolated fundus strips

Stavros Katsoulis, [1]Wolfgang E. Schmidt, Harald Schwörer & Werner Creutzfeldt

Division of Gastroenterology and Endocrinology, Department of Medicine, University of Göttingen, Robert-Koch-Str. 40, D-3400 Göttingen, F.R.G.

1 Rat and porcine galanin (rGal and pGal) produced dose-dependent contraction of rat fundus strips in a concentration range of 6 nM–100 nM.

2 The stimulatory effect of rGal on rat fundus strips was not modified in the presence of somatostatin (250 nM), naloxone (1 μM), guanethidine (10 μM), a mixture of propranolol (3 μM) and phentolamine (3 μM), tetrodotoxin (1 μM), indomethacin (10 μM), atropine (1 μM), a mixture of methysergide (2.5 μM) and ketanserin (2.5 μM), a mixture of mepyramine (10 μM) and cimetidine (10 μM), and saralasin (10 μM) or when strips were desensitized to substance P and neurotensin.

3 These results suggest the localization of specific Gal receptors on the surface of smooth muscle cells of rat fundus.

4 The galanin analogues [D-Trp^2]-rGal, [NLe^4]-rGal, [D-Ala^7]-rGal, [D-Trp^2-NLe^4-D-Ala^7]-rGal and fragments [Cys^{23}]-Gal (1–23), Gal (1–18) were fully active. In contrast, rGal (3–29) was completely inactive and showed no antagonistic properties to the contractile effect of intact galanin.

5 The order of potency of the galanin peptides, analogues and fragments to contract rat fundus strips was: pGal > rGal > [NLe^4]-rGal > [Cys^{23}]-Gal (1-23) > Gal (1-18) > [D-Ala^7]-rGal > [Trp^2]-rGal > [D-Trp^2-NLe^4-D-Ala^7]-rGal.

6 The data originating from our structure-activity study suggest that the C-terminal portion of Gal contributes mainly to the affinity of Gal receptors whereas the N-terminal portion of Gal is responsible for the full activation of Gal receptors in this tissue. In particular the amino acids in position 1 and 2 of Gal (Gly-Trp) appear to be essential for binding and intrinsic activity.

Introduction

Porcine galanin (pGal) is a 29 amino acid peptide isolated from porcine intestine by Tatemoto *et al.* (1983). Galanin occurs in the brain, spinal cord, urogenital tract and gastrointestinal tract of several mammalian species including man (Rokaeus *et al.*, 1984; Ekblad *et al.*, 1985a; Melander *et al.*, 1985; Bishop *et al.*, 1986; Furness *et al.*, 1987; Rokaeus, 1987). *In vitro* studies have shown that pGal evokes a contractile action on rat fundus, jejunum, ileum, colon and urinary bladder (Tatemoto *et al.*, 1983; Ekblad *et al.*, 1985b), human small intestine (Maggi *et al.*, 1989), human appendix (Ekblad *et al.*, 1989) and mouse colon (Fontaine & Lebrun, 1989). By contrast pGal relaxes canine ileum (Fox *et al.*, 1986) and has a neuromodulatory action on rat deferens (Ohhashi & Jacobowitz, 1985), guinea-pig taenia coli (Ekblad *et al.*, 1985b), guinea-pig small intestine (Palmer *et al.*, 1986; Yau *et al.*, 1986; Tamura *et al.*, 1987; 1988; Kuwahara *et al.*, 1989), and human urinary bladder (Maggi *et al.*, 1987).

Recently, some studies have been presented concerning the structure-activity relationship of galanin by use of galanin fragments and substitutions in rat isolated islets (Gregersen *et al.*, 1989), isolated perfused dog pancreas (Hermansen *et al.*, 1989) and in the pancreatic β-cell line Rin m 5F (Amiranoff *et al.*, 1989). So far there is little information about structure-activity relationships on smooth muscle tissues. Ekblad and his colleagues (1985b) reported that the N-terminal galanin fragment 1–10 was able to contract rat jejunum but was inactive, in contrast to intact galanin, in neuromodulation of guinea-pig taenia coli and rabbit iris sphincter.

The aim of this study was to compare the effects of pGal and the recently identified rat galanin (rGal) which differs at 3 positions in the C-terminal part (residue 23, 26 and 29, Table 1) (Vrontakis *et al.*, 1987; Kaplan *et al.*, 1988). Moreover, we analysed the mode of action of galanin on rat isolated fundus strips and used galanin analogues and fragments (Table 1) to

[1] Author for correspondence.

Table 1 Primary structure of porcine and rat galanin and various analogues and fragments

	1	5	10	15	20	25	29
Galanin (porcine):	Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-His-Ala-Ile-Asp-Asn-His-Arg-Ser-Phe-His-Asp-Lys-Tyr-Gly-Leu-Ala-NH_2						
Galanin (rat):	.....				Ser	His	Thr-NH_2
[D-Trp^2]-galanin (rat):	... DTrp	.....			Ser	His	Thr-NH_2
[NLe^4]-galanin (rat):	.....	NLe	.....		Ser	His	Thr-NH_2
[D-Ala^7]-galanin (rat):	.....	.....	DAla	.....	Ser	His	Thr-NH_2
[D-Trp^2-NLe^4-D-Ala^7]-galanin (rat):	... DTrp..	NLe.....	.DAla	.....	Ser	His	Thr-NH_2
[Cys^{23}]-galanin (1-23):	.....				Cys-NH_2		
Galanin (1-18):	.....						
Galanin (3-29) (rat):	.....				Ser	His	Thr-NH_2

Dots indicate conserved amino acid residues, compared with the porcine galanin sequence.

identify the molecular domains responsible for binding and activation of the receptor in this tissue. Data derived from structure-activity studies should be very useful for constructing specific galanin antagonists which are not yet available.

Methods

Animals and tissue preparations

Albino Wistar rats of either sex (weighing 150–300 g) were killed by a blow on the head; the abdomen was opened via an incision in the midline. The stomach was taken out and the fundus was separated from the whole organ. Longitudinal muscle strips were prepared from the fundic region as previously described (Katsoulis & Conlon, 1989). The strips were kept at a resting tension of 2.0 g in oxygenated (95% O_2 and 5% CO_2) Krebs solution maintained at 37°C. The composition of the Krebs solution was as follows (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.18, $NaHCO_3$ 25, KH_2PO_4 1.18 and glucose 5.55. Responses were measured isometrically with Grass FT03 force displacement transducers connected to a Grass multichannel polygraph (model 79D). Tissue was allowed to equilibrate for 30–60 min and the superfusion buffer was changed every 15–20 min.

Concentration-response curves

Experiments were begun when reproducible contractile responses to carbachol (10 μM) were obtained. Then, conventional dose-response curves were constructed. No more than two concentration-response studies (one for rat galanin and one for a galanin analogue or galanin fragment, applied alternately) were performed on each strip. Peptides were injected in increasing concentrations by non-cumulative additions, until maximum muscular responses occurred. The maximal effect was defined as the contractile response that could not be further increased by higher concentrations of peptide. The tissue was washed as soon as the peak contraction had developed. The contact time of the peptides with muscle strips was 1–3 min. Agonist applications were performed at 20–30 min intervals to avoid tachyphylaxis. Results are expressed as a percentage of the maximum response induced by each peptide. Potencies (ED_{50}) were calculated by interpolation from the appropriate dose-response curve. Effectiveness refers to the maxium response produced by intact rat galanin compared with the maximum effect produced by galanin analogues or galanin fragments.

Characterization of the contracting effect of galanin on rat fundus strips

In order to establish whether the stimulatory action of galanin is due to specific receptor-activation located on the smooth muscle cell or mediated by the release of endogenous substances, galanin and control agonists were tested in the absence and presence of various antagonists. The concentrations used for the antagonists were sufficiently high to block the corresponding agonists (see Table 3). The rat stomach strips were incubated with the inhibitors for 15–20 min before addition of test agents. The contractile effect of galanin was also investigated on preparations desensitized with substance P and neurotensin for which no potent antagonists were available. Tachyphylaxis to substance P was obtained by the method described by Holzer-Petsche *et al.* (1989). To induce tachyphylaxis to neurotensin five test concentrations of peptide (10 nM) were applied at 6 min intervals without washout; 1 min after the administration of the last test concentration, galanin was added to the bath. Test doses of [D-Trp^2-NLe^4-D-Ala^7]-rGal and rGal (3-29) were injected 5 min before application of rGal to examine their antagonistic properties to the effect of rGal.

Statistical analysis

All data are expressed as means ± s.e.mean. Student's t test for paired data was used for statistical evaluation of differences. A P value of less than 0.05 was taken to indicate a significant difference.

Drugs

The following drugs were used: atropine sulphate, cimetidine maleate, carbachol, indomethacin, histamine hydrochloride, 5-hydroxytryptamine creatinine sulphate, tetrodotoxin, naloxone hydrochloride, (±)-propranolol and guanethidine (all from Sigma Chemie). Galanin (porcine), angiotensin II, neurotensin, substance P and somatostatin (Peninsula Laboratories Europe). Saralasin (gift from Rhöm Pharma, F.R.G.), ketanserin tartrate (gift from Janssen, Belgium), methysergide hydrogenmaleate (gift from Sandoz, Switzerland), phentolamine methansulphate (gift from Ciba Geigy, Switzerland) and mepyramine maleate (gift from May and Baker, U.K.). Rat galanin and its analogues and fragments were synthesized by Fmoc solid phase peptide synthesis on a LKB Biolynx peptide synthesizer and purified to homogeneity by high performance liquid chromatography.

Results

Effects of galanin, its analogues and fragments on rat fundus strips

Both rGal and pGal elicited concentration-dependent contractions of rat fundus strips. Reproducible effects were observed at concentrations as low as 6 nM. Maximum contraction was obtained with pGal and rGal at a concentration of 100 nM; larger concentrations failed to produce a greater response. The potencies and effectiveness of rGal and pGal were not significantly different (Table 2) and the slopes of the dose-response curves were nearly identical (Figure 1). The maximum contraction induced by rGal (100 nM) and pGal (100 nM) was 51.5% and 50.5% respectively of that achieved with carbachol (30 μM) in this tissue. The dose-response curves produced by the galanin fragments and analogues are shown in Figure 1. Effectiveness, ED_{50} values and relative potencies are presented in Table 2. The concentration-response curves to [NLe^4]-rGal, [Cys^{23}]-Gal (1-23) and Gal (1-18) were displaced slightly to the right of that to rGal and pGal but remained parallel and exhibited the same maximum. Only minor losses (13 to 55%) of potency were observed with these compounds when compared to native galanin. On the other hand [D-Ala^7]-rGal, [D-Trp^2]-rGal and [D-Trp^2-NLe^4-D-

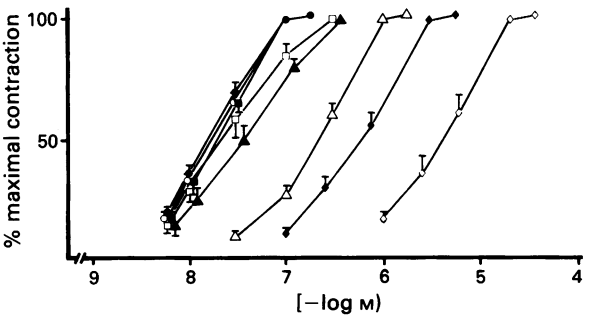

Figure 1 Dose-response curves for the contractile effect of porcine galanin (●), rat galanin (○), (NLe^4)-(rat) galanin (■), (Cys^{23})-galanin (1-23) (□), galanin (1-18) (▲), (D-Ala^7)-(rat) galanin (△), (D-Trp^2)-(rat) galanin (◆) and (D-Trp^2-NLe^4-D-Ala^7)-(rat) galanin (◇) on the rat fundus *in vitro*. Results are expressed as the percentage of the maximum effect induced by each peptide. Each point represents the mean and vertical bars show s.e.mean. The number of individual determinations is given in Table 2.

Table 2 A comparison of the effectiveness, potencies and relative potencies of galanin, its analogues and fragments on the contraction of the rat fundus

	Effectiveness (%)	ED_{50} (nM)	*Relative potency*	*Number of experiments*
Galanin (porcine)	99 ± 2.5	16.7 ± 1.0	100	5
Galanin (rat)	100.0	18.2 ± 0.8	92	65
[NLe^4]-galanin (rat)	101 ± 7.0	19.2 ± 1.3	87	8
[Cys^{23}]-galanin (1-23)	98 ± 7.0	27.6 ± 5.3*	61	12
Galanin (1-18)	97 ± 4.0	36.9 ± 5.7**	45	8
[D-Ala^7]-galanin (rat)	97 ± 3.5	180.0 ± 11.0***	9	8
[D-Trp^2]-galanin (rat)	103 ± 5.0	514.0 ± 47.0***	3	8
[D-Trp^2-NLe^4-D-Ala^7]-galanin (rat)	96 ± 5.0	3810.0 ± 500.0***	0.4	8
Galanin (3-29) (rat)	No effect	—	—	8

Data are expressed as means ± s.e.mean. Effectiveness refers to the maximum response produced by the test peptides compared with the maximum response produced by rat galanin. Potencies (ED_{50}) were calculated by interpolation from the appropriate dose-response curves. Relative potency refers to the ED_{50} of each peptide compared to the ED_{50} of porcine galanin.
* $P < 0.5$; ** $P < 0.01$; *** $P < 0.001$ compared to the ED_{50} of rat galanin.

Ala^7]-rGal showed the same maximum to intact Gal and pGal but their dose-response curves were shifted strongly to the right. [D-Ala^7]-rGal, [D-Trp^2]-rGal and [D-Trp^2-NLe^4-D-Ala^7]-rGal were 11, 33 and 250 times less potent compared to Gal in rat fundus strips (Figure 1, Table 2). rGal (3-29) was completely inactive as an agonist in these preparations even in concentrations up to 10 μM (Table 2).

Effects of various agents on the action of rGal in rat fundus strips

The contractions induced by rGal were not significantly modified in the presence of atropine, a mixture of methysergide and ketanserin, a mixture of mepyramine and cimetidine, and saralasin while those produced by their respective agonists (carbachol, 5-HT, histamine, angiotensin II) were abolished. Somatostatin, naloxone, guanethidine, a mixture of propranolol and phentolamine, TTX and indomethacin also failed to influence the contractile effect of rGal. Preparations desensitized to neurotensin or substance P remained fully responsive to rGal. None of the rGal derivatives rGal (3-29) and [D-Trp^2-NLe^4-D-Ala^7]-rGal behaved as an antagonist of rGal in rat fundus strips. These results are summarized in Table 3.

Discussion

A preliminary account has been published of the contractile effect of pGal in rat stomach strips (Tatemoto *et al.*, 1983). So far a pharmacological characterization of this effect has not been performed. The present study suggests that Gal contracts rat fundus by a direct interaction with specific galanin receptors located on the smooth muscle cells. This is supported by the observation that the action of Gal was unmodified by TTX, atropine, a mixture of methysergide and ketanserin, a mixture of mepyramine and cimetidine, saralasin, a mixture of propranolol and phentolamine, guanethidine, naloxone, somatostatin and indomethacin. Rat fundus strips desensitized with neurotensin or substance P were found to maintain their sensitivity to galanin. The existence of specific galanin receptors makes the rat fundus strip an appropriate bioassay preparation for investigating structure-function relationships of galanin and its myotropic activity.

Table 3 Myotropic effects of rat galanin (rGal) and other agonists on rat isolated stomach strips, as obtained in the absence and presence of specific antagonists, autacoids and peptides

Antagonist (M)	*Agonist*	*Dose* (M)	*Contraction (%)* *In the absence of the antagonist*	*In the presence of the antagonist*
Rat galanin (3-29) (10^{-5})	rGal	3×10^{-8}	100	111 ± 9
[D-Trp^2-NLe^4-D-Ala^7]-rat galanin (10^{-6})	rGal	3×10^{-8}	100	98 ± 9
Somatostatin (2.5×10^{-7})	rGal	10^{-7}	100	97 ± 3.5
Naloxone (10^{-6})	rGal	10^{-7}	100	106 ± 8
Guanethidine (10^{-5})	rGal	10^{-7}	100	101 ± 5
Propranolol (3×10^{-6}) + phentolamine (3×10^{-6})	rGal	10^{-7}	100	103 ± 7
TTX (10^{-6})	rGal	10^{-7}	100	93 ± 3
Indomethacin (10^{-5})	rGal	10^{-7}	100	96 ± 7
Atropine (10^{-6})	Carbachol	10^{-5}	100	0*
	rGal	10^{-7}	100	104 ± 2
Methysergide (2.5×10^{-6}) + ketanserin (2.5×10^{-6})	5-Hydroxytryptamine	10^{-5}	100	0*
	rGal	10^{-7}	100	95 ± 8
Mepyramine (10^{-5}) + cimetidine (10^{-5})	Histamine	10^{-4}	100	0*
	rGal	10^{-7}	100	97 ± 5
Saralasin (10^{-5})	Angiotensin II	10^{-6}	100	0*
	rGal	10^{-7}	100	107 ± 4
Substance P (tachyphylaxis)	Substance P	5×10^{-7}	100	43 ± 4.5*
	rGal	10^{-7}	100	100 ± 10
Neurotensin (tachyphylaxis)	Neurotensin	10^{-8}	100	0*
	rGal	10^{-7}	100	105 ± 9

The results are expressed as means ± s.e.mean of 8 experiments. The incubation time of the antagonists with the tissues was 15–20 min except rGal (3-29) and [D-Trp^2-NLe^4-D-Ala^7]-rGal which were injected 5 min before repeating application of agonists. The desensitizing procedures performed with substance P and neurotensin are described in methods. M is molar concentration. P values < 0.05 were considered significant (see asterisk).

Both rGal and pGal were equipotent and equally effective in producing contractions of rat fundus strips. Using galanin fragments we have found that deletion of the C-terminal portion of the molecule led only to a minor loss of the potency but the effectiveness was fully conserved. In contrast, substitutions in the N-terminal part of the molecule led to a major loss of potency. Nevertheless, although considerably less potent than Gal, the N-terminal modified galanin peptides were able to elicit a comparable maximum response to Gal. The present findings support earlier conclusions that the C-terminal residue of the galanin molecule contributes mainly to the affinity of Gal for its receptor whereas the N-terminal portion is responsible for binding and ability to convey biological effects (Amiranoff *et al.*, 1989; Gregersen *et al.*, 1989). Particularly the two first amino acids (Gly-Trp) at the N-terminus appear to be crucial for binding and/or receptor activation. Whether these residues interact directly with the receptor binding site or are essential for the conformation of the ligand, cannot be decided on the basis of this study. This conclusion is consistent with the absence of effect of rGal (3-29) on rat fundus strips. Moreover, binding studies with Gal and galanin fragments and analogues performed on RIN m 5F cells in our laboratory have revealed that rGal (3-29) does not bind to the galanin receptor (W.E. Schmidt, B. Gallwitz, R. Schwarzhoff, personal communication). In this light, the lack of antagonistic properties of rGal (3-29) to the contractile action of Gal is not unexpected.

Earlier reports on discrepancies in pGal action on rat smooth muscle compared to other species have been attributed to the supposedly different amino acid sequence of pGal and rGal (Rokaeus, 1987). However, the present work suggests that reported differences in responses to galanin may be species and tissue-dependent and not a consequence of the difference in primary structures of the galanins. The fact that intact Gal, unlike N-terminal galanin fragments, reduced the cholinergic contractile response to electrical stimulation on guinea-pig taenia coli and rabbit iris sphincter (Ekblad *et al.*, 1985b) and inhibited somatostatin secretion of the endocrine pancreas of the dog (Hermansen *et al.*, 1988) raises the possibility of the existence of different galanin receptor subtypes. Further studies to support this hypothesis are in progress at present.

In conclusion, the C-terminal region contributes mainly to the affinity of Gal for its smooth muscle receptors while the N-terminal region plays a critical role for its biological activity, particularly the first two amino acids (Gly-Trp), which seem crucial for receptor activation.

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References

AMIRANOFF, B., LORINET, A.-M., YANAIHARA, N. & LABURTHE, M. (1989). Structural requirement for galanin action in the pancreatic β-cell line Rin m 5F. *Eur. J. Pharmacol.*, **163**, 205–207.

BAUER, F.E., ADRIAN, T.E., CHRISTOFIDES, N.D., FERRI, G.-L., YANAIHARA, N., POLAK, J.M. & BLOOM, S.R. (1986). Distribution and molecular heterogeneity of galanin in human, pig, guinea pig, and rat gastrointestinal tracts. *Gastroenterology*, **91**, 877–883.

BISHOP, A.E., POLAK, J.M., BAUER, F.E., CHRISTOFIDES, N.D., CARLEI, F. & BLOOM, S.R. (1986). Occurrence and distribution of a newly discovered peptide, galanin, in the mammalian enteric nervous system. *Gut*, **27**, 849–857.

EKBLAD, E., ROKAEUS, A., HÅKANSON, R. & SUNDLER, F. (1985a). Galanin nerve fibers in the rat gut: distribution, origin and projections. *Neuroscience*, **16**, 355–363.

EKBLAD, E., HÅKANSON, R., SUNDLER, F. & WAHLSTEDT, C. (1985b). Galanin: neuromodulatory and direct contractile effects on smooth muscle preparations. *Br. J. Pharmacol.*, **86**, 241–246.

EKBLAD, E., ARNBJÖRNSSON, E., EKMAN, R., HÅKANSON, R. & SUNDLER, F. (1989). Neuropeptides in the human appendix. Distribution and motor effects. *Dig. Dis. Sci.*, **34**, 1217–1230.

FONTAINE, J. & LEBRUN, P. (1989). Galanin: Ca^{2+}-dependent contractile effects on the isolated distal mouse colon. *Eur. J. Pharmacol.*, **164**, 583–586.

FOX, J.E.T., McDONALD, T.J., KOSTOLANSKA, F. & TATEMOTO, K. (1986). Galanin: an inhibitory neural peptide in the canine small intestine. *Life Sci.*, **39**, 103–110.

FURNESS, J.B., COSTA, M., ROKAEUS, A., McDONALD, T.J. & BROOKS, B. (1987). Galanin-immunoreactive neurons in the guinea pig small intestine: their projections and relationships to other enteric neurons. *Cell Tiss. Res.*, **250**, 607–615.

GREGERSEN, S., AHREN, B. & HERMANSEN, K. (1989). Galanin fragments and substitutions; effects on glucose stimulated insulin secretion from isolated rat islets. *Diabetologia*, **32**, 493A.

HERMANSEN, K., AHREN, B. & YANAIHARA, N. (1989). On the nature of galanin action on the endocrine pancreas. *Diabetologia*, **32**, 49A.

HOLZER-PETSCHE, U., SEITS, H. & LEMBECK, F. (1989). Effect of capsaicin on gastric corpus smooth muscle of the rat *in vitro*. *Eur. J. Pharmacol.*, **162**, 29–36.

KAPLAN, L.M., SPINDEL, E.R., ISSELBACHER, K.J. & CHIN, W.W. (1988). Tissue-specific expression of the rat galanin gene. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 1065–1069.

KATSOULIS, S. & CONLON, J.M. (1989). Calcitonin gene-related peptides relax guinea pig and rat gastric smooth muscle. *Eur. J. Pharmacol.*, **161**, 129–134.

KUWAHARA, A., OZAKI, T. & YANAIHARA, N. (1989). Galanin suppresses neurally evoked contractions of circular muscle in the guinea-pig ileum. *Eur. J. Pharmacol.*, **164**, 175–178.

MAGGI, C.A., SANTICIOLI, P., PATACCHINI, R., TURINI, D., BARBANTI, G., BENEFORTI, P., GIULIANI, S. & MELI, A. (1987). Galanin: a potent modulator of excitatory neurotransmission in the human urinary bladder. *Eur. J. Pharmacol.*, **143**, 135–137.

MAGGI, C.A., PATACCHINI, R., SANTICIOLI, P., GIULIANI, S., TURINI, D., BARBANTI, G., BENEFORTI, P., MISURI, D. & MELI, A. (1989). Human isolated small intestine: motor responses of the longitudinal muscle to field stimulation and exogenous neuropeptides. *Naunyn Schmiedebergs Arch. Pharmacol.*, **339**, 415–423.

MELANDER, T., HÖKFELT, T., ROKAEUS, A., FAHRENKRUG, J., TATEMOTO, K. & MUTT, V. (1985). Distribution of galanin-like immunoreactivity in the gastro-intestinal tract of several mammalian species. *Cell Tiss. Res.*, **239**, 253–270.

OHHASHI, T. & JACOBOWITZ, D.M. (1985). Galanin potentiates electrical stimulation and exogenous norepinephrine-induced contractions in the rat deferens. *Regul. Pept.*, **12**, 163–171.

PALMER, J.M., SCHEMANN, M., TAMURA, K. & WOOD, J.D. (1986). Galanin mimics slow synaptic inhibition in myenteric neurons. *Eur. J. Pharmacol.*, **124**, 379–380.

ROKAEUS, A. (1987). Galanin: a newly isolated biologically active neuropeptide. *Trends Neurosci.*, **10**, 158–164.

ROKAEUS, A., MELANDER, T., HÖKFELT, T., LUNDBERG, J.M., TATEMOTO, K., CARLQUIST, M. & MUTT, V. (1984). A galanin-like peptide in the central nervous system and intestine of the rat. *Neurosci. Lett.*, **47**, 161–166.

TAMURA, K., PALMER, J.M., WINKELMANN, C.K. & WOOD, J.D. (1988). Mechanism of action of galanin on myenteric neurons. *J. Neurophysiol.*, **60**, 966–979.

TAMURA, K., PALMER, J.M. & WOOD, J.D. (1987). Galanin suppresses nicotinic synaptic transmission in the myenteric plexus of guinea pig small intestine. *Eur. J. Pharmacol.*, **136**, 445–446.

TATEMOTO, K., ROKAEUS, A., JÖRNVALL, H., McDONALD, T.J. & MUTT, V. (1983). Galanin – a novel biologically active peptide from porcine intestine. *FEBS Lett.*, **164**, 124–128.

VRONTAKIS, M.E., PEDEN, L.M., DUCKWORTH, M.L. & FRIESEN, H.G. (1987). Isolation and characterization of a complementary DNA (Galanin) clone from estrogen-induced pituitary tumor messenger RNA. *J. Biol. Chem.*, **262**, 16755–16758.

YAU, W.M., DORSETT, J.A. & YOUTHER, M.L. (1986). Evidence for galanin as an inhibitory neuropeptide of myenteric cholinergic neurons in the guinea pig small intestine. *Neurosci. Lett.*, **72**, 305–308.

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The pharmacokinetics of γ-glutamyl-L-dopa in normal and anephric rats and rats with glycerol-induced acute renal failure

*[1]Yaw A. Boateng, *Harold E. Barber, *[2]Thomas M. MacDonald, *James C. Petrie, †Michael R. Lee & ‡Paul H. Whiting

*Clinical Pharmacology Unit and †Department of Clinical Biochemistry, University of Aberdeen, Foresterhill, Aberdeen AB9 2ZD and ‡Department of Clinical Pharmacology, The Royal Infirmary, Edinburgh EH3 9YW

1 The pharmacokinetics of γ-glutamyl-L-dopa (gludopa) and its metabolite, L-dopa, have been studied in normal rats at three dose levels of gludopa: 2 mg kg^{-1}, 5 mg kg^{-1} and 7.5 mg kg^{-1}. The extent of metabolism in normal rats, and the pharmacokinetics in anephric rats and rats with glycerol-induced acute renal failure (ARF) were also studied at a gludopa dose of 2 mg kg^{-1}.

2 Gludopa was extensively metabolised to L-dopa with only about 10% of an injected dose being excreted unchanged. Normal rats had a rapid gludopa clearance of 50.9 ± 9.6 ml min^{-1} kg^{-1} and elimination rate constant of 2.99 ± 0.27 h^{-1}. The mean residence time and half-life were 20.9 ± 1.4 and 14.4 ± 1.0 min, respectively. The apparent volume of distribution at steady state was 1.05 ± 0.18 l kg^{-1}.

3 No statistically significant differences were found in the main pharmacokinetic parameters between ARF and controls for either gludopa or its metabolite L-dopa.

4 In anephric rats and controls the kidneys were found to contribute about 68.5% and 67.2% to the elimination of gludopa and the metabolite L-dopa, respectively.

5 These results confirm that gludopa is an efficient pro-drug for L-dopa, and that the kidneys are the major site of gludopa metabolism. It seems likely that the renal specificity of gludopa persists in ARF.

Introduction

γ-Glutamyl-L-dopa (gludopa) is a synthetic dipeptide pro-drug for L-dopa and dopamine. It is unique in being metabolised almost exclusively in the kidney by the sequential actions of γ-glutamyl transpeptidase (γ-GT) and aromatic L-amino acid decarboxylase (AAAD) in the brush border and within the cells, respectively, of the proximal tubules of the kidney (Wilk *et al.*, 1978; Worth *et al.*, 1985). The generation of dopamine leads to an increase in effective renal plasma flow and glomerular filtration rate as well as a natriuresis both in animals (Wilk *et al.*, 1978) and man (Worth *et al.*, 1985; Jeffrey *et al.*, 1988; MacDonald *et al.*, 1988).

The relative renal specificity of gludopa stems from the fact that the kidney has by far the greatest concentration of γ-GT compared to any other tissue in the body (Orlowski & Szewczuk, 1961; Wilk *et al.*, 1978). Gludopa ameliorates the effects of glycerol-induced acute renal failure (ARF), and it has been postulated that patients at risk of developing ARF may benefit from gludopa treatment (Casson *et al.*, 1983). Its diuretic and natriuretic actions could mean that it would also find use as an adjunct in the treatment of oedematous states (Lee, 1982). Despite this there is little information on the disposition of gludopa.

We present the first documentation of the pharmacokinetics of gludopa in normal rats. We have measured the fraction metabolised to L-dopa, the immediate precursor of renal dopamine (Baines & Chan, 1980; Brown & Allison, 1981), and calculated the relevant first order rate constants associated with this process.

Gludopa is metabolized predominantly by the kidney. Thus it is of interest to know what effect renal failure has on the disposition of gludopa. Studies on the influence of renal failure on the disposition of drugs have mainly concentrated on effects on hepatic drug metabolising enzymes (Van Peer & Belpaire, 1977; Leber *et al.*, 1978; Patterson & Cohn, 1984) and on the problems associated with drugs excreted mostly unchanged by the kidneys (Jackson *et al.*, 1988; Kraan *et al.*, 1988; Williams *et al.*, 1988). Little is known about the effect of uraemia on the disposition of drugs metabolised mostly by the kidney. We have investigated whether glycerol-induced ARF alters the pharmacokinetics of gludopa. In addition, using functionally nephrectomised rats, we have studied the role of the kidney in the elimination of gludopa.

Methods

Experiment 1

Twelve male Sprague-Dawley rats (350–530 g) were randomised into three groups, A, B and C to receive 2 mg kg^{-1}, 5 mg kg^{-1} and 7.5 mg kg^{-1} gludopa, respectively. On the study day rats were anaesthetized with urethane (15% w/v, 10 ml kg^{-1}, i.p.) and the jugular vein and contralateral carotid artery cannulated with Portex tubing for drug administration and blood sample collection respectively. Gludopa was obtained from UCB Bioproducts (Rue Berkendael 68, 1060 Brussels, Belgium) and was 92% pure peptide, the balance being acetic acid carried over from freeze drying. It was prepared for use in 0.5% bovine serum albumin in saline as carrier. After injection of heparinised (1000 iu l^{-1}) saline and collection of a sample at zero time, the appropriate dose of gludopa was injected rapidly as an intravenous bolus. Carefully timed blood samples (0.5 ml) were collected from 0.5 to 90 min; half the withdrawn blood was replaced with 0.9% (w/v) saline. The blood samples were centrifuged immediately and the plasma transferred to a separate tube containing sodium metabisulphite as an antioxidant and stored at −70°C to await assay for gludopa and L-dopa.

Experiment 2

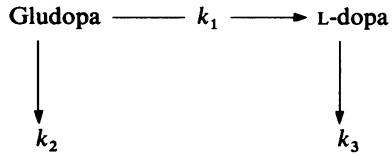
Eight male Sprague-Dawley rats (265–380 g) were randomised into two groups, X and Y to receive either 2 mg kg^{-1} gludopa or an equimolar (1.2 mg kg^{-1}) dose of L-dopa. Pharmacokinetic experiments were carried out as described above. The fraction of the dose of gludopa metabolised to L-dopa (Fm) was calculated:

$$Fm = AUC(1, g)/AUC(1)$$

[1] Author for correspondence.
[2] Present address: Department of Clinical Pharmacology, Ninewells Hospital, Dundee DD1 9SY.

where, AUC (1, g) and AUC (1) are the area under the plasma concentration versus time plot, from zero to infinity, of L-dopa following the administration of gludopa and equimolar L-dopa respectively. All AUCs were estimated by numerical integration with the trapezoidal rule.

The following scheme was devised, based on that of Kaplan *et al.* (1973), for the calculation of first order rate constants.



where

$$k_1 = \text{Fm} \times K$$

$$k_2 = K - k_1$$

$$k_3 = K \text{ (L-dopa)}$$

and where: k_1 is the rate constant of formation of L-dopa after the administration of gludopa; k_2 is the sum of the rate constants for the simultaneous processes of excretion and/or biotransformation to products other than L-dopa; K and K (L-dopa) is the total elimination rate constant for gludopa and the metabolite L-dopa respectively.

Experiment 3

Ten male Sprague-Dawley rats (180–340 g) were randomised into treatment and control groups. Rats in both groups were deprived of water for 24 h but were allowed food. In the treated group ARF was induced by the injection of glycerol (50% v/v; 10 ml kg^{-1}) in two divided doses into the hind limb musculature (Oken *et al.*, 1966). The control group received 0.9% (w/v) saline instead of glycerol. Drinking water was restored immediately and both groups of rats were used 48 h after injection. The extent of renal failure was assessed by determination of serum urea and creatinine concentrations (Dimension Discrete Analyser, Du Pont Ltd, Wilmington, DE 19898, U.S.A.) on the day of the experiment. On the study day rats were anaesthetized and, pharmacokinetic experiments carried out as above after injecting gludopa (2 mg kg^{-1}) as an intravenous bolus.

Experiment 4

Eight male Sprague-Dawley rats (270–315 g) were randomised into treatment and control groups. Those in the treatment group underwent functional nephrectomy on the day of the experiment. This is a procedure by which the rats were rendered anephric by ligating all the renal vessels and ureters on both sides. After urethane anaesthesia, a ventral midline incision was made on the abdomen. The kidneys were identified and the renal vessels and the ureters on both sides ligated completely at two points. In all cases an immediate renal infarction was produced, evidenced by complete darkening of both kidneys. Control rats underwent laparotomy where the abdomen was opened for a similar length of time and the kidneys handled. After 5 ml of sterile 0.9% (w/v) saline had been instilled into the abdomen, it was closed with Michel clips. Pharmacokinetic experiments were carried out immediately on both groups of animals as described above.

Analytical methods

Gludopa and its metabolite L-dopa were adsorbed onto alumina and eluted with 0.2 M perchloric acid by the method of Anton & Sayre (1964), with dihydroxybenzylamine (DHBA, 0.5 μg ml^{-1}) as internal standard. Plasma (0.1 ml) was diluted 1:10 with distilled water, extracted with 0.1 g of activated alumina and eluted with 0.2 ml perchloric acid, giving a final effective dilution of 1:5. The concentrations of the three substances were determined by high performance liquid chromatography using a Waters NOVAPAK C18 (3.9 mm i.d. × 15 cm) steel column without radial compression. The mobile phase solvent system consisted of 0.1 M sodium acetate and 0.1 M citric acid (in a ratio of approximately 1:1, pH 3.5), with 1.5% methanol and 50 g l^{-1} octane sulphonic acid as an ion-pairing agent and was run at a flow rate of 0.7 ml min^{-1}. A Shimadzu fluorescent spectrometer was used (excitation 284 nm and emission 314 nm) and peaks integrated by a Hewlett Packard 3390 A integrator. All test samples were analysed in duplicate. Gludopa, L-dopa and internal standard peaks were identified by injection of known standards and had retention times of 4.0, 5.5 and 11.5 min respectively.

The limit of detection of gludopa and L-dopa was 1 ng ml^{-1}. The efficiency of extraction was 64% and 75% for gludopa and L-dopa, respectively. The intra- and inter-assay coefficient of variation was 3.1% and 4.8% respectively for gludopa; and 2.2% and 4.3% respectively for L-dopa.

Pharmacokinetic analysis

Experiment 1 and Experiment 3 From the plasma concentration versus time profiles of both gludopa and L-dopa, pharmacokinetic parameters were estimated by the non-compartmental approach of the statistical moment theory (Yamaoka *et al.*, 1978; Gibaldi & Perrier, 1982; Chan, 1982).

The following parameters were estimated: (1) Clearance, CL = dose/AUC; (2) mean residence time MRT = AUMC/AUC; (3) half-life, $t_{1/2}$ = MRT × 0.693; (4) steady state volume of distribution, V_{ss} = CL × MRT; (5) elimination rate constant (of gludopa), K = 1/MRT; (6) elimination rate constant (of metabolite L-dopa), K = 1/(MRT 1 − MRT g); where: AUC is area under the plasma concentration versus time curve from zero to infinity (calculated by numerical integration by use of the trapezoidal rule with extrapolation to infinity); AUMC is the area under the product of plasma concentration and time versus time plot; dose is the dose of gludopa given; and MRT g and MRT 1 are the MRT of gludopa and the metabolite L-dopa respectively.

Experiment 4 The main pharmacokinetic parameters were estimated as described above. The elimination rate constant of functionally anephric rats represents non-renal elimination, K_{nr}, while that of the control group (laparotomies) represents total elimination, K_t. The renal component of elimination, K_r, was estimated as follows:

$$K_r = K_t - K_{nr} \qquad \text{(Moravek } et\ al.\text{, 1987)}$$

Statistical analysis

Results are expressed as mean ± s.d. Student's non-paired t test was used to test for statistical significance in pharmacokinetic parameters between ARF and control rats and between anephric rats and controls. Significance was taken as a P value of less than 0.05. Statistical analysis of the kinetic parameter in the three dosage groups in experiment 1 was by one-way analysis of variance and Scheffe's (1953) multiple comparison test.

Results

Experiment 1

Figure 1 shows the mean plasma concentration versus time plot for gludopa and the metabolite L-dopa for group A (gludopa 2 mg kg^{-1}). The mean plasma concentration of gludopa at 90 min was about 2.8% of the maximum concentration at 0.5 min. For groups B and C, these values were 1.7% and 2.8% respectively, showing a very rapid decline of injected gludopa at all three doses. The plasma concentration versus time profiles for groups B and C were similar to that of group A and are not shown. L-Dopa was the principal metabolite of gludopa detected in plasma. Its concentrations also fell rapidly (see Figure 1). Dopamine was not measured in this study.

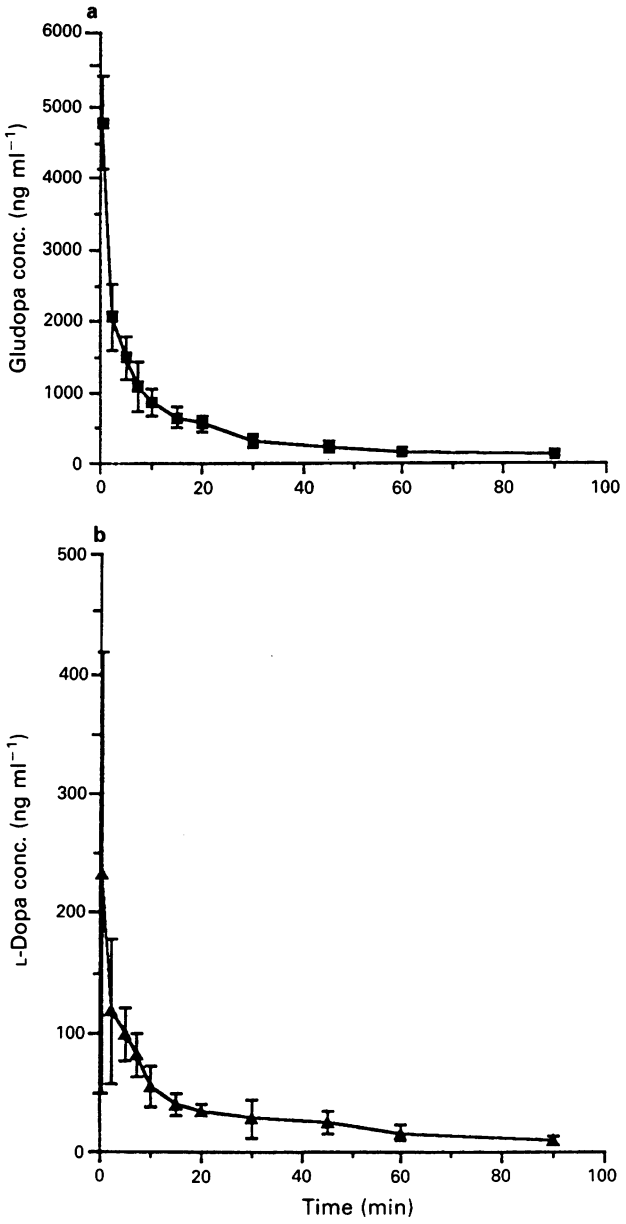


Figure 1 Mean plasma concentration versus time profiles of (a) gludopa and (b) its metabolite, L-dopa after injection of gludopa 2 mg kg^{-1} (group A). The vertical lines show s.d., $n = 4$.

A summary of the main pharmacokinetic parameters of gludopa and the metabolite L-dopa for the three groups is shown in Table 1. Analysis of variance and Scheffe's test showed no significant differences in the pharmacokinetic parameters of gludopa between the three groups. The kinetics of gludopa were, therefore, linear across the dose range studied. The total elimination rate constant of L-dopa was significantly less in rats in group C (gludopa 7.5 mg kg^{-1}) compared to those in group A (gludopa 2 mg kg^{-1}).

Experiment 2

The calculated AUCs were as follows: AUC (1, g) was 3779 ± 417 (ng ml^{-1}) min and AUC (1) was 6966 ± 1471 (ng ml^{-1}) min ($n = 4$ in both cases). According to the scheme mentioned earlier and the formulae and definitions given, Fm was 0.54; k_1, 1.55 h^{-1}; k_2, 1.32 h^{-1} and k_3 3.05 h^{-1}.

Experiment 3

The ARF group had a serum urea (mean ± s.d.) of 64.9 ± 38.5 mmol l^{-1} (393 ± 233 mg dl^{-1}) compared to the control group with 10.9 ± 2.4 mmol l^{-1} (66 ± 14 mg dl^{-1}) ($P < 0.02$). The mean serum creatinine values for ARF and control groups were 369.4 ± 176.7 μmol l^{-1} (4.2 ± 2.0 mg dl^{-1}) and 51.6 ± 15.5 μmol l^{-1} (0.6 ± 0.2 mg dl^{-1}) respectively ($P < 0.01$).

Figure 2 shows the mean plasma concentration versus time profiles of gludopa and L-dopa for ARF and controls. The concentrations of gludopa in plasma fell rapidly in both groups in a similar manner. The metabolite L-dopa fell much more slowly in ARF and with greater variation between rats.

Table 2 gives a summary of the main pharmacokinetic parameters for ARF versus controls for gludopa and metabolite L-dopa. There were no statistically significant differences in any of the parameters for gludopa or L-dopa between ARF and control rats.

Table 1 Main pharmacokinetic parameters for gludopa and the metabolite L-dopa for group A (gludopa 2 mg kg^{-1}), group B (gludopa 5 mg kg^{-1}) and group C (gludopa 7.5 mg kg^{-1})

Parameter	*Group A* ($n = 4$)	*Group B* ($n = 4$)	*Group C* ($n = 4$)
Gludopa			
Cl (ml min^{-1} kg^{-1})	53.6 ± 9.6	58.9 ± 11.0	40.3 ± 15.3
MRT (min)	21.4 ± 3.6	19.6 ± 6.1	22.0 ± 4.4
$t_{1/2}$ (min)	14.8 ± 2.4	13.3 ± 4.3	15.3 ± 3.0
V_{ss} (l kg^{-1})	1.14 ± 0.27	1.17 ± 0.57	0.84 ± 0.15
K (h^{-1})	2.87 ± 0.55	3.31 ± 0.86	2.80 ± 0.59
L-Dopa			
MRT (min)	46.2 ± 5.0	56.7 ± 19.2	62.1 ± 6.8
$t_{1/2}$ (min)	32.0 ± 3.5	39.4 ± 13.3	43.0 ± 4.8
K(h^{-1})	2.87 ± 0.69*	1.78 ± 0.72	1.53 ± 0.2*

The results are given as mean ± s.d.
* Statistically significant difference by Scheffe's test ($P < 0.05$). Cl = clearance of gludopa, MRT = mean residence time, $t_{1/2}$ = half-life, V_{ss} = apparent volume of distribution at steady state and K = total elimination rate constant.

Experiment 4

The mean plasma concentrations of gludopa and L-dopa versus time in anephric rats is compared to those in controls in Figure 3. A slower fall, with a plateau at 30 min, occurred in the anephric rats for gludopa and L-dopa.

Table 3 shows a comparison of the main pharmacokinetic parameters of gludopa and L-dopa between anephrics and controls. There were highly significant differences in the clearance and elimination rate constants but no difference in the V_{ss}.

Table 2 Comparison of the main pharmacokinetic parameters of gludopa and the metabolite L-dopa of rats with acute renal failure (ARF) with controls after 2 mg kg^{-1} gludopa

Parameter	*ARF* ($n = 5$)	*Control* ($n = 5$)
Gludopa		
Cl (ml min^{-1} kg^{-1})	42.4 ± 18.1	40.2 ± 26.1
MRT (min)	32.6 ± 6.9	34.2 ± 12.0
$t_{1/2}$ (min)	22.6 ± 4.8	23.6 ± 8.3
V_{ss} (l kg^{-1})	1.45 ± 0.75	1.31 ± 0.75
K (h^{-1})	1.90 ± 0.42	1.91 ± 0.58
L-Dopa		
MRT (min)	67.7 ± 18.0	76.9 ± 28.0
$t_{1/2}$ (min)	46.9 ± 12.5	53.3 ± 19.5
K (h^{-1})	1.95 ± 0.62	1.55 ± 0.50

The results are given as mean ± s.d. There were no significant differences in any of the parameters between ARF and controls by Student's non-paired t test. For key to abbreviations used see Table 1.

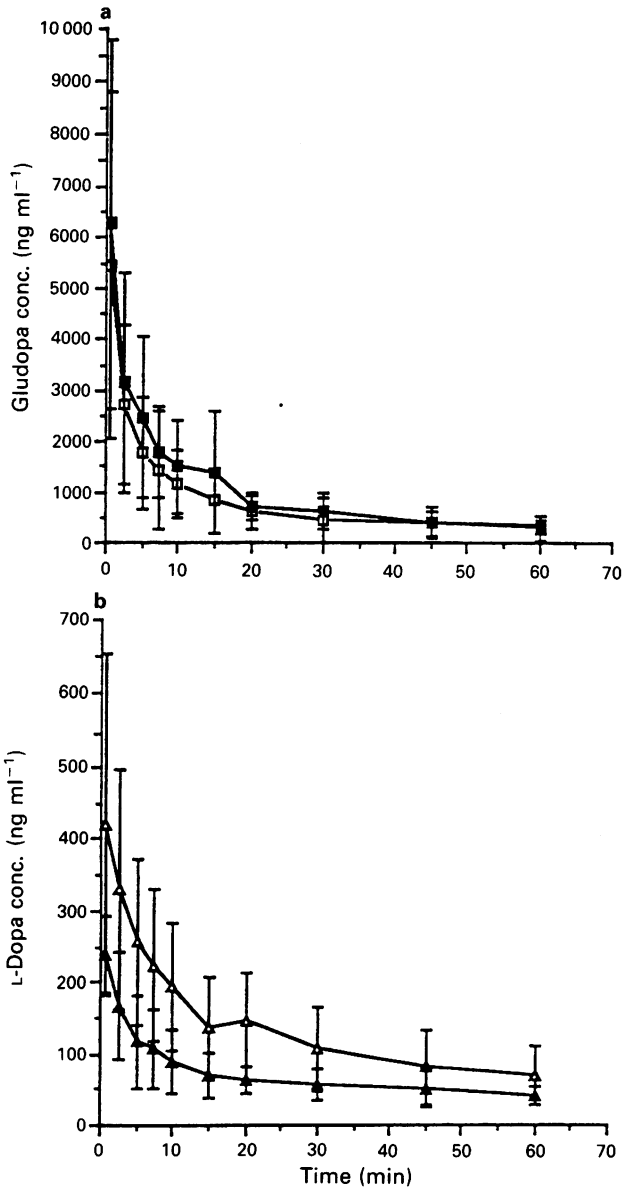


Figure 2 Mean plasma concentration versus time profiles for (a) gludopa and (b) the metabolite L-dopa for controls (■, ▲) compared to rats with acute renal failure (□, △). The vertical lines show s.d., $n = 5$.

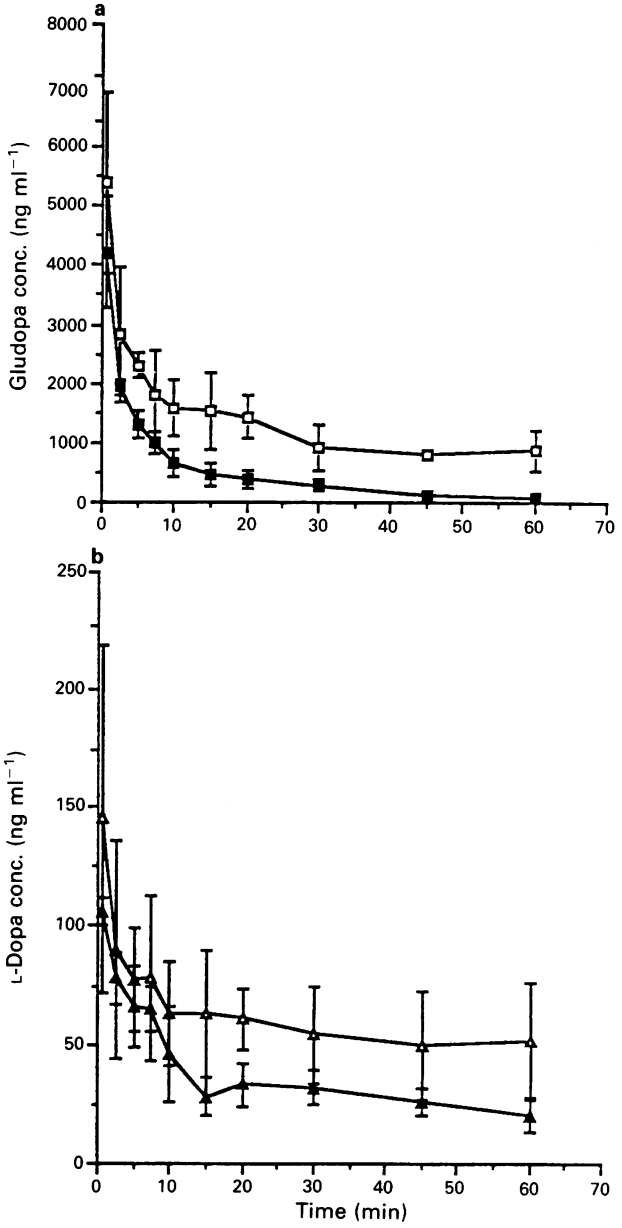


Figure 3 Mean plasma concentration versus time profiles for (a) gludopa and (b) the metabolite L-dopa for controls (■, ▲) compared with anephric rats (□, △). The vertical lines show s.d., $n = 4$.

Table 3 Comparison of the main pharmacokinetic parameters of gludopa and the metabolite L-dopa of anephric rats with controls after 2 mg kg^{-1}

Parameter	*Anephric* ($n = 4$)	*Control* ($n = 4$)
Gludopa		
Cl (ml min^{-1} kg^{-1})	17.1 ± 5.3	63.4 ± 8.3****
MRT (min)	73.0 ± 28.7	20.9 ± 3.9**
$t_{1/2}$ (min)	50.6 ± 19.9	14.5 ± 2.7**
V_{ss} (l kg^{-1})	1.15 ± 0.25	1.30 ± 0.15
K (h^{-1})	0.93 ± 0.40	2.95 ± 0.57***
L-Dopa		
MRT (min)	171.7 ± 64.1	51.2 ± 13.4**
$t_{1/2}$ (min)	118.5 ± 44.3	35.4 ± 9.3**
K (h^{-1})	0.73 ± 0.42	2.23 ± 0.95*

The results are given as mean ± s.d. Kinetic parameters were compared by Student's non-paired t test. * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$; **** $P < 0.001$. For key to abbreviations used see Table 1.

From Table 3, total elimination rate constant for gludopa gave a K_t of 2.95 h^{-1} while the non-renal elimination rate constant, K_{nr} was 0.93 h^{-1} (i.e. the K values for controls and anephrics respectively). By difference the renal elimination rate constant, K_r was 2.02 h^{-1}. The kidney would appear to contribute about 68.5% of the elimination of gludopa.

For L-dopa, K_t was 2.23 h^{-1} and K_{nr} 0.73 h^{-1} (i.e. the K values for controls and anephrics respectively). By difference K_r was 1.50 h^{-1}. The kidney seems to contribute about 67.2% of the elimination of the metabolite L-dopa.

Discussion

The high Cl and K, and short MRT show that gludopa is rapidly cleared from plasma. This is a measure both of the high V_{ss} and the efficiency with which γ-GT hydrolyses gludopa to L-dopa. L-Dopa also had a short MRT, suggesting that the decarboxylation of formed L-dopa to dopamine is also a rapid process, though dopamine was not measured in this study. The high V_{ss} suggests that gludopa is either concentrated in a particular tissue (most probably the kidney), or widely distributed in total body water. Since anephric rats did

not have a significantly reduced V_{ss}, the latter is likely to be the more probable explanation.

The K of L-dopa was found to be significantly reduced in rats in group C (gludopa 7.5 mg kg^{-1}) compared to that in group A (gludopa 2 mg kg^{-1}), while the corresponding values for gludopa showed no difference. It is possible that at higher doses the conversion of L-dopa to dopamine may be rate-limiting in the sequential conversion of gludopa to dopamine. The kidney is rich in AAAD (Lovenberg *et al.*, 1962), the enzyme being located in both proximal and distal tubules (Goldstein *et al.*, 1972). Since decarboxylation does not appear to be rate-limiting (Brown & Dollery, 1981), it is more likely that it is the active reabsorption of generated L-dopa from the tubules (Chan, 1976) which is rate-limiting after the highest dose of gludopa. Though this saturation of the uptake would lead to an increase in the excretion of unchanged L-dopa, it would also lead to a decrease in the decarboxylation of L-dopa to dopamine within the cells. Since the latter is the major route of L-dopa elimination, an overall decrease in the elimination rate constant of L-dopa results.

The fraction of injected gludopa metabolised to L-dopa in our studies was 0.54 or 54%. Complete hydrolysis of gludopa would give a ratio of 0.6 or 60% since L-dopa constitutes about 60% of the molecular weight of the gludopa molecule (the balance of 40% being the glutamyl group). For every mol of L-dopa formed, a mol of glutamate is released. Thus an Fm of 0.54 for L-dopa will give an Fm of 0.36 for glutamate. So it would appear that most of the injected dose of gludopa (at 2 mg kg^{-1}) was hydrolysed to L-dopa (and glutamate) and that approximately 10% was excreted unchanged.

The finding that there seems to be no significant differences in the main pharmacokinetic parameters of gludopa between ARF and control rats was surprising. The structural damage caused by glycerol-induced ARF is largely in the proximal tubules (Finckh, 1957; Carroll *et al.*, 1965), where the γ-GT responsible for gludopa metabolism is also predominantly located (Orlowski & Szewczuk, 1961). It is probable that the concentration of renal γ-GT is so high that even in acute tubular necrosis there is enough residual activity to convert gludopa at normal rates. Indeed Finckh (1957) found that after glycerol injection, those proximal tubules which survived the widespread necrosis had normal brush border, where γ-GT is thought to be located. Under these circumstances, since the kidney is also rich in AAAD, the active uptake of the metabolite L-dopa and its subsequent metabolism could be normal, explaining perhaps why the disposition of L-dopa also remained unchanged in ARF. These findings may suggest that if gludopa were given to man to correct incipient ARF, as has been suggested, (Casson *et al.*, 1983) its disposition could remain normal, ensuring that dopamine generated in the kidney might exert a corrective effect.

There were some differences in the pharmacokinetic parameters of the control rats in the study on ARF compared to those in experiment 1 on normal rats. It is difficult to explain the greater variability in the parameters of control rats in the ARF study. However, the mean values obtained are within the normal range established in experiment 1 for the three dosage groups (see Table 1).

The highly significant differences in the kinetics of both gludopa and L-dopa betewen anephrics and controls was predictable. There was a 4 fold reduction in plasma clearance of these substances in anephrics compared to controls, with corresponding increases in MRT and $t_{1/2}$ and reductions in elimination rate constant. The renal component of the elimination rate constant for gludopa, K_r was 2.02 h^{-1}, representing about 68.5% of total elimination. This is a mean value and the variability in the K values (see Table 2) suggests that K_r values could have ranged from about 45% to 85%, though K_r values could not be calculated for individual rats. In man the kidney contributes about 86% of γ-GT activity compared to about 14% for all other tissues (Orlowski & Szewczuk, 1961; Wilk *et al.*, 1978). Our data are similar to this, supporting the hypothesis that the site of gludopa metabolism is dictated by the distribution of γ-GT, and that the kidney is the principal organ of gludopa metabolism. The K_r of metabolite L-dopa was 1.50 h^{-1}, representing about 67.2% of total elimination. This figure parallels that for gludopa since elimination of L-dopa is closely linked to its formation from gludopa.

In conclusion, the pharmacokinetics of gludopa and its metabolite L-dopa suggest that gludopa is an efficient prodrug for L-dopa and dopamine. The kinetics remain unaltered in ARF with possible important implications for the use of gludopa in this condition. The kidney contributes nearly 70% to the elimination of gludopa and it seems that this renal specificity of gludopa persists in acute renal failure.

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References

ANTON, A.H. & SAYRE, D.F. (1964). The distribution of dopamine and dopa in various animals and a method for their determination in diverse biological material. *J. Pharmacol. Exp. Ther.*, **145**, 326–336.

BAINES, R.W. & CHAN, W. (1980). Production of urine free dopamine from DOPA; a micropuncture study. *Life Sci.*, **26**, 253–259.

BROWN, M.J. & ALLISON, D.J. (1981). Renal conversion of plasma dopa to urine dopamine. *Br. J. Clin. Pharmacol.*, **12**, 251–253.

BROWN, M.J. & DOLLERY, C.T. (1981). A specific radioenzymatic assay for dihydroxyphenylalanine (DOPA). Plasma dopa may be the precursor of urine free dopamine. *Br. J. Clin. Pharmacol.*, **1**, 79–83.

CARROLL, R., KOVAKS, K. & TAPP, E. (1965). The pathogenesis of glycerol-induced renal tubular necrosis. *J. Path. Bact.*, **89**, 573–580.

CASSON, I.F., CLAYDEN, D.A., COPE, G.F. & LEE, M.R. (1983). The protective effect of gamma glutamyl L-dopa on the glycerol treated rat model of acute renal failure. *Clin. Sci.*, **65**, 159–164.

CHAN, K.K. (1982). A simple integrated method for drug and derived metabolite kinetics – An application of the statistical moment theory. *Drug. Metab. Dispos.*, **10**, 474–479.

CHAN, Y.L. (1976). Cellular mechanisms of renal tubular transport of L-dopa and its derivatives in the rat: microperfusion studies. *J. Pharmacol. Exp. Ther.*, **199**, 17–24.

FINCKH, E.S. (1957). Experimental acute tubular nephrosis following subcutaneous injection of glycerol. *J. Path. Bact.*, **73**, 69–85.

GIBALDI, M. & PERRIER, D. (1982). *Pharmacokinetics*, 2nd edn. pp. 409–417. New York: Marcel Dekker Inc.

GOLDSTEIN, M., FUXE, K. & HOKFELT, T. (1972). Characterisation and tissue localisation of catecholamine synthesizing enzymes. *Pharmacol. Rev.*, **24**, 293–309.

JACKSON, B., CUBELA, R.B., CONWAY, E.L. & JOHNSTON, C.I. (1988). Lisinopril pharmacokinetics in chronic renal failure. *Br. J. Clin. Pharmacol.*, **25**, 719–724.

JEFFREY, R.F., MACDONALD, T.M., MARWICK, K. & LEE, M.R. (1988). The effect of carbidopa and indomethacin on the renal response to gamma glutamyl L-dopa in normal man. *Br. J. Clin. Pharmacol.*, **25**, 195–201.

KAPLAN, S.A., JACK, M.L., COTLER, S. & ALEXANDER, K. (1973). Utilisation of area under the curve to elucidate the disposition of an extensively biotransformed drug. *J. Pharmacokinet. Biopharm.*, **1**, 201–216.

KRAAN, J., JONKMAN, J.H.G., KOETER, G.H., GIPS, C.H., DE JONG P.E., VAN DER MARK, TH. W., EKMAN, I. & DE ZEEUW, R.A. (1988). The pharmacokinetics of theophylline and enprofylline in patients with liver cirrhosis and in patients with chronic renal disease. *Dur. J. Clin. Pharmacol.*, **35**, 357–362.

LEBER, H.W., LUDWIG, G. & SCHUTTERLE, G. (1978). Enzyme induction in uremic liver. *Kidney Int.*, **13**, (Suppl. 8): S43–S48.

LEE, M.R. (1982). Dopamine and the kidney. *Clin. Sci.*, **62**, 439–448.

LOVENBERG, W., WEISSBACH, H. & UDENFRIEND, S. (1962). Aromatic L-amino acid decarboxylase. *J. Biol. Chem.*, **237**, 89–93.

MacDONALD, T.M., JEFFREY, R.F., FREESTONE, S. & LEE, M.R. (1988). (+)-Sulpiride antagonises the renal effects of gamma glutamyl L-dopa in man. *Br. J. Clin. Pharmacol.*, **25**, 203–212.

MORAVEK, J., SCHUCK, O., HATALA, M. & PRIBORSKY, J. (1987). Preclinical modelling of changes in drug kinetics caused by acute renal failure in rats. *J. Pharmacokinet. Biopharm.*, **15**, 15–23.

OKEN, D.E., ARCE, M.L. & WILSON, D.R. (1966). Glycerol-induced hemoglobinuric acute renal failure in the rat. I. Micropuncture studies of the development of oliguria. *J. Clin. Invest.*, **45**, 724–735.

ORLOWSKI, M. & SZEWCZUK, A. (1961). Colorimetric determination of gamma-glutamyl transpeptidase activity in human serum and tissues with synthetic substrates. *Acta Biochem. Polon.*, **8**, 189–199.

PATTERSON, S.E. & COHN, V.H. (1984). Hepatic drug metabolism in rats with experimental chronic renal failure. *Biochem. Pharmacol.*, **33**, 711–716.

SCHEFFE, H. (1953). A method for judging all contrasts in the analysis of variance. *Biometrika*, **40**, 87–104.

VAN PEER, A.P. & BELPAIRE, F.M. (1977). Hepatic oxidative drug metabolism in rats with experimental renal failure. *Arch. Int. Pharmacodyn.*, **228**, 180–183.

WILK, S., MIZOGUCHI, H. & ORLOWSKI, M. (1978). Gamma glutamyl L-dopa: A kidney specific dopamine precursor. *J. Pharmacol. Exp. Ther.*, **206**, 227–232.

WILLIAMS, A.J., McQUINN, R.L. & WALLS, J. (1988). Pharmacokinetics of flecainide acetate in patients with severe renal impairment. *Clin. Pharmacol. Ther.*, **43**, 449–455.

WORTH, D.P., HARVEY, J.N., BROWN, J. & LEE, M.R. (1985). Gamma glutamyl L-dopa is a dopamine pro-drug relatively specific for the kidney in normal subjects. *Clin. Sci.*, **69**, 207–214.

YAMAOKA, K., NAKAGAWA, T. & UNO, T. (1978). Statistical moments in pharmacokinetics. *J. Pharmacokinet. Biopharm.*, **6**, 547–558.

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The effects of substance P analogues on the scratching, biting and licking response induced by intrathecal injection of N-methyl-D-aspartate in mice

[1]Tsukasa Sakurada, Yoichi Manome, Koichi Tan-No, Shinobu Sakurada & Kensuke Kisara

Department of Pharmacology, Tohoku College of Pharmacy, 4-4-1 Komatsushima, Sendai 981, Aoba-ku, Japan

1 Intrathecal (i.t.) administration of N-methyl-D-aspartate (NMDA) elicited a dose-dependent behavioural response consisting of licking, biting and scratching in mice.

2 Repeated i.t. injections of 0.4 nmol NMDA, at 5 min intervals, resulted in the rapid development of desensitization to this NMDA-induced behavioural phenomenon.

3 The NMDA-induced response was dose-dependently inhibited by the simultaneous injection of a selective NMDA-receptor antagonist, D-2-amino-5-phosphonovaleric acid.

4 The substance P (SP) analogues [D-Pro^2, D-Trp^{7,9}] SP and [D-Arg^1, D-Trp^{7,9}, Leu^{11}] SP (spantide) inhibited NMDA-induced behavioural responses in a dose-dependent manner. However, [D-Phe^7, D-His^9] SP (6–11), a SP analogue selective for neurokinin$_1$ (NK_1) receptors, failed to inhibit NMDA-induced responses even at a dose of 4.0 nmol.

5 These results indicate that NMDA-induced behavioural responses are mainly mediated through NMDA receptors without affecting NK_1 receptors in the spinal cord.

Introduction

In recent years, much attention has been directed toward the study of the pharmacological and physiological effects of excitatory amino acids (EAAs) in the mammalian central nervous system (CNS). The EAAs are considered to function as neurotransmitters in the CNS. It is most likely that glutamate is one of the EAAs under discussion. Receptors for EAAs have recently been classified into three different types, N-methyl-D-aspartate (NMDA), quisqualate and kainic acid receptors, based on their sensitivity to these three agonists (Davies *et al.*, 1980; McLennan, 1981; Watkins & Evans, 1981). These three receptors are mostly characterized by using pharmacological treatments (Davies *et al.*, 1980; Watkins & Evans, 1981) and are ubiquitously but unevenly distributed in the mammalian CNS (Cotman *et al.*, 1987).

Several lines of evidence indicate that EAAs may be involved in the neurotransmission of painful stimuli in the spinal cord (Anis *et al.*, 1983; Aanonsen & Wilcox, 1987). This suggestion is supported by the fact that NMDA can induce biting and scratching behaviour resembling that of substance P (SP) when administered intrathecally (i.t.) in the behavioural assay (Aanonsen & Wilcox, 1986). The behavioural effects indicative of activation of sensory pathways are brought about by i.t. injections of not only EAAs, but also tachykinins (Hylden & Wilcox, 1981; 1983; Vaught *et al.*, 1984), somatostatin (Seybold *et al.*, 1982), bombesin (O'Donohue *et al.*, 1984), 5-hydroxytryptamine (5-HT) (Fasmer & Post, 1983), and muscarinic agonists (Raffa *et al.*, 1987).

The main purpose of the present investigation was to examine the effects of SP analogues on NMDA-induced behavioural responses in order to see the interaction with SP receptors in the mouse spinal cord.

Methods

Male ddY-strain mice weighing between 22–26 g were used for all experiments. Mice were housed in colony cages with food and water continuously available. Testing took place during the light period of a 12–12 h light-dark cycle. All i.t. injections were adapted from the method of Hylden & Wilcox (1980). The lumbar puncture was made in unanaesthetized mice at the L5 and L6 intervertebral space with a 28 gauge needle on a 50 μl Hamilton syringe. NMDA was dissolved in sterile artificial cerebrospinal fluid containing (g 1000 ml^{-1}): NaCl 7.4, KCl 0.19, $MgCl_2$ 0.19 and $CaCl_2$ 0.14 and administered in a total volume of 5 μl, with the exception of repeatedly injected NMDA. For repeated injections of NMDA, 0.4 nmol/5 μl was delivered twice at 5 min or 15 min intervals. Before the i.t. injection, the mice were adapted for 1 h to an individual plastic cage (22.0 × 15.0 × 12.5 cm) which served as the observation chamber. Immediately after the i.t. injection, the mice were placed in the transparent cage and the accumulated response time of scratching, biting, forepaw and hindpaw licking was measured for 5 min. The inhibitory actions of SP analogues and D-(−)-2-amino-5-phosphonovalerate (D-APV), an NMDA antagonist, were determined by co-administration of NMDA (0.4 nmol).

Drugs administered were NMDA (Nakarai Tesque, Inc., Kyoto, Japan), D-APV (Cambridge Research Biochemicals, Cambridge, England), [D-Pro^2, D-Trp^{7,9}] SP (DPDT-SP) and [Arg^1, D-Trp^{7,9}, Leu^{11}] SP (spantide) (Peptide Institute, Inc., Osaka, Japan). [D-Phe^7, D-His^9] SP (6–11) was synthesized by solid phase peptide methodology (Sakurada *et al.*, 1989).

Statistical analysis of the results included determination of ED_{50} values by the method of Litchfield & Wilcoxon (1949) and Dunnett's test for multiple comparisons after analysis of variance (ANOVA). All values are expressed as the mean ± s.e.mean.

Results

Behavioural response induced by intrathecally administered N-methyl-D-aspartate

Intrathecal administration of NMDA resulted in a characteristic behavioural response consisting of vigorous biting, licking and scratching, which peaked at 0–5 min and had disappeared at 15 min post-injection. As seen in Figure 1, a dose-dependent increase in the total time of biting, licking and scratching was observed following i.t. administration of

[1] Author for correspondence.

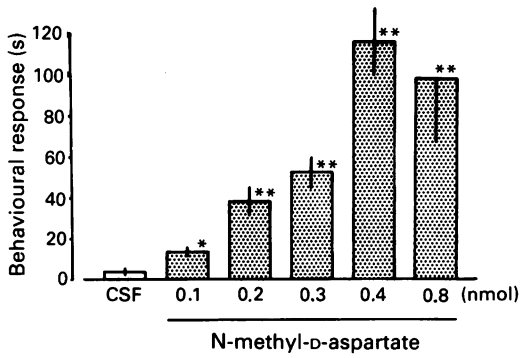

Figure 1 The effect of varying doses (0.1–0.8 nmol per mouse) of N-methyl-D-aspartate (NMDA) administered i.t. in the mouse. The duration of scratching, biting and licking induced by NMDA was determined over a 5 min period starting immediately after injection. * $P < 0.05$, ** $P < 0.01$ when compared with CSF-controls.

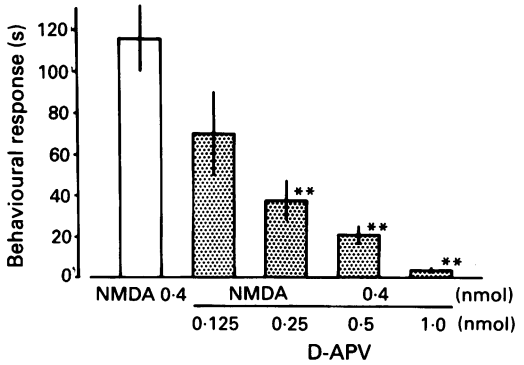

Figure 3 Effect of D-(−)-2-amino-5-phosphonovalerate (D-APV) on N-methyl-D-aspartate (NMDA)-induced scratching, biting and licking response in mice. D-APV was co-administered i.t. with NMDA in a 5 μl volume. The duration of scratching, biting and licking induced by NMDA was determined over a 5 min period starting immediately after injection. ** $P < 0.01$ when compared to NMDA (0.4 nmol) alone.

NMDA (0.1–0.8 nmol). The highest dose (0.8 nmol) of NMDA tested produced seizures in 80% of mice. NMDA-induced seizures were seen immediately after administration and subsided in 2–3 min. In further experiments, 0.4 nmol of NMDA was therefore used in combination with D-APV and SP analogues to test their antagonistic actions. Intrathecal injection of artificial CSF (5 μl) had no apparent effect on the behaviour of animals.

Mice were injected i.t. with 0.4 nmol of NMDA at 5 or 15 min intervals twice to assess the reproducibility of scratching, biting and licking response produced by NMDA. Desensitization to the behavioural response produced by i.t. administration of NMDA (0.4 nmol) was observed when administered at 5 min intervals (Figure 2); injection of 0.4 nmol of NMDA resulted in a significant decrease in the response to a second injection at 5 min intervals.

Effects of D-(−)-2-amino-5-phosphonovalerate and substance P analogues on N-methyl-D-aspartate-induced behavioural response

The selective NMDA receptor antagonist, D-APV (0.125–1.0 nmol), co-administered i.t. with NMDA (0.4 nmol) caused a dose-dependent inhibition of the NMDA-induced response (Figure 3). The ID_{50} for D-APV was 0.195 (0.126–0.303) nmol.

Interaction of putative tachykinin antagonists with the NMDA receptor agonist was investigated. When co-administered i.t. with DPDT-SP (1.0–4.0 nmol), the NMDA-induced response was reduced dose-dependently (Figure 4a).

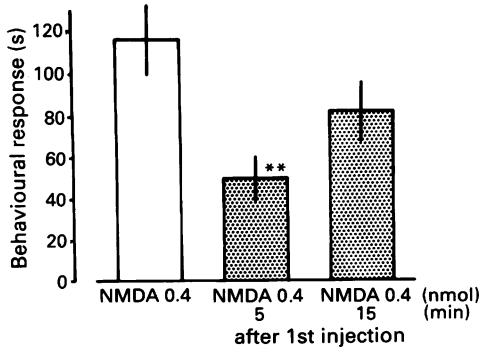

Figure 2 Comparison of the scratching, biting and licking response induced in the mouse by two i.t. injections of N-methyl-D-aspartate (NMDA) 5 or 15 min apart. The duration of scratching, biting and licking induced by NMDA was determined over a 5 min period starting immediately after injection. ** $P < 0.01$ when compared to a single injection of NMDA.

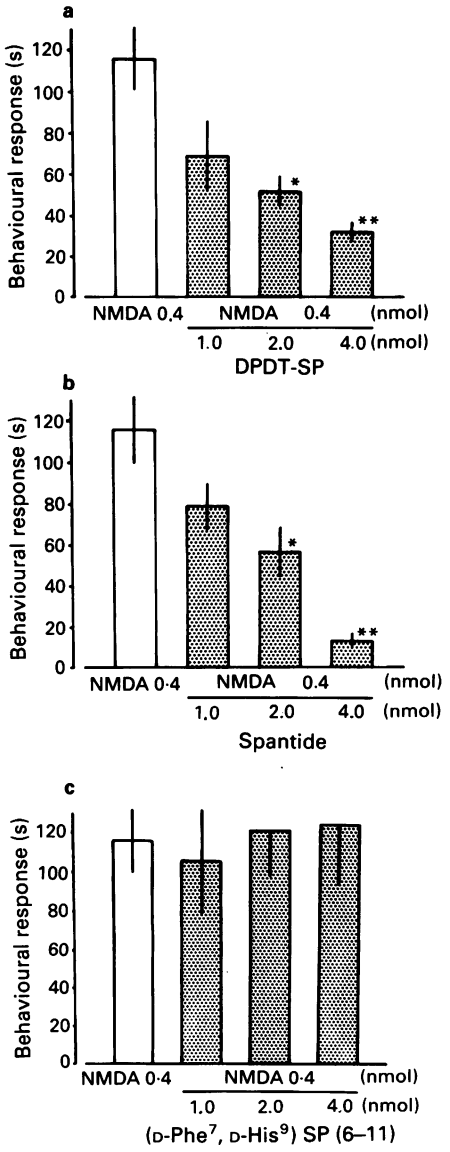

Figure 4 Effect of [D-Pro^2, D-Trp^{7,9}] substance P (DPDT-SP) (a), spantide (b) and (D-Phe^7, D-His^9)-SP (6–11) (c) on N-methyl-D-aspartate (NMDA)-induced scratching, biting and licking response in mice. The duration of scratching, biting and licking induced by NMDA was determined over a 5 min period starting immediately after injection. * $P < 0.05$, ** $P < 0.01$ when compared to NMDA (0.4 nmol) alone.

Spantide, a putative tachykinin antagonist, yielded results similar to those observed for DPDT-SP (Figure 4b). The ID_{50} for DPDT-SP and spantide was 1.66 (0.66–3.88) nmol and 1.70 (1.03–2.81) nmol, respectively. In order to characterize more fully tachykinin receptors, a shortened analogue of SP, (D-Phe^7, D-His^9) SP (6–11) with greater selectivity for spinal NK_1 receptors was used. However, (D-Phe^7, D-His) SP (6–11), in doses ranging from 1.0–4.0 nmol did not antagonize NMDA-induced behavioural response (Figure 4c).

Discussion

The principal finding of the present experiments is that these NMDA-induced behavioural responses are not mediated through spinal NK_1 receptors in mice. Furthermore, desensitization occurs to the behavioural response induced by repeatedly administered NMDA, suggesting that NMDA may act on the NMDA receptors directly in the spinal cord.

The present experiments showed that NMDA injected i.t. produced a dose-dependent behavioural response consisting of biting, licking and scratching. This NMDA-induced behaviour was dose-dependently blocked by co-administration of the NMDA antagonist, D-APV. These data are consistent with the hypothesis that NMDA may have a nociceptive action in the spinal cord (Aanonsen & Wilcox, 1986). It was previously reported that SP and NMDA are able to elicit a behavioural syndrome indicative of nociceptive behavioural response such as biting, licking and scratching (Hylden & Wilcox, 1981; Aanansen & Wilcox, 1986; Sakurada *et al.*, 1987). Both substances can also produce hyperalgesia as measured by the tail-flick and pressure tests in rats or mice (Yasphal *et al.*, 1982; Moochhala & Sawynok, 1984; Matsumura *et al.*, 1985b; Aanonsen & Wilcox, 1986). Desensitization to the actions of i.t. administered SP in eliciting scratching and biting response has been found in mice (Larson, 1988). Similarly, apparent desensitization has been reported to occur to the hyperalgesic effect of i.t. administered SP in rats in the tail-flick assay (Moochhala & Sawynok, 1984). In the present experiments, a similar phenomenon was observed with repeated i.t. injections of NMDA. Taking these results into consideration, i.t. administered NMDA is involved in the transmission of nociceptive information by acting on the spinal NMDA receptors.

The characteristic behavioural response produced by i.t. administered SP was inhibited by a number of SP analogues and opioid peptides (Hylden & Wilcox, 1983; Post & Folkers, 1985; Takahashi *et al.*, 1987; Sakurada *et al.*, 1987; 1988; 1989) when co-administered with SP. Of these SP analogues, DPDT-SP and spantide have often been used as pharmacological and physiological tools to study the participation of SP, though the specificity of SP analogues as antagonists has been discussed (Salt *et al.*, 1982; Stoppinin *et al.*, 1983; Wiesenfeld-Hallin *et al.*, 1987). DPDT-SP has been found to block the 5-hydroxytryptamine induced behavioural syndrome which is similar to that seen after injection of SP (Fasmer & Post, 1983; Vaught & Scott, 1988). Subsequently, we have reported that co-administered spantide resulted in a reduction of the behavioural response produced by not only SP but also by eledoisin, neurokinin A and somatostatin (Sakurada *et al.*, 1989). These results suggest that the two SP analogues do not have a selective action on the NK_1 receptor, at least, in the mouse spinal cord. In addition to the antagonistic effect at tachykinin receptors, neurotoxic actions of DPDT-SP have been reported: (1) I.t. administered DPDT-SP produced long-lasting hindlimb paralysis (Åkerman *et al.*, 1982; Rodgriguez *et al.*, 1983; Matsumura *et al.*, 1985a). (2) Microinjected DPDT-SP into the ventral tegmentum area led to wide-spread neuronal necrosis (Hökfelt *et al.*, 1981). (3). I.t. administered DPDT-SP produced a profound reduction in glutamate, glycine, γ-aminobutyric acid and aspartate (Sakurada *et al.*, 1990) as well as SP (Matsumura *et al.*, 1985a) in the spinal cord of paralysed rats. However, there were no significant changes in the level of amino acids and SP in non-paralysed rats. These alterations may be attributed to the neurotoxic action on the spinal cord (Post & Paulsson, 1985).

In the present study, i.t. co-administration of DPDT-SP produced a dose-dependent inhibition of NMDA-induced behavioural responses. However, this result is not consistent with the previous report that NMDA-induced behaviour was not significantly changed by co-administration of DPDT-SP (DeLander & Wahl, 1989). The difference in the results may be due to the difference in the dose of DPDT-SP used, and of the procedure for behavioural observation. However, the inhibitory effects of DPDT-SP and spantide on the NMDA-induced behavioural response is not due to their selectivity for NMDA receptors, as mentioned above. It is possible that NMDA-induced behaviour is mediated through NK_1 receptors in the spinal cord, since the behavioural response produced by tachykinins has been shown to be mediated mainly by NK_1 receptors (Sakurada *et al.*, 1989). The NMDA-induced behavioural response was also inhibited by co-administration of spantide, a putative tachykinin antagonist. Recently, we have found that a novel peptide (D-Phe^7, D-His^9) SP (6–11) is a selective NK_1 receptor antagonist (Sakurada *et al.*, 1989). SP-induced behavioural response was decreased to less than 20% by 2.0 nmol of this antagonist with an ED_{50} of 0.47 (0.23–0.95) nmol. Thus, this has a relatively low ED_{50} as compared to the ED_{50} values of DPDT-SP and spantide which were 1.88 and 1.00 nmol, respectively, when assayed against SP-induced behavioural responses. It is therefore probable that (D-Phe^7, D-His^9) SP (6–11) is a highly selective and potent NK_1 receptor antagonist in the spinal cord as compared to DPDT-SP and spantide. Moreover, this SP analogue is able to inhibit the SP-induced response without affecting the behavioural responses produced by NK_2 agonists such as neurokinin A and eledoisin, somatostatin and bombesin (Sakurada *et al.*, 1989). The results presented in this paper with the NK_1 receptor antagonist show that it did not antagonize the NMDA-induced behavioural response. Therefore, it seems evident that i.t. administered NMDA acts independently of SP receptors in the mouse spinal cord.

Our investigations have demonstrated the capacity for DPDT-SP and spantide to inhibit NMDA-induced responses. The finding that a selective antagonist for spinal NK_1 receptors, (D-Phe^7, D-His^9) SP (6–11) failed to antagonize NMDA-induced action reveals that i.t. administered NMDA does not interact with the NK_1 receptor to induce scratching, biting and licking responses.

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References

AANONSEN, L.M. & WILCOX, G.L. (1986). Phencyclidine selectively blocks a spinal action of N-methyl-D-aspartate in mice. *Neurosci. Lett.*, **67**, 191–197.

ÅKERMAN, B., ROSELL, S. & FOLKERS, K. (1982). Intrathecal (D-Pro^2, D-Trp^{7,9})-SP elicits hypoalgesia and motor blockade in the rat and antagonizes noxious responses induced by substance P. *Acta Physiol. Scand.*, **114**, 631–633.

ANIS, N.A., BERRY, S.C., BURTON, N.R. & LODGE, D. (1983). The dissocative anesthetics, ketamine and phencyclidine, selectively reduce excitation of central neurons by N-methyl-aspartate. *Br. J. Pharmacol.*, **79**, 564–575.

COTMAN, C.W., MONAGHAN, D.T., OTTERSEN, O.P. & STORM-MATHISEN, J. (1987). Anatomical organization of excitatory amino acid receptors and their pathways. *Trends Neurosci.*, **10**, 273–274.

DAVIES, J., FRANSIS, A.A., JONES, A.W. & WATKINS, J.C. (1980). 2-Amino-5-phosphonovalerate (2-APV), a highly potent and specific antagonist at spinal NMDA receptors. *Br. J. Pharmacol.*, **70**, 52p–53.

DELANDER, G.E. & WAHL, J.J. (1989). In vivo characterization of phenycyclidine/σ agonist-mediated inhibition of nociception. *Eur. J. Pharmacol.*, **159**, 149–156.

FASMER, O.B. & POST, C. (1983). Behavioural responses induced by intrathecal injection of 5-hydroxytryptamine in mice are inhibited by a substance P antagonist, D-Pro^2, D-Trp^{7,9}-substance P. *Neuropharmacology*, **22**, 1397–1400.

HÖKFELT, T., VINCENT, S., HELLSTEN, I., ROSELL, S., FOLKERS, K., MARKEY, K., GOLDSTEIN, M. & CUELLO, C. (1981). Immunohistochemical evidence for a neurotoxic action of [D-Pro^2, D-Trp^{7,9}]-substance P, an analogue with substance P antagonistic activity. *Acta Physiol. Scand.*, **113**, 571–573.

HYLDEN, J.L.K. & WILCOX, G.L. (1980). Intrathecal morphine in mice: a new technique. *Eur. J. Pharmacol.*, **67**, 313–316.

HYLDEN, J.L.K. & WILCOX, G.L. (1981). Intrathecal substance P elicits a caudally-directed biting and scratching behaviour in mice. *Brain Res.*, **217**, 212–215.

HYLDEN, J.L.K. & WILCOX, G.L. (1983). Intrathecal opioids block a spinal action of substance P in mice: functional importance of both μ- and δ-receptors. *Eur. J. Pharmacol.*, **86**, 95–98.

LARSON, A.A. (1988). Desensitization to intrathecal substance P in mice: possible involvement of opioids. *Pain*, **32**, 367–374.

LITCHFIELD, J.T. & WILCOXON, F. (1949). A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Ther.*, **96**, 99–113.

MATSUMURA, H., SAKURADA, T., HARA, A., SAKURADA, S. & KISARA, K. (1985a). Intrathecal substance P analogue causes a motor dysfunction in the rat. *Neuropharmacology*, **24**, 811–813.

MATSUMURA, H., SAKURADA, T., HARA, A., SAKURADA, S. & KISARA, K. (1985b). Characterization of the hyperalgesic effect induced by intrathecal injection of substance P. *Neuropharmacology*, **24**, 421–426.

McLENNAN, H. (1981). On the nature of the receptors for various excitatory amino acids in the mammalian central nervous system. In *Glutamate as a Neurotransmitter*. ed. DiChiara, G. & Gessa, G.L., pp. 253–262. New York: Raven Press.

MOOCHHALA, S.M. & SAWYNOK, J. (1984). Hyperalgesia produced by intrathecal substance P and related peptides: desensitization and cross desensitization. *Br. J. Pharmacol.*, **82**, 381–388.

O'DONOHUE, T.L., MASSARI, V.J., PAZOLES, C.J., CHRONWALL, B.M., SHULTS, C.W., QUIRION, R., CHASE, T.N. & MOODY, T.W. (1984). A role for bombesin in sensory processing in the spinal cord. *J. Neurosci.*, **4**, 2956–2962.

POST, C. & FOLKERS, K. (1985). Behavioural and antinociceptive effects of intrathecally injected substance P analogues in mice. *Eur. J. Pharmacol.*, **113**, 335–343.

POST, C. & PAULSSON, I. (1985). Antinociceptive and neurotoxic actions of substance P analogues in the rat's spinal cord after intrathecal administration. *Neurosci. Lett.*, **57**, 159–164.

RAFFA, R.B., MATHIASEN, J.R., GOODE, T.L. & VAUGHT, J.L. (1987). Evidence that reciprocal hindlimb scratching elicited in mice by intrathecal administration of muscarinic agonists is mediated through M1 type receptors. *Life Sci.*, **41**, 1831–1836.

RODRIGUEZ, R.E., SALT, T.E., CAHUSAC, P.M.B. & HILL, R.G. (1983). The behavioural effects of intrathecally administered (D-Pro^2, D-Trp^{7,9})-substance P, an analogue with presumed antagonist actions. *Neuropharmacology*, **22**, 173–176.

SAKURADA, T., KUWAHARA, H., SAKURADA, S., KISARA, K., OHBA, M. & MUNEKATA, E. (1987). Behavioural assessment as substance P antagonists in mice. *Neuropeptides*, **9**, 197–206.

SAKURADA, T., TAKAHASHI, K., SAKURADA, S., KISARA, K., FOLKESSON, R. & TERENIUS, L. (1988). Enkephalins interact with substance P-induced aversive behaviour in mice. *Brain Res.*, **442**, 191–194.

SAKURADA, T., YAMADA, T., SAKURADA, S., KISARA, K. & OHBA, M. (1989). Substance P analogues containing histidine antagonize the behavioural effects of intrathecally co-administered substance P in mice. *Eur. J. Pharmacol.*, **174**, 153–160.

SAKURADA, T., HARA, A., MATSUMURA, H., YAMADA, H., SAKURADA, S. & KISARA, K. (1990). A substance P analogue reduces amino acid contents in the rat spinal cord. *Pharmacol. Toxicol.*, **66**, 75–76.

SALT, T.E., DE VRIES, G.J., RODRIGUEZ, R.E., CAHUSAC, P.M.B., MORRIS, R. & HILL, R.G. (1982). Evaluation of (D-Pro^2, D-Trp^{7,9})-substance P as an antagonist of substance P responses in the rat central nervous system. *Neurosci. Lett.*, **30**, 291–295.

SEYBOLD, V.S., HYLDEN, J.L.K. & WILCOX, G.L. (1982). Intrathecal substance P and somatostatin in rats: Behaviors indicative of sensation. *Peptides*, **3**, 49–54.

STOPPINI, L., BAERTSCHI, A.J., MATHISON, R. & BARJA, F. (1983). Neuronal actions of several substance P antagonists in the rat spinal cord. *Neurosci. Lett.*, **37**, 279–283.

TAKAHASHI, K., SAKURADA, T., SAKURADA, S., KUWAHARA, H., YONEZAWA, A., ANDO, R. & KISARA, K. (1987). Behavioural characterization of substance P-induced nociceptive response in mice. *Neuropharmacology*, **26**, 1289–1293.

VAUGHT, J.L., POST, L.J., JACOBY, H.I. & WRIGHT, D. (1984). Tachykinin-like central activity of neuromedin K in mice. *Eur. J. Pharmacol.*, **103**, 355–357.

VAUGHT, J.L. & SCOTT, R. (1988). Interactions of substance P antagonists with serotonin in the mouse spinal cord. *Peptides*, **9**, 909–913.

WATKINS, J.C. & EVANS, R.H. (1981). Excitatory amino acid transmitters. *Annu. Rev. Pharmacol. Toxicol.*, **21**, 165–204.

WIESENFELD-HALLIN, Z. & DURANTI, R. (1987). D-Arg^1, D-Trp^{7,9}, Leu^{11}-substance P (spantide) does not antagonize substance P-induced hyperexcitability of nociceptive flexion withdrawal reflex in the rat. *Acta Physiol. Scand.*, **129**, 55–59.

YASPHAL, K., WRIGHT, D. & HENRY, J.L. (1982). Substance P reduces tail-flick latency: Importance for chronic pain syndrome. *Pain*, **14**, 155–167.

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The role of cyclic AMP and its protein kinase in mediating acetylcholine release and the action of adenosine at frog motor nerve endings

[2]Jody K. Hirsh, [1]Eugene M. Silinsky & [3]Carles S. Solsona

Department of Pharmacology, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, Illinois 60611, U.S.A.

1 The importance of adenosine 3':5'-cyclic monophosphate (cyclic AMP) and its protein kinase (protein kinase A, PKA) in promoting acetylcholine (ACh) release was studied at frog motor nerve endings. The effects of cyclic AMP-dependent protein phosphorylation on the action of adenosine receptor agonists were also investigated.

2 Cyclic AMP was delivered to a local region of the cytoplasm just beneath the plasma membrane of motor nerve endings using phospholipid vesicles (liposomes) as a vehicle. Cyclic AMP in liposomes produced a parallel reduction in the mean level of evoked ACh release (m) and spontaneous ACh release (miniature endplate potential frequency; $m.e.p.p._f$) in most experiments. These inhibitory effects of cyclic AMP on quantal ACh release resemble the action of adenosine.

3 The effects of global increases in cytoplasmic cyclic AMP concentrations using lipophilic cyclic AMP analogues were generally different from those observed with cyclic AMP. 8-(4-Chlorophenylthio) cyclic AMP (CPT cyclic AMP) produced approximately two fold increases in m and $m.e.p.p._f$. Dibutyryl cyclic AMP (db cyclic AMP) also increased m and $m.e.p.p._f$, with the effect on m being smaller and more variable.

4 All three cyclic AMP analogues reduced the effects of adenosine receptor agonists on spontaneous and evoked ACh release.

5 The roles of protein phosphorylation in mediating ACh release and the inhibitory effects of adenosine were studied with the protein kinase inhibitor H7. H7 (30–100 μM) produced no consistent effect on evoked or spontaneous ACh release. At these concentrations, however, H7 exerted an unfortunate inhibitory action on the nicotinic ACh receptor/ion channel.

6 H7 prevented the increases in spontaneous ACh release produced by CPT cyclic AMP (250 μM). Thus H7 is likely to inhibit PK A in frog motor nerve endings.

7 H7 did not alter the inhibitory effect of adenosine on evoked and spontaneous ACh release.

8 The results suggest: (i) that the adenylyl cyclase-cyclic AMP-PK A system is compartmentalized within the motor nerve terminal, (ii) that phosphorylation does not play a major role in ACh release and (iii) the cyclic AMP-PK A system modulates rather than mediates the inhibitory effects of adenosine.

Introduction

Adenosine 3':5'-cyclic monophosphate (cyclic AMP) is a ubiquitous modulator that transduces the activation of specific cell surface receptors into a biological response (Dunwiddie & Hoffer, 1982; North, 1989). At peripheral cholinergic nerve endings, the role of cyclic AMP in coupling presynaptic receptor activation to changes in transmitter release is controversial; cyclic AMP has been suggested to increase (Goldberg & Singer, 1969; Standaert & Dretchen, 1979; Branisteanu *et al.*, 1988; Dryden *et al.*, 1988), decrease (Kuba *et al.*, 1981; Silinsky, 1984), or have no effect (Miyamoto & Breckenridge, 1974) on acetylcholine (ACh) secretion.

One possible explanation for these disparate results could be compartmentation of the effects of cyclic AMP in the nerve ending (Harper *et al.*, 1985). Specifically, it has been suggested that increases in cyclic AMP concentration at local submembrane regions could inhibit ACh release (Moskowitz & Puszkin, 1985; Silinsky, 1986) whilst more global increases in cyclic AMP at synaptic vesicles (Moskowitz & Puszkin, 1985; Silinsky, 1985b) or Ca storage sites (Cocks *et al.*, 1984; Silinsky & Vogel, 1986; Harper, 1988) could increase ACh release. One objective of this paper is to test the compartmentation hypothesis by comparing the effects of the localized delivery of cyclic AMP to the cytoplasm (using lipid vesicles as a vehicle) with more widespread increases in cytoplasmic cyclic AMP concentrations (using stable, membrane permeant cyclic AMP analogues).

Adenosine has been implicated as a negative feedback modulator of ACh release and its receptors are frequently coupled to adenylyl cyclase. Unfortunately, the coupling of adenosine receptor activation to presynaptic adenylyl cyclase is also controversial, with positive coupling, negative coupling, or no coupling through this enzyme being suggested (for review see Silinsky, 1989). Another objective of this paper is to evaluate the contribution of cyclic AMP-dependent processes to the inhibitory effects of adenosine. In this regard, the effects of increases in cyclic AMP concentrations and of inhibition of cyclic AMP-dependent protein kinase (protein kinase A, PK A) on the action of adenosine will be evaluated. H7 (1-(5-isoquinolinylsulphonyl)-2-methylpiperazine) has been found to inhibit PK A, protein kinase C (PK C), and protein kinase G (PK G) with apparent equilibrium dissociation constants (K_is) in the low micromolar range (Hidaka *et al.*, 1984). If it can be shown that H7 fails to alter the presynaptic effects of adenosine in preparations where H7 has been shown to act as a PK A inhibitor, then it is likely that adenosine acts independently of cyclic AMP-dependent protein phosphorylation.

The results of this study support the hypothesis that the effects of cyclic AMP are compartmentalized in the nerve

[1] Author for correspondence.
[2] Present address: Department of Physiology, Rush Medical College, 1750 West Harrison Street, Chicago, IL 60612, U.S.A.
[3] Present address: Department of Cellular Biology and Pathology, University of Barcelona, Casanova 143, 08036 Barcelona, Spain.

ending. They also suggest that while the action of adenosine may be modified by changes in cyclic AMP levels, adenosine inhibits ACh release independently of protein kinase A at frog motor nerve endings. Brief accounts of these results have appeared in abstract form (Hirsh *et al.*, 1988; Hirsh & Silinsky, 1989).

Methods

Electrophysiological procedures

Frog pectoralis proprius nerve-cutaneous pectoris muscle preparations (*Rana pipiens*) were dissected and superfused with flowing Ringer solution. Conventional electrophysiological techniques for stimulation and recording were employed (see Silinsky, 1984; 1987). Continuous intracellular recordings of endplate potentials (e.p.ps) and miniature endplate potentials (m.e.p.ps) were made from individual endplates, with each fibre serving as its own control. In the majority of instances, experiments were carried out with the assistance of an LSI 11/73 minicomputer (Cambridge Digital) and 125 kHz 14 bit A/D-12 bit D/A converter on line with a hard copy of the digitalized traces being made on an XY plotter (Hewlett Packard 7470A). In a few experiments, e.p.ps were averaged by a computer of average transients (Nicolet) in which case the average was displayed on a pen recorder or XY plotter. In most experiments, the frequency of m.e.p.ps (m.e.p.p.$_f$) and m.e.p.p. amplitudes were determined by an automated analysis using the LSI computer in conjunction with a circulating buffer and a FORTRAN programme that uses an algorithm based upon the initial slope of the rising phase to detect an event ('mepp-o-matic'). The m.e.p.ps were first recorded on magnetic tape (Indec IR2 Instrumentation Recorder) before analysis by minicomputer. In some experiments, ACh potentials were produced by applying puffs of ACh (100–500 μM dissolved in Ringer) from a 1–3 MΩ microelectrode connected to a source of pressurized gas (Picospritzer II, General Valve Corporation).

Measurements of quantal ACh release

In the absence of (+)-tubocurarine (TC), the mean number of ACh quanta released synchronously by a nerve impulse (m) was determined from the ratio of the mean computer averaged e.p.p. to the mean m.e.p.p. amplitude (del Castillo & Katz, 1954). In the experiments in which cyclic AMP analogues or adenosine was studied in curarized preparations, changes in e.p.p. amplitudes were used as a measure of the presynaptic effects of these agents (for justification see Ginsborg & Hirst, 1972; Silinsky, 1984).

Preparation of cyclic adenosine monophosphate-containing lipid vesicles

Dispersions of cyclic AMP and phosphatidylcholine (egg PC) were subjected to ultrasonic irradiation, forming unilamellar lipid vesicles (liposomes) with an entrapped core of cyclic AMP (for precise details of preparation, see Mellow *et al.*, 1982). Liposomes were suspended in Ringer and applied to the preparation by superfusion. In half of these experiments 100 mM cyclic AMP as the free acid was employed as the formation solution for the liposomes with KOH added to establish pH 7.2–7.4; KCl-containing liposomes with the formation solution equimolar to the added KOH served as the control. In the remaining experiments, the Na salt of cyclic AMP was used with equimolar NaCl as the control. Liposomes formed in this manner have been shown to deliver their entrapped contents reversibly to the cytoplasm of the motor nerve ending, i.e. when Ringer containing liposomes is exchanged for liposome-free solution ACh release returns to the control level (Rahamimoff *et al.*, 1978; Kharasch *et al.*, 1981; Mellow *et al.*, 1982). Moreover, lipid vesicles containing cyclic AMP have been used successfully in the past to deliver this membrane-impermeable nucleotide to the cytoplasm of living cells (Papahadjopoulos *et al.*, 1974).

Statistical methods

Statistical procedures were identical to those described previously (see Silinsky, 1984; 1987). Statistically significant differences were generally observed at $P \ll 0.01$. In instances where significance was at the $P < 0.05$ level, this is stated in the text. Corrections for non-linear summation were employed when necessary (McLachlan & Martin, 1981).

Composition of solutions and chemicals

All Ringer solutions were applied by superfusion using a flow inducer and removed by suction. Normal frog Ringer contained (mM): NaCl 115, KCl 2, $CaCl_2$ 1.8, $NaHCO_3$ 2 (pH 7.2–7.4). TC (4–5 mg l^{-1}) was used in some experiments to reduce the e.p.p. below threshold for muscle contraction. Normal frog Ringer containing TC was used for all studies performed at normal levels of ACh release. At low levels of ACh release, low Ca-high Mg Ringer (which contained 0.35–4 mM $CaCl_2$, 3–6 mM $MgCl_2$, and 1 mg l^{-1} neostigmine methyl sulphate to increase the size of the m.e.p.ps) was employed. Ca-free Ringer contained no added Ca and 1–3 mM $MgCl_2$. In the experiments with cyclic AMP-containing liposomes, 2 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid), pH 7.1, was employed as the buffer. Most chemicals were purchased from the Sigma Chemical Company (St. Louis, MO, U.S.A.) including H7 (free acid and dihydrochloride). In three experiments, H7 (dihydrochloride) was obtained instead from Seikagaku America, Inc. (St. Petersburg, FL, U.S.A.) and was found to be equivalent to the product purchased from Sigma.

Results

The effects of cyclic AMP analogues on ACh release

Unilammelar phospholipid vesicles (liposomes) have been shown to deliver their entrapped contents locally to a compartment just beneath the plasma membrane (Theoharides & Douglas, 1978; see also Discussion). Thus cyclic AMP in liposomes would be expected to produce increases in cyclic AMP in a restricted cytoplasmic region near the membrane of the nerve ending. In contrast, continuous superfusion with stable, lipophilic cyclic AMP derivatives would produce higher concentrations at more distal sites in the nerve terminal. Based upon the above hypothesis of compartmentation of cyclic AMP effects presented in the Introduction (Moskowitz & Putzkin, 1985; Silinsky, 1986), it might be predicted that cyclic AMP delivered in liposomes would inhibit ACh release whilst lipophilic cyclic AMP analogues might stimulate ACh release.

Cyclic AMP-containing liposomes Figure 1 shows the typical result of four experiments in which cyclic AMP was delivered directly to the cytoplasm of the motor nerve terminals when liposomes were used as a vehicle. In this experiment, the increase in cytoplasmic cyclic AMP produced a reversible reduction in m (Figure 1) and m.e.p.p.$_f$ (see figure legend) by approximately 50%. These effects are similar to the published effects of adenosine from this and other laboratories, namely a maximal inhibitory effect on m and m.e.p.p.$_f$ of 50% of the control (see Ginsborg & Hirst, 1972; Silinsky, 1984 and Table 2 in this paper). For a further discussion of these experiments, see legend to Figure 1.

It might be argued that the leakage of cyclic AMP to the extracellular fluid and its subsequent effects on extracellular adenosine receptors was producing the behaviour shown in Figure 1. Figure 2 shows that this is not the case; cyclic AMP-containing liposomes retained their ability to reduce ACh release with the adenosine receptor blocker theophylline

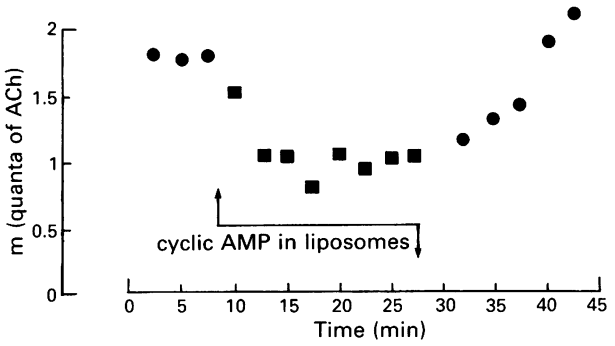

Figure 1 Inhibitory effects of increases in cellular cyclic AMP concentrations on acetylcholine (ACh) release. The cyclic AMP was delivered to the cytoplasm via phospholipid vesicles (liposomes). Each symbol is the averaged response to 64 stimuli delivered at a frequency of 0.5 Hz. Low Ca, high Mg Ringer was employed in this experiment and the experiment of Figure 2. Control (●) consisted of KCl in liposomes (see Methods). Cyclic AMP in liposomes (■) consisted of 100 mM cyclic AMP with KOH added to pH 7.2. Highly significant declines in both m (shown as symbols) and m.e.p.p.$_f$ (not shown) of approximately 50% were observed. M.e.p.p. frequencies (s^{-1}): control (0.69 ± 0.14, $n = 49$), cyclic AMP-containing liposomes (0.35 ± 0.09, $n = 55$), post-cyclic AMP control (0.79 ± 0.13, $n = 38$). Note that the level of ACh release continued to increase during the post-cyclic AMP control solution (not shown). This figure shows the typical result of four experiments. In two other experiments, either no change in ACh release occurred (despite inhibition of the effects of adenosine by cyclic AMP) or a modest (less than 20%) increase in ACh release was produced by cyclic AMP liposomes.

(1.8 mM) present in the bathing fluid throughout the entire experiment.

Lipophilic cyclic AMP analogues Cellular cyclic AMP levels may be elevated in a technically less demanding manner by use of membrane permeant cyclic AMP analogues. We employed two membrane permeant cyclic AMP analogues in this study, 8-(4-chlorophenylthio) cyclic AMP (CPT cyclic AMP) and dibutyryl cyclic AMP (db cyclic AMP). The results will focus on CPT cyclic AMP as this analogue is the most potent activator of PK A available commercially (Beebe *et al.*, 1988). The results with db cyclic AMP are presented in an abridged manner for the purpose of comparison with other studies; indeed, this agent is not a potent activator of kinase A and been suggested that it produces its intracellular effects, in part, by acting as a phosphodiesterase (PDE) inhibitor rather than by directly stimulating PK A (Harper, 1988).

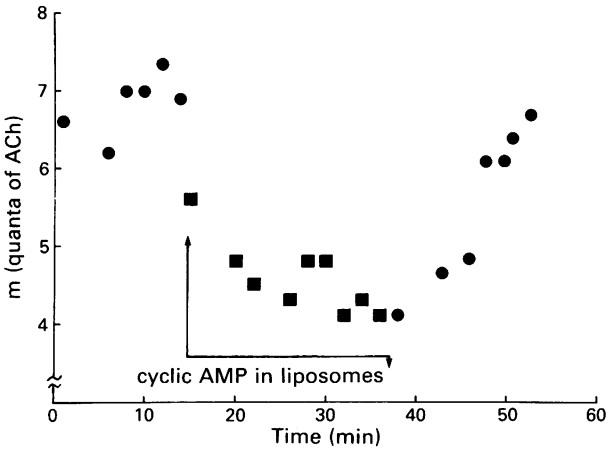

Figure 2 Inhibitory effects of cyclic AMP-containing liposomes on acetylcholine (ACh) release in the presence of the adenosine receptor blocker theophylline. Results and conditions were similar to Figure 1, except that the adenosine receptor blocker theophylline (1.8 mM) was included in the Ringer solution (i.e. external to the liposomes) throughout the entire experiment. Control (●) consisted of KCl in liposomes. Cyclic AMP in liposomes (■) consisted of 100 mM cyclic AMP + KOH to pH 7.2. The m declined to 63% of the control value in the presence of cyclic AMP liposomes. M.e.p.p. frequencies (s^{-1}, $n = 47$ for all): control (1.15 ± 0.17); cyclic AMP-containing liposomes (0.81 ± 0.13); post-cyclic AMP control (1.00 ± 0.11). A steady-state in the post-cyclic AMP control was not achieved due to the limited volume of the control (KCl-containing) liposome suspension. It should be stressed that at these low levels of ACh release, whilst theophylline antagonizes the action of adenosine, it has little consistent influence on m in contrast to the stimulating effect on m of selective phosphodiesterase inhibitors (unpublished, see also Dryden *et al.*, 1988). Thus the theophylline is unlikely to be inhibiting phosphodiesterase locally near the plasma membrane.

Figure 3 shows the typical effects of 250 μM CPT cyclic AMP on spontaneous ACh release: a 2.4 fold increase in m.e.p.p.$_f$ that only slowly declined upon return to control Ringer. In a total of 11 experiments, CPT cyclic AMP produced 2.36 ± 0.29 fold increases in m.e.p.p.$_f$ (range 1.78–5.0). (The effects of adenosine after treatment with this cyclic AMP analogue are illustrated in this figure and are discussed below).

CPT cyclic AMP consistently increased evoked ACh release in parallel with its effects on m.e.p.p.$_f$. Specifically, at low levels of ACh release, 250 μM CPT cyclic AMP increased m 1.69 ± 0.67 fold ($n = 4$, range 1.21 to 2.68 fold). At normal levels of release, m was elevated 2.37 ± 0.08 fold ($n = 2$).

The effects of db cyclic AMP were qualitatively similar to those found with CPT cyclic AMP. Increases in both m.e.p.p.$_f$ and m generally occurred but the effect of db cyclic AMP on m was less pronounced and more variable. Specifically, m.e.p.p.$_f$ was elevated from 1.9–3.2 fold ($n = 4$) in 3–4 mM db cyclic AMP. These concentrations of db cyclic AMP also increased m in six of eight experiments but the increases were modest (less than two fold). In the remaining two experiments, db cyclic AMP either had no effect on ACh release or produced inhibition of ACh release during exposure to this analogue. A rebound increase in ACh release upon washout of the drug was seen in both experiments (cf. Kuba *et al.*, 1981).

To summarize the results so far, cyclic AMP generally decreased ACh release whilst the membrane permeant cyclic AMP analogues promoted ACh secretion. We next attempted to evaluate the suggestions that local changes in cyclic AMP concentrations might mediate the inhibitory effect of adenosine (Silinsky, 1986; Chen *et al.*, 1989) by examining the interactions between cyclic AMP analogues and adenosine. If adenosine exerts its inhibitory effects by altering cyclic AMP levels, then exogenous cyclic AMP analogues should reduce the effects of adenosine.

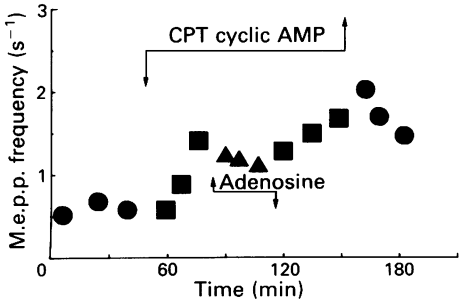

Figure 3 The effects of 8-(4-chlorophenylthio) (CPT) cyclic AMP on spontaneous acetylcholine (ACh) release and the action of adenosine. Solutions were sequentially superfused over the preparation (left to right): control solution (0.35 mM Ca, 3.0 mM Mg Ringer; ●); 250 μM CPT cyclic AMP (■); control, CPT cyclic AMP plus adenosine (250 μM each, ▲). Evoked ACh release in this fibre was reversibly increased 2.69 fold by CPT cyclic AMP and adenosine produced a typical effect (56.1% inhibition; data shown in Table 1). The absence of effects of adenosine on miniature endplate potential frequency (m.e.p.p.$_f$) in this experiment is not likely to be due to blockade of adenosine receptors by CPT cyclic AMP as the inhibition of evoked ACh release by adenosine proceeded normally.

Elevation of cellular cyclic AMP levels reduces the inhibitory effects of adenosine receptor agonists

The typical experimental result with CPT cyclic AMP is shown in Figure 3. Note that after this analogue produced its characteristic stimulating effect (namely a two fold increase in m.e.p.p.$_f$), adenosine (250 μM) failed to inhibit significantly spontaneous ACh release. In seven of eight experiments in which concentrations of adenosine between 25 and 250 μM were employed, CPT cyclic AMP reduced or completely occluded the effects of adenosine on evoked or spontaneous ACh release (Table 1). db Cyclic AMP (3 mM) either reduced or prevented the inhibitory effects of adenosine receptor analogues (n = 5, data not shown). Cyclic AMP liposomes also reduced the effectiveness of adenosine in inhibiting ACh release (n = 2, data not shown).

The results of this section suggest that increasing cellular cyclic AMP concentrations by cyclic AMP or by lipophilic analogues of cyclic AMP impairs the ability of adenosine receptor agonists to inhibit ACh release. These results are consistent with cyclic AMP mediating the effects of adenosine. (For a summary of other results in accordance with this hypothesis, see Silinsky & Vogel, 1986; 1987.) However, the results presented below suggest that cyclic AMP-dependent protein kinase does not mediate the inhibitory effects of adenosine.

Protein kinase inhibition by H7

General observations on the action of H7 at frog neuromuscular junctions H7 at concentrations ranging from 60–100 μM produced no consistent effect on either evoked or spontaneous ACh release (Table 2).

Unfortunately, at these concentrations H7 produced postjunctional depressant effects, reflected as parallel decreases in e.p.p. and m.e.p.p. amplitudes, generally to about 50% of the control level (compare Figure 4a with 4b). These effects were associated with an acceleration of the time course of the rising phase and a pronounced biphasic decay of the endplate responses (Figure 4c). Such changes in e.p.p. and m.e.p.p. configurations are reminiscent of open channel block at the nicotinic receptor (Peper *et al.*, 1982) and were a disappointing impediment in these presynaptic studies. These depressant effects were postjunctional in origin; ACh potentials decreased in the presence of 60 μM H7 in a manner comparable to the depression of e.p.p. and m.e.p.p. amplitudes (data not shown). Effects of H7 on e.p.ps and m.e.p.ps could be reversed by washing the drug out of the bath with control solution, albeit incompletely at high concentrations.

Table 2 The effect of H7 on spontaneous and evoked acetylcholine (ACh) release

Ringer (mM)	*Spontaneous ACh release (% change)*	*Evoked ACh release (% change)*
0.35 Ca, 3.0 Mg	NS	ND
0.35 Ca, 3.0 Mg	NS	−2.8
0.35 Ca, 3.0 Mg	NS	28.1
0.35 Ca, 4.0 Mg*	NS	−5.7
1.8 Ca	−26.1	ND
Mean ± s.e.mean:	−5.2 ± 5.8	6.5 ± 9.4

* H7 100 μM; otherwise 60 μM H7.
NS = not significant; ND = not determined.

H7 blocks cyclic AMP-dependent protein kinase in frog motor nerve endings Previous studies in the frog have shown that H7 (10–60 μM) prevented the increases in spontaneous ACh release produced by 12-O-tetradecanoylphorbol-13-acetate (TPA), thus suggesting that H7 can inhibit protein kinase C activity (Branisteanu *et al.*, 1988; Caratsch *et al.*, 1988). As described earlier, CPT cyclic AMP (250 μM) consistently produced approximately two fold increases in the rate of spontaneous ACh release. In the presence of H7 (60 μM), however,

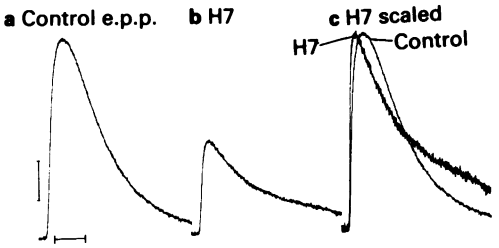

Figure 4 H7 blocks the acetylcholine (ACh) receptor/ion channel. The average response to 64 stimuli is shown for a frog nerve-cutaneous pectoris muscle that was sequentially superfused in (a) control (0.35 mM Ca, 3.0 mM Mg Ringer) and (b) 60 μM H7. In (c) the averaged endplate potential (e.p.p.) in H7 (heavier trace) was scaled up to and superimposed upon the averaged control e.p.p. H7 reduced the mean amplitude of the e.p.p. (above) and m.e.p.p. (not shown) to a similar extent. For example, in this experiment the mean e.p.p. amplitude was reduced by 48.3%, and the mean m.e.p.p. amplitude was reduced by 46.9% in H7 as compared to control. In addition, H7 accelerated the time course of the rising phase and produced pronounced biphasic decay of these potentials (c). These effects of H7 were reversed when the drug was washed out of the bath (not shown). Calibration bars: 1 mV and 5 ms.

Table 1 The effect of 8-(4-chlorophenylthio) (CPT) cyclic AMP treatment on the action of adenosine

Ringer (mM)	*Adenosine concentration* (μM)	*Spontaneous ACh release (% inhibition by adenosine)*	*Evoked ACh release (% inhibition by adenosine)*
0 Ca, 3 Mg	25	NS	ND
0 Ca, 3 Mg	25	32.6	ND
0 Ca, 3 Mg	250	NS	ND
0.35 Ca, 3 Mg	25	NS	21.3
0.35 Ca, 3 Mg	25	NS	NS
0.35 Ca, 3 Mg*	250	NS	56.1
1.8 mM Ca, 4 mg l^{-1} TC	25	ND	33.8
1.8 mM Ca, 4 mg l^{-1} TC	250	ND	26.6

In all experiments, 250 μM CPT cyclic AMP was used. This concentration of CPT cyclic AMP (250 μM) was chosen for all experiments based upon preliminary studies in which lower concentrations (e.g. 25 μM) did not alter ACh release (data not shown).
NS = adenosine produced no significant inhibition of ACh release.
ND = not determined.
* Experiment shown in Figure 3. TC = (+)-tubocurarine.

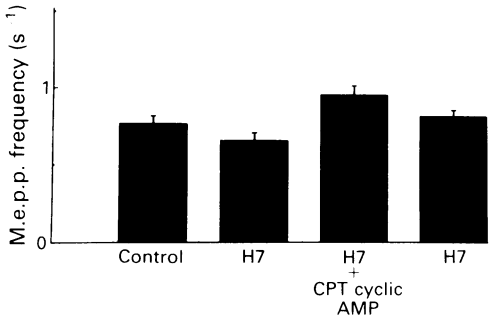

Figure 5 H7 occludes the stimulating effects of 8-(4-chlorophenylthio) (CPT) cyclic AMP on acetylcholine (ACh) release. Solutions were sequentially superfused over the preparation as follows: control (0.35 mM Ca, 4.0 mM Mg Ringer); 60 μM H7, 60 μM H7 and 250 μM CPT cyclic AMP; 60 μM H7. Each column represents the mean miniature endplate potential frequency (m.e.p.p.$_f$) at a time when the superfusion solution had attained a steady-state effect. (n = 120 s for all.)

Table 3 Inhibitory effects of adenosine on acetylcholine (ACh) release in the presence of H7

Ringer (mM)	*Spontaneous ACh release (% inhibition by adenosine)*	*Evoked ACh release (% inhibition by adenosine)*
0.35 Ca, 3.0 Mg	41.4	43.9
0.35 Ca, 3.0 Mg	67.6	63.9
0.35 Ca, 3.0 Mg	NS	ND
0.35 Ca, 4.0 Mg*†	59.8	58.1
1.8 Ca†	41.4	ND
1.8 Ca, 4 mg l^{-1} TC†	ND	49.0
1.8 Ca, 4 mg l^{-1} TC	ND	40.0
1.8 Ca, 4 mg l^{-1} TC	ND	60.8
Mean ± s.e.mean:	42.0 ± 13.0	52.6 ± 4.3

* H7 100 μM; otherwise 60 μM H7.
† Adenosine 25 μM; otherwise 50 μM adenosine.
NS = not significant; ND = not determined.

this increase was occluded (Figure 5). In a total of 4 such experiments performed in the presence of 30–60 μM H7, CPT cyclic AMP (250 μM), produced submaximal increases in m.e.p.p. frequency (mean 28.3 ± 11.8%; range 0 to 45.4%, n = 4). It thus appears that H7 is an inhibitor of PK A in frog motor nerve endings.

H7 does not impair the inhibitory effect of adenosine at frog motor nerve endings As H7 apparently inhibits PK A and PK C in frog neuromuscular preparations, we next examined whether H7 could modify the ability of adenosine to inhibit ACh release. As is illustrated in Figure 6, H7 (60 μM) did not alter the typical effect of adenosine. Specifically, adenosine (25–50 μM) reduced evoked (Figure 6a) and spontaneous (Figure 6b) ACh release by approximately 50% in the presence or absence of H7 (Ginsborg & Hirst, 1972; Silinsky, 1984). These data are presented in Table 3.

The results of this section suggest that phosphorylation by A, C, G and perhaps other protein kinases does not play a major role in the control of resting ACh release or in the modulation of ACh release by adenosine.

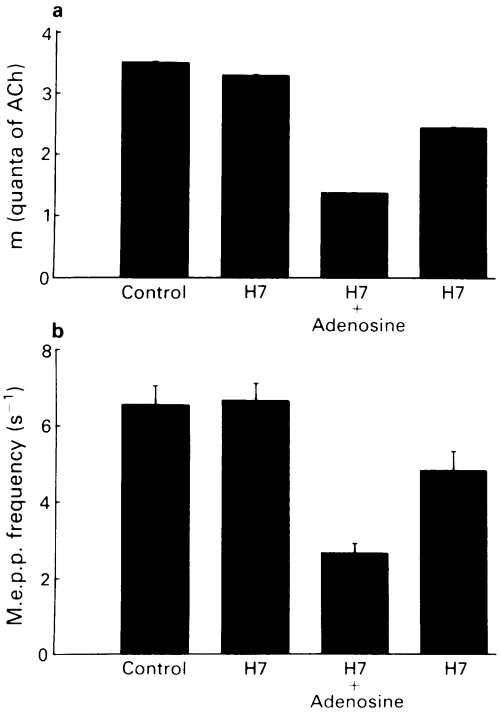

Figure 6 H7 does not alter the effect of adenosine. Solutions were sequentially superfused from left to right as follows: Control (0.35 mM Ca 3.0 mM Mg Ringer); 60 μM H7; 60 μM H7 and 50 μM adenosine; 60 μM H7. Each column represents the m (a, n = 256 to 640 sweeps) or the mean miniature endplate potential frequency (m.e.p.p.$_f$) (b, n = 30 to 60) at a time when the superfusion solution had attained a steady-state effect. Note that adenosine produced a typical effect, specifically a 58.1% decline in m, and a 59.9% reduction in m.e.p.p.$_f$ in the presence of H7. release (m) vs treatment; n = 384–448 sweeps, 1.0 Hz.

Discussion

Cyclic AMP and compartmentation

The results demonstrate that CPT cyclic AMP, a stable lipophilic cyclic AMP analogue with a high potency as a PK A activator exclusively produces increases in ACh, whilst the local delivery of the membrane impermeant natural messenger cyclic AMP via liposomes inhibits ACh release. db Cyclic AMP, a lipophilic analogue with low potency as a PK A activator, produced smaller increases in evoked ACh release and on occasion may even have inhibited transmitter secretion. The most plausible explanation for these results is that multiple cellular compartments for cyclic AMP exist within the nerve terminal (Silinsky & Vogel, 1986; see also Dunwiddie & Hoffer, 1982; Phillis & Barraco, 1985). Compartmentation of second messenger action is an established phenomenon (Harper *et al.*, 1985; Harper, 1988) and has even been suggested to explain the complex effects of cyclic nucleotides at nerve endings (Moskowitz & Puszkin, 1985; Silinsky & Vogel, 1986). Specifically, it has been suggested that local submembraneous increases in cyclic AMP (as would be produced by cyclic AMP in liposomes) inhibit ACh release whilst more global increases in cyclic AMP concentrations (such as those produced by lipophilic cyclic AMP analogues) phosphorylate vesicular proteins or Ca translocation sites and stimulate secretion. Support for the hypothesis of local delivery of liposomal contents just beneath the plasma membrane in secretory cells may be found in a number of different studies. Ca delivered in liposomes evokes highly-localized exocytotic responses at the sites of liposome fusion in mast cells (Theoharides & Douglas, 1978). Moreover, Ca- and Sr-containing liposomes produce selective effects on m relative to spontaneous ACh release (Mellow *et al.*, 1982) and studies with the Ca buffer BAPTA suggest m is dependent on local, submembraneous Ca rather than bulk, cytoplasmic Ca (Kijima & Tanabe, 1988).

With respect to the stimulating effects of cyclic AMP analogues, it has been suggested that phosphorylation of proteins

associated with synaptic vesicles (e.g Synapsin I) increase the availability of the transmitter for release (Silinsky, 1985a, b; Miller, 1985; Bähler & Greengard, 1987; Moskowitz & Puszkin, 1985; Harper, 1988; Dryden *et al.*, 1988). These stimulating effects could also arise secondarily as a result of increases in cellular Ca levels caused by cyclic AMP accumulation (Moskowitz & Puszkin, 1985; Tomlinson *et al.*, 1985; Silinsky & Vogel, 1986; Harper, 1988). The excitatory actions of cyclic AMP analogues are unlikely to occur exclusively by the stimulation of Ca entry through voltage-gated Ca channels; excitatory effects of these analogues on m.e.p.p.$_f$ were observed in Ca-free solution.

With respect to decreases in ACh release, it has been suggested that phosphorylation of a Ca binding protein intimately associated with the secretory apparatus might yield a decreased affinity for Ca and thus inhibit release (see Silinsky, 1984; 1986 for discussion of this mechanism). Alternatively, cyclic AMP could stimulate Ca uptake into cellular storage sites near the plasma membrane; cyclic AMP-driven Ca uptake has been found in lysed brain synaptosomes (Mekhail-Ishak *et al.*, 1987). In this regard, cyclic AMP-dependent phosphorylation of inositol trisphosphate receptors has been found to decrease Ca release (Supattapone *et al.*, 1988). Regardless of the precise mechanism, the suggestion of opposing effects of cyclic AMP at different parts of the secretory apparatus appears to provide the simplest explanation for the results presented herein. Specifically, a balance between phosphorylation near the membrane and phosphorylation of more distal sites appears to determine whether a cyclic AMP analogue will produce excitation or inhibition. Based upon these results and previous results with PDE inhibitors (Silinsky, 1984; Silinsky & Vogel, 1987; Dryden *et al.*, 1988), it is likely that the more distal of these mechanisms, i.e. the one responsible for the excitatory effects of cyclic AMP, possesses the greatest capacity to influence the secretory process.

When compared to the results of others using lipophilic cyclic AMP analogues, the present results generally agree with those of Dryden *et al.* (1988) who obtained stimulating effects of 8-Br-cyclic AMP on mouse motor nerve terminals. The stimulating effects of db cyclic AMP in the present study in the frog also agree with most previous studies in the frog (Branisteanu *et al.*, 1988), the cat (Standaert & Dretchen, 1979) and the rat (Goldberg & Singer, 1969), but do not accord with other studies in the rat (Miyamoto & Breckenridge, 1974) or the mouse (Dryden *et al.*, 1988) where no effects were demonstrated. It should be noted that biphasic effects of db cyclic AMP, an initial inhibition during exposure to this analogue and a rebound increase in ACh release after washing with drug-free solution, were consistently observed in bullfrog sympathetic ganglia (Kuba *et al.*, 1981). We also observed such an effect in two of the six experiments with db cyclic AMP. Biological variability and species specificity could explain why DB cyclic AMP fails to produce uniform effects at peripheral cholinergic nerve endings.

Cyclic AMP and the interactions with adenosine

The observations that local increases in cyclic AMP mimic the inhibitory effects of adenosine, and that cyclic AMP and its congeners decrease the inhibitory effects of adenosine, support the suggestion that adenosine could be increasing local cyclic AMP levels to inhibit ACh release (Silinsky, 1984). Because this adenosine receptor is apparently linked to a pertussis toxin-sensitive G-protein (Silinsky *et al.*, 1989; Chen *et al.*, 1989), and blockade of PK A by H7 does not alter the effects of adenosine, it appears more likely that phosphorylation via PK A modulates rather than mediates the actions of adenosine.

What are some phosphorylation sites that may impair the action of adenosine? One possibility is that phosphorylation of the adenosine receptor itself could decrease the effects of adenosine in a manner similar to the heterologous desensitization produced by increases in cellular cyclic AMP levels (see Huganir & Greengard, 1987 for a review). Alternatively, nerve terminal calcium binding proteins could be phosphorylated and reduce the effectiveness of adenosine (see above). Whatever the mechanism, it is unlikely to be related to extracellular effects of cyclic AMP analogues on adenosine receptors as: (i) the effects of cyclic AMP in liposomes were not blocked by high concentrations of the adenosine receptor blocker, theophylline (Figure 2); (ii) in the experiment shown in Figure 3, CPT cyclic AMP antagonized the effect of adenosine only on spontaneous ACh release and not evoked ACh secretion (see figure legend), (iii) in several experiments in which db cyclic AMP, inhibited the action of adenosine, this analogue was washed out of the bathing fluid before the addition of adenosine, and (iv) cyclic AMP in liposomes inhibited the action of adenosine under conditions in which cyclic AMP liposomes in themselves did not alter ACh release ($n = 1$, data not shown).

Effects of protein kinase inhibition

With respect to the control of presynaptic function by protein kinases, consistent changes in evoked and spontaneous ACh release failed to occur in the presence of H7. This suggests that phosphorylation by H7-sensitive kinases does not play a substantial role in ACh release in the unperturbed system. Modulation of ACh release by these kinases can still occur, as shown by the effects of cyclic AMP derivatives and phorbol esters. With respect to the action of adenosine, the results of this study suggest that adenosine inhibits ACh release from frog motor nerve endings independently of protein phosphorylation by any kinases which are affected by H7. H7 is likely to be inhibiting PK A in this species, as it can prevent increases in spontaneous ACh release produced by the potent PK A stimulator CPT cyclic AMP. As H7 has been found to prevent TPA-induced increases in spontaneous ACh release (Branisteanu *et al.*, 1988; Caratsch *et al.*, 1988), PK C also appears to be inhibited under conditions where adenosine exerts its typical effects on ACh release.

A comparison of our results with previous data in the literature reveals considerable controversy. Chen *et al.* (1989) showed that 50 μM H7 blocked the effects of 2-chloroadenosine and decreased basal levels of spontaneous ACh release in the mouse phrenic nerve-hemidiaphragm. It is possible that species heterogeneity could account for this observation. Most puzzling, however, is the result of Branisteanu *et al.* (1988) where the presence of 10 μM H7 converted the effect of 50 μM adenosine into stimulation of spontaneous ACh release in cutaneous pectoris nerve-muscle preparations from *Rana ridibunda*. We have no reasonable explanation for this discrepancy, especially since stimulating effects of adenosine on ACh release are believed to be mediated by stimulation of adenylyl cyclase (see Silinsky, 1989 for a review). However, in partial accordance with our results Branisteanu *et al.* (1988) showed that H7 alone did not alter the m.e.p.p. frequency.

With respect to the postjunctional action of H7, the apparent blockade of the nicotinic receptor/ionic channel by this drug has not been widely demonstrated in neuromuscular preparations (Caratsch *et al.*, 1988; Branisteanu *et al.*, 1988; Chen *et al.*, 1989; but see Sebastiao, 1989). It is of interest that H7 decreases the mean open time of single channel currents induced by ACh at nicotinic receptors expressed in *Xenopus* oocytes (Reuhl *et al.*, 1989). These effects at nicotinic receptors are likely to be produced by channel block (Peper *et al.*, 1982), although the precise kinetic model awaits studies under voltage clamp and at the level of single ionic channels. Regardless of mechanism, these postjunctional effects could potentially lead to the erroneous conclusion that ACh release is decreased in the presence of H7. This is especially true if the channel block is use-dependent and repetitive nerve stimulation is employed.

In conclusion, it appears that the cyclic AMP-PK A system is compartmentalized at motor nerve endings and modulates the effects of adenosine. Adenosine, however, inhibits ACh

release from motor nerve endings by a mechanism independent of PK A, PK C, and possibly other kinases in the frog. These results do not exclude the remote possibility that cyclic AMP may mediate the effects of adenosine independently of protein phosphorylation. However, a more conventional interpretation is that the adenosine receptor at motor nerve endings is coupled directly through a G protein to its cellular effector, as has been shown for Ca channels and potassium channels in some neuronal soma (see Silinsky, 1989 for review). At frog motor nerve endings, this cellular effector is likely to be a Ca-sensitive component of the secretory apparatus (Silinsky, 1986; Silinsky *et al.*, 1989).

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References

BÄHLER, M. & GREENGARD, P. (1987). Synapsin I bundles F-actin in a phosphorylation-dependent manner. *Nature*, **326**, 704–707.

BEEBE, S.J., BLACKMORE, P.F., CHRISMAN, T.D. & CORBIN, J.D. (1988). Use of synergistic pairs of site-selective cAMP analogs in intact cells. In *Methods in Enzymology*, Vol. 159, ed. Corbin, J.D. & Johnson, R.A., pp. 118–139. San Diego: Academic Press.

BRANISTEANU, D.D., POPESCU, L.M., BRANISTEANU, D.D. & HAULICA, I.D. (1988). Cyclic GMP and protein kinase G inhibit the quantal transmitter release induced by protein kinase C. *Mol. Brain Res.*, **4**, 263–266.

CARATSCH, C.G., SCHUMACHER, S., GRASSI, F. & EUSEBI, F. (1988). Influence of protein kinase C-stimulation by a phorbol ester on neurotransmitter release at frog end-plates. *Naunyn-Schmeidebergs Arch. Pharmacol.*, **337**, 9–12.

CHEN, H., SINGH, Y.N. & DRYDEN, W.F. (1989). Transduction mechanism involving the presynaptic adenosine receptor at mouse motor nerve terminals. *Neurosci. Lett.*, **96**, 318–322.

COCKS, T.M., JENKINSON, D.H. & KOLLER, K. (1984). Interactions between receptors that increase cytosolic calcium and cyclic AMP in guinea-pig liver cells. *Br. J. Pharmacol.*, **83**, 281–291.

DEL CASTILLO, J. & KATZ, B. (1954). Quantal components of the end-plate potential. *J. Physiol.*, **124**, 560–573.

DRYDEN, W.F., SINGH, Y.N., GORDON, T. & LAZARENKO, G. (1988). Pharmacological elevation of cyclic AMP and transmitter release at the mouse neuromuscular junction. *Can. J. Physiol. Pharmacol.*, **66**, 207–212.

DUNWIDDIE, T.V. & HOFFER, B.J. (1982). The role of cyclic nucleotides in the nervous system. In *Cyclic Nucleotides* Part II *Physiology and Pharmacology*, ed. Kebabian, J.W. & Nathanson, J.A., pp. 389–463. Berlin: Springer-Verlag.

GINSBORG, B.L. & HIRST, G.D.S. (1972). The effect of adenosine on the release of the transmitter from the phrenic nerve of the rat. *J. Physiol.*, **224**, 629–645.

GOLDBERG, A.L. & SINGER, J.J. (1969). Evidence for a role of cyclic AMP in neuromuscular transmission. *Proc. Natl. Acad. Sci. U.S.A.*, **64**, 134–141.

HARPER, J.F. (1988). Stimulus-secretion coupling: second messenger-regulated exocytosis. In *Advances in Second Messenger and Phosphoprotein Research*, Vol. 22. ed. Greengard, P. & Robison, G.A., pp. 193–318. New York: Raven Press.

HARPER, J.F., HADDOX, M.K., JOHANSON, R.A., HANLEY, R.M. & STEINER, A.L. (1985). Compartmentation of second messenger action: immunocytochemical and biochemical evidence. In *Vitamins and Hormones. Advances in Research and Applications*, Vol. 42. ed. Aurbach, G.D. & McCormick, D.B., pp. 197–252. Orlando: Academic Press.

HIDAKA, H., INAGAKI, M., KAWAMOTO, S. & SASAKI, Y. (1984). Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry*, **23**, 5036–5041.

HIRSH, J.K. & SILINSKY, E.M. (1989). Signal transduction and the adenosine receptor inhibitory to acetylcholine release in frog endings. *Soc. Neurosci.*, **15**, 484.

HIRSH, J.K., SOLSONA, C.S. & SILINSKY, E.M. (1988). Evidence for compartmentation of cyclic AMP effects in the motor nerve terminal. *The Pharmacologist*, **30**, A85.

HUGANIR, R.L. & GREENGARD, P. (1987). Regulation of receptor function by protein phosphorylation. *Trends Pharmacol. Sci.*, **8**, 472–477.

KHARASCH, E.D., MELLOW, A.M. & SILINSKY, E.M. (1981). Intracellular magnesium does not antagonize calcium-dependent acetylcholine secretion. *J. Physiol.*, **314**, 255–263.

KIJIMA, H. & TANABE, N. (1988). Calcium-independent increase of transmitter release at frog end-plate by trinitrobenzene sulfonic acid. *J. Physiol.*, **403**, 135–149.

KUBA, K., KATO, E., KUMAMOTO, E., KOKETSU, K. & HIRAI, K. (1981). Sustained potentiation of transmitter release by adrenaline and dibutyryl cyclic AMP in sympathetic ganglia. *Nature*, **291**, 654–656.

McLACHLAN, E.M. & MARTIN, A.R. (1981). Non-linear summation of end-plate potentials in the frog and mouse. *J. Physiol.*, **311**, 307–324.

MEKHAIL-ISHAK, K., LAVOIE, P. & SHARKAWI, M. (1987). Effects of caffeine and cyclic adenosine 3′,5′-monophosphate on adenosine triphosphate-dependent calcium-uptake by lysed brain synaptosomes. *Brain Res.*, **426**, 62–68.

MELLOW, A.M., PERRY, B.D. & SILINSKY, E.M. (1982). Effects of calcium and strontium in the process of acetylcholine release from motor nerve endings. *J. Physiol.*, **328**, 547–562.

MILLER, R.J. (1985). Second messengers, phosphorylation and neurotransmitter release. *Trends Neurosci.*, **8**, 463–465.

MIYAMOTO, M.D. & BRECKENRIDGE, B. McL. (1974). A cyclic adenosine monophosphate link in the catecholamine enhancement of transmitter release at the neuromuscular junction. *J. Gen. Physiol.*, **63**, 609–624.

MOSKOWITZ, N. & PUSZKIN, S. (1985). A unified theory of presynaptic neurotransmission. *J. Theor. Biol.*, **112**, 513–534.

NORTH, R.A. (1989). Neurotransmitters and their receptors: from the clone to the clinic. *Semin. Neurosci.*, **1**, 81–90.

PAPAHADJOPOULOS, D., POSTE, G. & MAYHEW, E. (1974). Cellular uptake of cAMP captured within phospholipid vesicles and effect on cell-growth behaviour. *Biochem. Biophys. Acta*, **363**, 404–418.

PEPER, K., BRADLEY, R.J. & DREYER, F. (1982). The acetylcholine receptor at the neuromuscular junction. *Physiol. Rev.*, **62**, 1271–1340.

PHILLIS, J.W. & BARRACO, R.A. (1985). Adenosine, adenylate cyclase, and transmitter release. In *Advances in Cyclic Nucleotide and Protein Phosphorylation Research*, Vol. 19. ed. Cooper, D.M.F. & Seamon, K.B., pp. 243–257. New York: Raven Press.

RAHAMIMOFF, R., MEIRI, H., ERULKAR, S.D. & BARENHOLZ, Y. (1978). Changes in transmitter release induced by ion-containing liposomes. *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 5214–5216.

REUHL, T., PINKHAM, J., MOORMAN, J.R. & DANI, J.A. (1989). Compounds commonly used to alter phosphorylation act directly on the channels to decrease currents through nicotinic acetylcholine receptors. *Soc. Neurosci. Abstr.*, **15**, 827.

SEBASTIAO, A.M. (1989). On the transducing mechanism operated by the adenosine receptor mediating inhibition of transmitter release at the neuromuscular junction. In *Adenosine Receptors in the Nervous System*, ed. Ribeiro, J.A., pp. 131–140. London: Taylor & Francis.

SILINSKY, E.M. (1984). On the mechanism by which adenosine receptor activation inhibits the release of acetylcholine from motor nerve endings. *J. Physiol.*, **346**, 243–256.

SILINSKY, E.M. (1985a). Process by which purines inhibit transmitter release. In *Purines. Pharmacology and Physiological Roles.* ed. Stone, T.W., pp. 67–73. London: Macmillan Press.

SILINSKY, E.M. (1985b). The biophysical pharmacology of calcium-dependent acetylcholine secretion. *Pharmacol. Rev.*, **37**, 81–132.

SILINSKY, E.M. (1986). Inhibition of transmitter release by adenosine: are Ca^{2+} currents depressed or are the intracellular effects of Ca^{2+} impaired? *Trends Pharmacol. Sci.*, **7**, 180–185.

SILINSKY, E.M. (1987). Electrophysiological methods for studying acetylcholine secretion. In *In Vitro Methods for Studying Secretion*, ed. Poisner, A.M. & Trifaró, J.M., pp. 255–271. Amsterdam: Elsevier.

SILINSKY, E.M. (1989). Adenosine derivatives and neuronal function. *Semin. Neurosci.*, **1**, 155–164.

SILINSKY, E.M. & VOGEL, S.M. (1986). The effects of an adenylate cyclase inhibitor on the electrophysiological correlates of neuromuscular transmission in the frog. *Br. J. Pharmacol.*, **88**, 799–805.

SILINSKY, E.M. & VOGEL, S.M. (1987). Independent control of channel closure and block of open channels by methylxanthines at acetylcholine receptors in frog. *J. Physiol.*, **390**, 33–44.

SILINSKY, E.M., SOLSONA, C.S. & HIRSH, J.K. (1989). Pertussis toxin prevents the inhibitory effect of adenosine and unmasks adenosine-induced excitation of mammalian motor nerve endings. *Br. J. Pharmacol.*, **97**, 16–18.

STANDAERT, F.G. & DRETCHEN, K.L. (1979). Cyclic nucleotides and neuromuscular transmission. *Fedn. Proc.*, **38**, 2183–2192.

SUPATTAPONE, S., DANOFF, S.K., THEIBERT, A., JOSEPH, S.K., STEINER, J. & SNYDER, S.H. (1988). Cyclic AMP-dependent phosphorylation of a brain inositol trisphosphate receptor decreases its release of calcium. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 8747–8750.

THEOHARIDES, T.C. & DOUGLAS, W.W. (1978). Secretion in mast cells induced by calcium entrapped within phospholipid vesicles. *Science*, **201**, 1143–1145.

TOMLINSON, S., MACNEIL, S. & BROWN, B.L. (1985). Calcium, cyclic AMP, and hormone action. *Clin. Endocrinol.*, **23**, 595–610.

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Binding of [^{125}I]-endothelin-1 to rat cerebellar homogenates and its interactions with some analogues

[1]C. Robin Hiley, C. Richard Jones, John T. Pelton & Robert C. Miller

Merrell Dow Research Institute, B.P. 447 R/9, 16 rue d'Ankara, F-67009 Strasbourg Cedex, France

1 [^{125}I]-endothelin-1, over the concentration range 6 pM–10 nM, bound to a single site in homogenates of rat cerebellum with high affinity ($K_d = 2.8 \pm 0.6 \times 10^{-10}$ M). The site was present in a concentration of 321 ± 58 fmol mg^{-1} protein.

2 The rates of association and dissociation of [^{125}I]-endothelin-1 with the binding site were slow (at 25°C, $k_{+1} = 8.0 \pm 1.3 \times 10^5$ M^{-1}s^{-1}; $k_{-1} = 2.6 \times 10^{-4}$ s^{-1}) and, on addition of a maximally displacing concentration of endothelin-1 (100 nM), 94.0 ± 8.4% of the [^{125}I]-endothelin-1 was still bound after 14 h.

3 [^{125}I]-endothelin-1 binding was inhibited by a number of naturally occurring or genetically encoded members of the endothelin/sarafotoxin family of peptides. The order of potency was endothelin-3 = sarafotoxin S6b > endothelin-2 = endothelin-1 ≫ porcine proendothelin$_{1-39}$.

4 Binding was also inhibited by analogues in which either one or both of the cystine disulphide bridges had been replaced by substitution with 2 or 4 alanine residues. The tetra-alanyl substituted analogue, [Ala^{1,3,11,15}]endothelin-1, was equipotent with endothelin-1 at inhibiting the binding of [^{125}I]-endothelin-1. [Ala^{3,11}]endothelin-1 and [Ala^{1,15}]endothelin-1, analogues which each contained one of the disulphide bridges from the parent peptide, were respectively 3 and 14 times less potent than the parent peptide. An analogue in which the Glu^{10} residue had been anisylated was 25 fold less potent than endothelin-1.

5 It is concluded that the structural requirements for binding to the cerebellar sites for [^{125}I]-endothelin-1 do not require the presence of the disulphide bridges characteristic of the endothelin/sarafotoxin family. Rather, the binding may be more sensitive to the presence of bulky side chain substituents, at least in the smaller intramolecular loop.

Introduction

Yanagisawa *et al.* (1988b) isolated a very potent pressor peptide, which they named endothelin, from medium conditioned by pig aortic endothelial cells. It has since been established that this is one of a group of peptides, the endothelin/sarafotoxin family, that occur either naturally or may be found encoded in mammalian genomic libraries. Each member of this group consists of 21 amino acids and has 2 disulphide bridges which are between residues 1 and 15 and between residues 3 and 11. The three different peptides encoded in the human genome have been denoted endothelin-1, endothelin-2 and endothelin-3 (Inoue *et al.*, 1989). Of these peptides, coding for a sequence identical to human endothelin-1 has also been found in the pig (Yanagisawa *et al.*, 1988b), for endothelin-1 and endothelin-2 in the dog (Itoh *et al.*, 1989; Kimura *et al.*, 1989) and for endothelin-3 in the rat and rabbit (Yanagisawa *et al.*, 1988a; Ohkubo *et al.*, 1990). The sequence for a fourth mammalian peptide, vasoactive intestinal contractor, has been detected in the mouse genome (Saida *et al.*, 1989). In addition to these mammalian peptides, there exist closely homologous peptides, the sarafotoxins S6, which are found in the venom of the burrowing asp, *Atractaspis engaddensis* (Kloog *et al.*, 1988).

In view of the conservation of the disulphide bridges between the members of the endothelin/sarafotoxin family, interest has centred on their importance for biological activity. Kimura *et al.* (1988) found that peptides in which the cysteines which form the disulphide bridges had been left blocked by carboxamidomethyl groups were at least 1000 fold less active as vasoconstrictor agents on pig coronary artery. However, the blocking groups are very bulky and may interfere with the structure of the peptides in ways other than those simply associated with the loss of the disulphide bridges. Accordingly, a series of analogues of human endothelin-1 have been synthesized in which the cystine residues have been replaced by pairs of alanines. Those analogues which contain only one of the disulphide bridges have been found to be only 10 to 40 fold less active than endothelin-1 itself not only in the rat aorta (Topouzis *et al.*, 1989) and rat mesenteric arterial bed (Hiley *et al.*, 1989a; Randall *et al.*, 1989) but also in preparations such as guinea-pig trachea (Pelton & Miller, unpublished observations) and the field stimulated guinea-pig ileum and rat vas deferens (Hiley *et al.*, 1989b).

The discovery that endothelin and its analogues could act on nerve/muscle preparations was particularly interesting in view of the observation that binding sites for [^{125}I]-endothelin-1 have been identified in many tissues, including brain and kidney, with distributions that do not suggest that they are exclusively associated with the blood vessels of those tissues (Davenport *et al.*, 1989a,b; Jones *et al.*, 1989a,b). In the rat brain, the greatest concentration of [^{125}I]-endothelin-1 binding sites was found in the hind brain, particularly in the cerebellum (Jones *et al.*, 1989b). It was therefore of interest to study the interactions between [^{125}I]-endothelin-1, its naturally encoded relations and its alanyl-substituted analogues in rat brain.

A preliminary communication of this work was made to the December 1988 Meeting of the British Pharmacological Society (Jones *et al.*, 1989c).

Methods

Membrane preparation

Male Sprague-Dawley rats (185–250 g; Charles Rivers France) were killed by stunning and decapitation. The cerebellum was then removed and homogenized in 10 vol. ice-cold 50 mM HEPES ({N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]}; Sigma Chimie, La Verpillière, France) buffer (pH 7.4) containing 1 mM 1,10 phenanthroline (Sigma). The

[1] Author for correspondence at permanent address: Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ.

homogenates were centrifuged at 1000 g for 10 min at 4°C and the binding studies were carried out on the resulting supernatant. Protein content was determined by the method of Lowry et al. (1951).

[^{125}I]-endothelin-1

Human endothelin-1 (1 nmol; Peptide Institute, Osaka, Japan) was iodinated by the method of McKee et al. (1986) using a 10 s reaction time with chloramine-T (Aldrich Chemical Co., Strasbourg, France) and sodium [^{125}I]-iodide (481–574 MBq μg^{-1}; Amersham, Les Ulis, France). The reaction was stopped by the addition of sodium metabisulphite. Bovine serum albumin (500 mg in 500 μl 50 mM Tris buffer, pH 7.6) was added to the reaction mixture as a carrier and the reaction mixture was then passed down a Sephadex G-15 column. Elution was with the Tris buffer and the product was then subjected to reverse phase high performance liquid chromatography using a Vydac (Hesperia, CA, U.S.A.) Type 218TP54 C-18 reverse phase column for protein and peptide separation; the mobile phase consisted of a linear gradient passing from 75% to 50% H_2O in CH_3CN (v/v) containing 0.1% trifluoroacetic acid (v/v) and the flow rate was 2 ml min^{-1}.

In later experiments, [^{125}I]-endothelin-1 was purchased from Amersham (specific activity 74 TBq $mmol^{-1}$) and no differences were found between the performance of this radioligand and that prepared in the laboratory.

Synthesis of analogues of endothelin-1

Analogues of endothelin-1 were synthesized by standard solid-phase synthetic techniques using an automated peptide synthesizer employing Boc/Bzl chemistry (Merrifield, 1963; Stewart & Young, 1984) and t-butyloxycarbonyltryptophyl-4-(oxymethyl)phenylacetamidomethyl resin. The peptides were deprotected and removed from the resin with the two-step 'low-high' HF method (Tam et al., 1983). After cyclization with 0.01 M $K_3Fe(CN)_6$ at pH 8.5 (Pelton et al., 1986), the analogues were purified by gel filtration and preparative reverse-phase high pressure liquid chromatography. Amino acid analysis after acid hydrolysis gave the proper molar ratios (± 8.0%) of the constituent amino acids. Molecular ions and fragmentation patterns determined by fast atom bombardment-mass spectroscopy were consistent with the amino acid composition and sequence.

Binding studies

Incubations were carried out in triplicate at 25°C in a volume of 0.5 ml in microcentrifuge tubes using 150–700 μg protein. For inhibition experiments with unlabelled ligands membranes were incubated with 20–50 pM [^{125}I]-endothelin-1; under these conditions the radiolabel occupies about 3–6% of the binding sites in the absence of a competing ligand. Non-specific binding was defined with 0.1 μM unlabelled endothelin-1 and the reaction was started in all cases by the addition of the cerebellar homogenate. An incubation time of 2 h was used for inhibition studies with unlabelled peptides and for saturation experiments. Other times of incubation were used as appropriate for kinetic experiments. The reaction was terminated by centrifugation in a Beckman Microfuge B for 2 min at 14000 g; the pellets were washed 3 times by dipping the microcentrifuge tubes into ice-cold incubation medium (50 mM HEPES, pH 7.4; 0.1% bovine serum albumin; 1 mM 1,10 phenanthroline; 140 $\mu g\,ml^{-1}$ bacitracin: all from Sigma) and then the residual radioactivity in the pellets was determined by counting in a Packard Autogamma 5000 γ-scintillation spectrometer.

Kinetic experiments were carried out by determining the amount of specific binding over several time points with 6 different concentrations of [^{125}I]-endothelin-1 ranging from 18–450 pM. The rate constant for the formation of the [^{125}I]-endothelin-1/binding site complex (k_{obs}) was calculated by use of LIGAND (see below) and the forward and backward rate constants (k_{+1} and k_{-1}) were determined by linear regression of k_{obs} against concentration of radioligand.

Analysis of data

Kinetic data were analysed by non-linear least squares fitting with the KINETIC program (McPherson, 1985) running on a Compaq 386 PC AT compatible computer. Saturation data were initially analysed with EBDA (McPherson, 1985) and then subjected to non-linear least squares curve fitting by use of LIGAND (Munson & Rodbard, 1980).

Inhibition data were also subjected to preliminary analysis with EBDA and then the resulting inhibition data were fitted to a logistic equation:

$$\%\ \text{inhibition of specific binding} = \frac{100}{\{1 - (IC_{50}/X)^{n_H}\}}$$

where IC_{50} is the concentration of the unlabelled ligand causing 50% inhibition of the binding of the radioligand, X is the concentration of the unlabelled ligand and n_H is the Hill slope of the curve. Non-linear curve-fitting was carried out with the RS/1 data analysis package (BBN Software Products Corporation, Cambridge, MA, U.S.A.). The K_i was calculated from the resulting IC_{50} by use of the procedure of Munson & Rodbard (1988).

Results

Rate of binding of [^{125}I]-endothelin-1 to cerebellar binding sites

The time course of labelling of binding sites by 30 pM [^{125}I]-endothelin-1 in rat cerebellar homogenates was very slow. From a series of experiments using 6 different concentrations of the radioligand, the forward rate constant was calculated to be 8.0 ± 1.3 × $10^5\,M^{-1}\,s^{-1}$ and the backward rate constant to be 2.6 ± 0.3 × $10^{-4}\,s^{-1}$; this gives an estimate for the binding constant of 3.2 × 10^{-10} M. In dissociation experiments, 100 nM endothelin-1 was added to the membrane/[^{125}I]-endothelin-1 reaction mix after the 2 h association phase, dissociation of the bound [^{125}I]-endothothelin-1 was very slow with 94.0 ± 8.4% (n = 3) remaining bound to the membranes after 14 h.

Saturation analysis of [^{125}I]-endothelin-1 binding to cerebellar sites

Figure 1 shows the saturation curve and the Scatchard analysis for an experiment in which cerebellar membranes were incubated with 6 pM–10 nM [^{125}I]-endothelin-1. The binding appears to be a single class of site and in 5 experiments the equilibrium dissociation constant was 2.8 ± 0.6 × 10^{-10} M, the maximal specific binding was 321 ± 58 fmol mg^{-1} protein and the Hill slope was 0.92 ± 0.04. Figure 1 shows the results from one of two saturation experiments in which 1 nM sarafotoxin S6b was present and it can be seen that, in its presence, the Scatchard plot for [^{125}I]-endothelin-1 is linear and the intercept on the abscissa scale is very close to that for the iodinated ligand alone. Analysis of these two competition experiments gave values of the dissociation constant for sarafotoxin S6b of 3.2 and 1.9 × 10^{-10} M and the Hill slopes for the binding of [^{125}]-endothelin-1 were 0.92 and 1.04. The maximal specific binding of [^{125}I]-endothelin-1 in the presence of sarafotoxin S6b in these two experiments was 510 and 394 fmol mg^{-1} protein as compared to the values of 473 and 340 fmol mg^{-1} protein found with [^{125}I]-endothelin-1 alone in the same two homogenates.

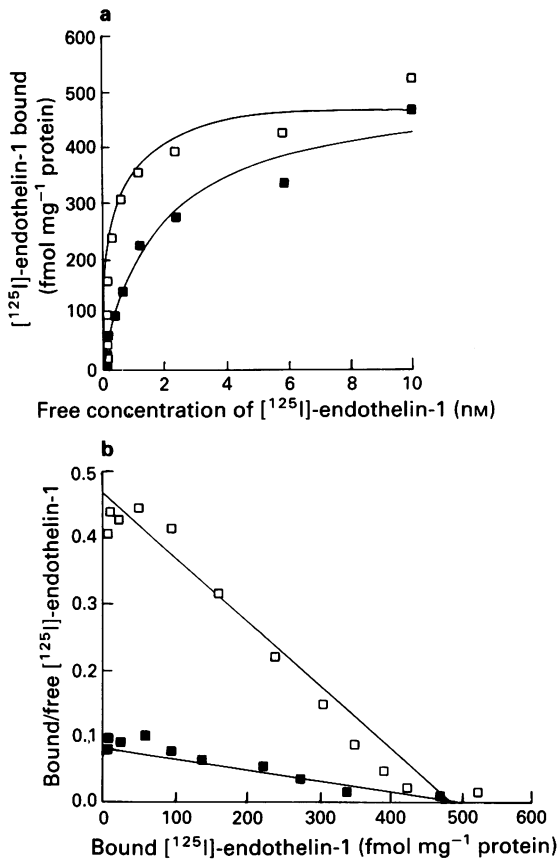

Figure 1 Results from a single experiment in which cerebellar membranes were incubated with 6 pM–10 nM [^{125}I]-endothelin-1 in the absence (□) and presence (■) of 1 nM sarafotoxin S6b. (a) Saturation curves with the lines showing the curves obtained from the fitting procedure. (b) Scatchard plots of the same data showing the linear least squares regression lines. The points are the mean of triplicate determinations.

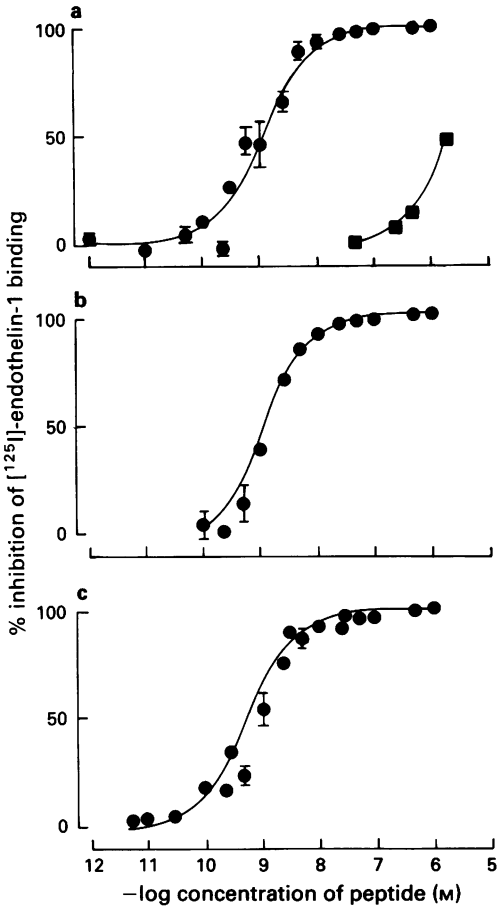

Figure 2 Inhibition of specific [^{125}I]-endothelin-1 binding to homogenates of rat cerebellum by members of the endothelin family. (a) Inhibition by endothelin-1 (●; $n = 6$) and porcine proendothelin$_{1-39}$ (■; $n = 4$); (b) inhibition by endothelin-2 ($n = 3$): (c) inhibition by endothelin-3 ($n = 6$). The points are the mean of n determinations on separate homogenates and the bars show s.e.mean; where no bar is shown the s.e.mean lies within the size of the symbol. The lines show the curves of best fit obtained from the fitting procedure.

Displacement of [^{125}I]-endothelin-1 by unlabelled analogues

Table 1 shows that [^{125}I]-endothelin-1 binding was inhibited by all the analogues used in the study; all of the peptides inhibited the binding of the radioligand to the same extent. The most potent inhibitors of its binding were the snake cardiotoxin sarafotoxin S6b and endothelin-3 with endothelin-1 itself and endothelin-2 having dissociation constants that were 3 to 4 fold greater (Figure 2). It is particularly interesting to note that all three of the alanyl-substituted analogues of endothelin-1 were able to inhibit specific [^{125}I]-endothelin-1 binding to sites in cerebellar homogenates (Figure 3). Thus, [Ala^{3,11}]endothelin-1 was about 3 fold less potent than the parent peptide and [Ala^{1,15}]endothelin-1 had an equilibrium dissociation constant 14 times greater than that of endothelin-1. However, the most interesting observation is that the ana-

Table 1 Binding constants and Hill slopes for inhibition of [^{125}I]-endothelin-1 by members of the endothelin/sarafotoxin family and by alanyl-substituted analogues of endothelin-1

	K_i (nM)	*Hill slope*	n
Endothelin-1	0.83 ± 0.15	1.1 ± 0.1	6
Endothelin-2	1.02 ± 0.16	1.0 ± 0.1	3
Endothelin-3	0.24 ± 0.05	0.9 ± 0.1	6
Sarafotoxin S6b	0.23 ± 0.06	1.1 ± 0.1	6
Porcine proendothelin$_{1-39}$	1250 ± 210	1.1 ± 0.2	4
[Ala^{1,15}]endothelin-1	11.5 ± 2.7	1.5 ± 0.1**	7
[Ala^{3,11}]endothelin-1	2.98 ± 0.3	1.3 ± 0.1**	8
[Ala^{1,3,11,15}]endothelin-1	0.72 ± 0.11	0.9 ± 0.1	7
[Acetamidomethyl-Cys^{3,11}]endothelin-1	8.1 ± 0.8	0.8 ± 0.1*	6
[Anisyl-Glu^{10}]endothelin-1	20.7 ± 2.1	0.8 ± 0.1*	3

K_i is the dissociation constant for the inhibition of [^{125}I]-endothelin-1 binding by the unlabelled peptide. Values are given as the mean ± s.e.mean and n represents the number of homogenates used. The values were determined by non-linear least squares curve fitting to a logistic equation.

Significant differences from 1 in Hill slopes are indicated by: * $P < 0.05$; ** $P < 0.01$. These were tested by carrying out curve-fitting with the Hill slope constrained to 1 and determining if the sum of squares of residuals was significantly greater than when the Hill slope was allowed to vary.

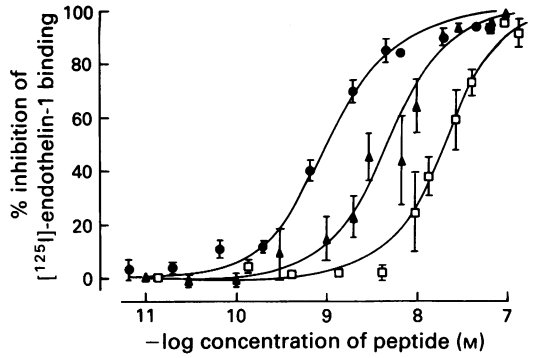

Figure 3 Inhibition of [^{125}I]-endothelin-1 binding to homogenates of rat cerebellum by three alanyl-substituted analogues of endothelin-1. (●) [Ala^{1,3,11,15}]endothelin-1 ($n = 7$); (▲) [Ala^{3,11}]endothelin-1 ($n = 8$); (□) [Ala^{1,15}]endothelin-1 ($n = 7$). The lines are the curves of best fit obtained from the fitting procedure. The points are the mean• of n determinations on separate homogenates and the bars show s.e.mean; where no bar is shown the s.e.mean lies within the size of the symbol.

logue which is devoid of disulphide bridges, [Ala^{1,3,11,15}]-endothelin-1, was equipotent with endothelin-1.

Comparison of [Ala^{3,11}]endothelin-1 with an analogue of endothelin-1 in which acetamidomethyl blocking groups were present on cysteine residues 3 and 11 shows that these bulky groups further reduced the inhibitory potency of the peptide by a factor of 2.7. Another analogue modified in the region of the disulphide bridge forming the smaller intramolecular loop, [anisyl-Glu^{10}]endothelin-1, was the least potent of the peptides studied being 25 fold less potent than endothelin-1.

The Hill slopes of the inhibition curves were not significantly different from 1 for endothelin-1, endothelin-2, endothelin-3, sarafotoxin S6b or the tetra-alanyl analogue of endothelin-1. The two dialanyl-substituted analogues of endothelin-1 had slopes which were significantly greater than unity whilst those of [acetamidomethyl-Cys^{3,11}]endothelin-1 and [anisyl-Glu^{10}]endothelin-1 were significantly less than 1.

Discussion

The rat cerebellum has previously been shown by autoradiography to contain high concentrations of binding sites for [^{125}I]-endothelin-1 (Davenport *et al.*, 1989b; Hoyer *et al.*, 1989; Jones *et al.*, 1989b). The binding site in rat cerebellar slices is of high affinity and the radioligand does not dissociate readily from the binding site. The present study shows that homogenates of rat cerebellum also contain a binding site for [^{125}I]-endothelin-1 which is of high affinity. Over the range 25 pM–10 nM the binding is to a single site and the affinity of this site, approximately 0.4 nM, is similar regardless of whether it is determined by saturation or by analysis of the kinetics of onset.

The interaction between the radioligand and its binding site can be inhibited by a number of peptide analogues of endothelin-1. The snake cardiotoxin, sarafotoxin S6b, and endothelin-3 were the most potent inhibitors of [^{125}I]-endothelin-1 binding, both being more potent than endothelin-1 itself. Endothelin-2, the third peptide encoded in the human genome (Inoue *et al.*, 1989), was approximately equipotent with the parent peptide; thus all four of these naturally occurring or genetically encoded peptides have approximately equal affinity at this binding site.

Mutual inhibition of endothelin-1 and sarafotoxin S6b specific binding in rat brain has also been demonstrated by Ambar *et al.* (1989), but they found that endothelin-1 was more potent than the toxin at inhibiting [^{125}I]-endothelin-1 binding in whole brain homogenates and they showed that the two peptides were somewhat less potent than was found in the present study. In other tissues, such as rat heart, sarafotoxin S6b and endothelin-1 have been found to be approximately equipotent at inhibiting [^{125}I]-endothelin-1 specific binding (Gu *et al.*, 1989). In the rat cerebellar homogenates, the presence of 1 nM sarafotoxin S6b in saturation experiments did not alter either the maximal binding or Hill slope for [^{125}I]-endothelin-1, which confirms that there was simple competition for a single site.

It has been suggested that the peptides may be produced from a 'pre-pro' form and the sequences for the pig and human forms of the putative precursors of endothelin-1 have been determined (Yanagisawa *et al.*, 1988b; Itoh *et al.*, 1988). Pig proendothelin-1 is a 39 amino acid peptide and it was found to be very weak at inhibiting the binding of [^{125}I]-endothelin-1, having an affinity 1000 fold less than that of endothelin-1 and the other 21 amino acid peptides. However, the putative human precursor, proendothelin-1_{1-38}, has vasoconstrictor actions in the rat isolated mesenteric bed (Douglas & Hiley, 1990), where it is at least 300 times less potent than endothelin-1, and in the rat aorta in which it is 100 fold less potent than the parent peptide (Kashiwabara *et al.*, 1989). Thus there may be differences in potency between the two proendothelins.

In view of the fact that the endothelin/sarafotoxin family of peptides are characterized by the possession of two disulphide bridges, between positions 1 and 15, and 3 and 11, it was of particular interest to determine the activities of peptide analogues of endothelin-1 which lack one or both of these bridges. Four of these peptides were investigated. One, [acetamidomethyl-Cys^{3,11}]endothelin-1, had the same amino acid sequence as the parent peptide but the formation of the 3-11 disulphide bridge was prevented by the use of acetamidomethyl blocking groups. In the other 3 compounds cysteine residues were replaced with alanine, which is pseudoisoteric with cysteine, in order to minimize the disruption to the secondary structure. All three of these compounds were able to inhibit the binding of [^{125}I]-endothelin-1 to its binding sites in cerebellum and they inhibited the radiolabelling to the same extent as did endothelin-1 itself and the other 21 amino acid peptides encoded in the human genome. [Ala^{3,11}]endothelin-1, which possesses a single disulphide bridge between positions 1 and 15, was approximately 3 fold less potent than endothelin-1 itself but was in turn 3 times more active than the compound in which the formation of the 3-11 bridge had been prevented by blocking groups. This suggests that the acetamidomethyl groups cause more disruption of the structure than can be attributed simply to the absence of the disulphide bond.

Indeed, [acetamidomethyl-Cys^{3,11}]endothelin-1 did not inhibit the binding of [^{125}I]-endothelin-1 to rat cultured vascular smooth cells in concentrations up to 0.1 μM, nor did it stimulate increases in intracellular calcium (Hirata *et al.*, 1989). Further indication of the sensitivity of the binding site to substitution in the smaller intramolecular loop near to position 11 came from the markedly lower potency at inhibiting the binding of the radiolabel found for [anisyl-Glu^{10}] endothelin-1. Thus it would seem that bulkiness of the amino acid residues in this region is of more consequence for binding potency than the 3-11 disulphide bridge itself. Another point of interest is that [anisyl-Glu^{10}]endothelin-1 and [acetamidomethyl-Cys^{3,11}]endothelin-1 were the only two of the agents investigated which had Hill slopes of interaction with [^{125}I]-endothelin-1 which were significantly less than unity; there is therefore the possibility that they do not interact with a single class of binding sites even though no heterogeneity of binding was observed with [^{125}I]-endothelin-1 itself.

Confirmation of the relative lack of importance of the disulphide bridges in determining binding potency came from the second peptide containing a single disulphide bridge due to alanine substitution; [Ala^{1,15}]endothelin-1 was 4 fold less potent than [Ala^{3,11}]endothelin-1 in inhibiting radioligand

binding. Thus, possession of a single disulphide bridge in itself does not greatly reduce the activity of this series of peptides at the cerebellar binding site. Further, perhaps the most surprising result was that the peptide devoid of disulphide bridges, [Ala^{1,3,11,15}]endothelin-1, was more potent than either of the single bridge compounds and was approximately equipotent with endothelin-1.

All the alanyl-substituted endothelin-1 analogues have been found to have biological activity. In rat aorta (Topouzis *et al.*, 1989), guinea-pig trachea (Pelton & Miller, unpublished observations) and rat perfused mesenteric arterial beds (Randall *et al.*, 1989) the order of potency at causing constriction is endothelin-1 > [Ala^{3,11}]endothelin-1 > [Ala^{1,15}]-endothelin-1. [Ala^{1,3,11,15}] endothelin-1 did not cause any constriction of the isolated Krebs-Henseleit perfused mesenteric bed, but it had agonist activity in guinea-pig trachea and acted as a partial agonist in the *in situ* blood perfused mesenteric bed where it had an ED_{50} equal to that of endothelin-1. It also had a pressor effect in the pithed rat in which it had one-fifth of the potency of the parent peptide (MacLean & Hiley, 1989). Thus the finding that it is equipotent at inhibiting [^{125}I]-endothelin-1 binding is consistent with this biological activity.

The results of this present study show that there is apparently a single class of binding sites for [^{125}I]-endothelin-1 in the rat cerebellum and that the radiolabel may be inhibited from binding to these sites by a number of peptide analogues. The structure-activity relationship for this displacement shows that the possession of disulphide bridges is not a strict requirement for binding to the site and this is consistent with observed biological activity in a number of tissues. Binding may, however, be sensitive to the possession of bulky substituents in the region of residues 3, 10 and 11.

References

AMBAR, I., KLOOG, Y., SCHVARTZ, I., HAZUM, E. & SOKOLOVSKY, M. (1989). Competitive interaction between endothelin and sarafotoxin: binding and phosphoinositides hydrolysis in rat atria and brain. *Biochem. Biophys. Res. Commun.*, **158**, 195–201.

DAVENPORT, A.P., NUNEZ, D.J. & BROWN, M.J. (1989a). Binding sites for 125I-labelled endothelin-1 in the kidneys: differential distribution in rat, pig and man demonstrated by using quantitative autoradiography. *Clin. Sci.*, **77**, 129–131.

DAVENPORT, A.P., NUNEZ, D.J., HALL, J.A., KAUMANN, A.J. & BROWN, M.J. (1989b). Autoradiographical localization of binding sites for porcine [^{125}I]endothelin-1 in humans, pigs and rats: functional relevance in humans. *J. Cardiovasc. Pharmacol.*, **13** (Suppl. 5), S166–S170.

DOUGLAS, S.A. & HILEY, C.R. (1990). Endothelium-dependent vascular activities of endothelin-like peptides in the isolated superior mesenteric arterial bed of the rat. *Br. J. Pharmacol.*, (in press).

GU, X.-H., CASLEY, D.J. & NAYLER, W.G. (1989). Sarafotoxin S6b displaces specifically bound ^{125}I-endothelin. *Eur. J. Pharmacol.*, **162**, 509–510.

HILEY, C.R., DOUGLAS, S.A. & RANDALL, M.D. (1989a). Pressor effects of endothelin and some analogs in the perfused superior mesenteric arterial bed of the rat. *J. Cardiovasc. Pharmacol.*, **13** (Suppl. 5), S197–S199.

HILEY, C.R., PELTON, J.T. & MILLER, R.C. (1989b). Effects of endothelin on field stimulated rat vas deferens and guinea-pig ileum. *Br. J. Pharmacol.*, **96**, 104P.

HIRATA, Y., YOSHIMI, H., EMORI, T., SHICHIRI, M., MARUMO, F., WATANABE, T.X., KUMAGAYE, S., NAKAJIMA, K., KIMURA, T. & SAKAKIBARA, S. (1989). Receptor binding activity and cytosolic free calcium response by synthetic endothelin analogs in cultured rat smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **160**, 228–234.

HOYER, D., WAEBER, C. & PALACIOS, J.M. (1989). [^{125}I]endothelin-1 binding sites: autoradiographic studies in the brain and periphery of various species including humans. *J. Cardiovasc. Pharmacol.*, **13** (Suppl. 5), S162–S165.

INOUE, A., YANAGISAWA, M., KIMURA, S., KASUYA, Y., MIYAUCHI, T., GOTO, K. & MASAKI, T. (1989). The human endothelin family: Three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 2863–2867.

ITOH, Y., YANAGISAWA, M., OHKUBO, S., KIMURA, C., KOSAKA, T., INOUE, A., ISHIDA, N., MITSUI, Y., ONDA, H., FUJINO, M. & MASAKI, T. (1988). Cloning and sequence analysis of cDNA encoding the precursor of a human endothelium-derived vasoconstrictor peptide, endothelin: identity of human and porcine endothelin. *FEBS Lett.*, **231**, 440–444.

ITOH, Y., KIMURA, C., ONDA, H. & FUJINO, M. (1989). Canine endothelin-2: cDNA sequence for the mature peptide. *Nucleic Acids Res.*, **17**, 5389.

JONES, C.R., HILEY, C.R., PELTON, J.T. & MILLER, R.C. (1989a). Autoradiographic localisation of endothelin binding sites in kidney. *Eur. J. Pharmacol.*, **163**, 379–382.

JONES, C.R., HILEY, C.R., PELTON, J.T. & MOHR, M. (1989b). Autoradiographic visualization of the binding sites for [^{125}I]endothelin in rat and human brain. *Neurosci. Lett.*, **97**, 276–279.

JONES, C.R., PELTON, J.T., MILLER, R.C. & HILEY, C.R. (1989c). Binding characteristics of [^{125}I]endothelin to rat brain and its displacement by peptide analogues. *Br. J. Pharmacol.*, **96**, 346P.

KASHIWABARA, T., INAGAKI, Y., OHTA, H., IWAMATSU, A., NOMIZU, M., MORITA, A. & NISHIKORI, K. (1989). Putative precursors of endothelin have less vasoconstrictor activity in vitro but a potent pressor effect in vivo. *FEBS Lett.*, **247**, 73–76.

KIMURA, S., KASUYA, Y., SAWAMURA, T., SHINMI, O., SUGITA, Y., YANAGISAWA, M., GOTO, K. & MASAKI, T. (1988). Structure-activity relationships of endothelin: importance of the C-terminal moiety. *Biochem. Biophys. Res. Commun.*, **156**, 1182–1186.

KIMURA, C., ITOH, Y., OHKUBO, S., OGI, K., ONDA, H. & FUJINO, M. (1989). Cloning and sequencing of a canine gene segment encoding mature endothelin. *Nucleic Acids Res.*, **17**, 3290.

KLOOG, Y., AMBAR, I., SOKOLOVSKY, M., KOCHVA, E., WOLLBERG, Z. & BDOLAH, A. (1988). Sarafotoxin, a novel vasoconstrictor peptide: phosphoinositide hydrolysis in rat heart and brain. *Science*, **242**, 268–270.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

MACLEAN, M.R. & HILEY, C.R. (1989). Blood pressure changes induced by endothelin and [Ala^{1,3,11,15}]endothelin in pithed rats: effect of lowering ventilation volume. *Br. J. Pharmacol.*, **97**, 529P.

MERRIFIELD, R.B. (1963). Solid phase peptide synthesis. 1. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.*, **85**, 2149–2154.

MCKEE, R.L., PELTON, J.T., TRIVEDI, D., JOLINSON, D.G., COY, D.H., SUIERAS-DIAZ, J. & HRUBY, V.J. (1986). Receptor binding and adenylate cyclase activities of glucagon analogues modified in the N-terminal region. *Biochemistry*, **25**, 1650–1656.

MCPHERSON, G.A. (1985). *KINETIC, EDBA, LIGAND, LOWRY A collection of radioligand binding analysis programs.* Amsterdam: Elsevier Science Publishers.

MUNSON, P.J. & RODBARD, D. (1989). LIGAND: a versatile computerized approach for the characterization of ligand binding systems. *Anal. Biochem.*, **107**, 220–236.

MUNSON, P.J. & RODBARD, D. (1988). An exact correction to the "Cheng-Prusoff" correction. *J. Receptor Res.*, **8**, 533–546.

OHKUBO, S., ITOH, Y., KIMURA, C., ONDA, H. & FUJINO, M. (1990). Nucleotide sequence of a rabbit genomic DNA encoding mature endothelin-3. *Nucleic Acids Res.*, **18**, 374.

PELTON, J.T., KAZMIERSKI, W., GULYA, K., YAMAMURA, H.I. & HRUBY, V.J. (1986). Design and synthesis of conformationally constrained somatostatin analogues with high potency and specificity for μ opioid receptors. *J. Med. Chem.*, **29**, 2370–2375.

RANDALL, M.D., DOUGLAS, S.A. & HILEY, C.R. (1989). Vascular activities of endothelin-1 and some alanyl substituted analogues in resistance beds of the rat. *Br. J. Pharmacol.*, **98**, 685–699.

SAIDA, K., MITSUI, Y. & ISHIDA, N. (1989). A novel peptide, vasoactive intestinal contractor, of a new (endothelin) peptide family. *J. Biol. Chem.*, **264**, 14613–14616.

STEWART, J.M. & YOUNG, J.D. (1984). *Solid Phase Peptide Synthesis*, 2nd edition, p. 121. Rockford: Pierce Chemical Co., U.S.A.

TAM, J.P., HEATH, W.F. & MERRIFIELD, R.B. (1983). Improved deprotection in solid phase synthesis: removal of protecting groups from synthetic peptides by an S_N2 mechanism with low concentrations of HF in dimethylsulfide. *J. Am. Chem. Soc.*, **105**, 6442–6455.

TOPOUZIS, S., PELTON, J.T. & MILLER, R.C. (1989). Effects of calcium

entry blockers on contractions evoked by endothelin, [Ala^{3,11}]endothelin-1 and [Ala^{1,15}]endothelin-1 in rat isolated aorta. *Br. J. Pharmacol.*, **98**, 669–677.

YANAGISAWA, M., INOUE, A., ISHIKAWA, T., KASUYA, Y., KIMURA, S., KUMAGAYE, S.-I., NAKAJIMA, K., WATANABE, T.X., SAKAKIBARA, S., GOTO, K. & MASAKI, T. (1988a). Primary structure, synthesis, and biological activity of rat endothelin, an endothelium-derived vasoconstrictor peptide. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 6964–6967.

YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988b). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332**, 411–415.

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Characterization of the L-arginine:nitric oxide pathway in human platelets

[1]M.W. Radomski, R.M.J. Palmer, [2]S. Moncada

Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS

1 The activation of the L-arginine:nitric oxide (NO) pathway during aggregation of human platelets by adenosine 5'-diphosphate (ADP), arachidonic acid, thrombin and the calcium ionophore A23187 and its inhibition by N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine methyl ester (L-NAME) and N-iminoethyl-L-ornithine (L-NIO) were studied. The inhibition of the cytosolic platelet NO synthase by these compounds was also examined.

2 Platelet aggregation induced by ADP (1–10 μM) and arachidonic acid (0.1–10 μM), but not that induced by thrombin (1–30 mu ml^{-1}) or A23187 (1–10 nM), was inhibited by L-, but not D-arginine (1–30 μM). However, in the presence of a subthreshold concentration of prostacyclin (0.1 nM) or of M & B 22948 (1 μM), a selective inhibitor of guanosine 3':5'-cyclic monophosphate (cyclic GMP) phosphodiesterase, L-arginine caused concentration-dependent inhibition of aggregation induced by all of these aggregating agents.

3 L-NMMA, L-NAME and L-NIO (all at 1–30 μM), but not their D-enantiomers, enhanced to the same extent platelet aggregation induced by ADP, arachidonic acid and thrombin without affecting that induced by A23187.

4 In the presence of 300 μM L-arginine, the NO synthase in platelet cytosol was inhibited by L-NMMA, L-NAME and L-NIO with IC_{50}s of 74 ± 9, 79 ± 8 and 8.5 ± 1.5 μM (n = 3), respectively.

5 These results indicate that the L-arginine:NO pathway in human platelets plays a role in the modulation of platelet aggregation.

Introduction

Synthesis of nitric oxide (NO) from L-arginine is now known to occur in vascular endothelial cells (Palmer *et al.*, 1988a), macrophages (Hibbs *et al.*, 1988; Marletta *et al.*, 1988; Kwon *et al.*, 1989), neutrophils (McCall *et al.*, 1989; Salvemini *et al.*, 1989), brain synaptosomes (Knowles *et al.*, 1989), adrenal glands (Palacios *et al.*, 1989) and a number of other tissues (for review see Moncada *et al.*, 1989). This pathway plays a role in the regulation of vascular tone and blood pressure (Rees *et al.*, 1989; Aisaka *et al.*, 1989) and in neurotransmission in the central nervous system (Garthwaite *et al.*, 1989; Bredt & Snyder 1989), in peripheral non-adrenergic, non-cholinergic neurones (Gillespie *et al.*, 1989; Gibson *et al.*, 1990) and possibly in the regulation of the entry of sensory stimuli into the peripheral nerve endings (Duarte *et al.*, 1990). In addition, NO released by phagocytic cells mediates some of their cytotoxic functions (Hibbs *et al.*, 1990). These findings led us to propose that the L-arginine:NO pathway is a widespread regulatory mechanism and the endogenous activator of the soluble guanylate cyclase (Moncada *et al.*, 1989).

Nitric oxide also inhibits platelet aggregation (Mellion *et al.*, 1981; Radomski *et al.*, 1987a). This effect is mediated via activation of the soluble guanylate cyclase and is potentiated by a selective inhibitor of the guanosine 3':5'-cyclic monophosphate (cyclic GMP) phosphodiesterase, 2-O-propoxyphenyl-8-azapurin-6-one (M&B 22948, Lugnier *et al.*, 1986) and by a subthreshold concentration of prostacyclin (Radomski *et al.*, 1987a). Furthermore, we have recently shown that when human platelets are stimulated with collagen, they synthesize NO from L-arginine. This NO activates the soluble guanylate cyclase and down-regulates the response of platelets to collagen (Radomski *et al.*, 1990).

There are at least two distinct NO synthases in different tissues; the endothelial/brain enzyme (Palmer & Moncada, 1989; Meyer *et al.*, 1989; Knowles *et al.*, 1989) and the phagocytic cell enzyme (Marletta *et al.*, 1988; McCall *et al.*, 1989). The former is NADPH- and Ca^{2+}-dependent and not affected by L-canavanine (Palmer & Moncada 1989; Meyer *et al.*, 1989; Knowles *et al.*, 1989) and the latter is not Ca^{2+}-dependent, is inhibited by L-canavanine and requires tetrahydrobiopterin in addition to NADPH (Marletta *et al.*, 1988; Kwon *et al.*, 1989). Our previous findings indicate that the platelet NO synthase is of the endothelial/brain type since it is Ca^{2+}-dependent (Radomski *et al.*, 1990).

N^G-monomethyl-L-arginine (L-NMMA) was originally described as an inhibitor of NO_2^- and NO_3^- synthesis from L-arginine by macrophages (Hibbs *et al.*, 1987) and of NO synthesis in vascular endothelial cells (Palmer *et al.*, 1988b). Furthermore, L-NMMA inhibits NO synthesis in platelets (Radomski *et al.*, 1990) and in a number of other tissues (for review see Moncada *et al.*, 1989). More recently, N^G-nitro-L-arginine or its methyl ester (L-NAME, Palacios *et al.*, 1989; Rees *et al.*, 1990; Moore *et al.*, 1990; Mulsch & Busse 1990) and N^G-iminoethyl-L-ornithine (L-NIO, Palacios *et al.*, 1989; Rees *et al.*, 1990; Knowles *et al.*, 1990) have also been shown to inhibit NO synthesis in adrenal glands, endothelial cells and brain synaptosomes.

In the present study we have examined the activation of the L-arginine:NO pathway in platelets stimulated with adenosine 5'-diphosphate (ADP), arachidonic acid, thrombin and A23187 and have characterized the activity of these L-arginine analogues as inhibitors of platelet NO synthase.

Methods

Platelet aggregation

Blood was collected from healthy volunteers into trisodium citrate (0.315%) and platelets were isolated and washed using prostacyclin as described previously (Radomski & Moncada, 1983). The platelets were finally resuspended in Tyrode solution at a concentration of 2×10^8 platelets ml^{-1}. Platelet aggregation induced by ADP, arachidonic acid, thrombin and

[1] Present address: Department of Cardiovascular Physiology, Medical Research Centre, Polish Academy of Sciences, Warsaw Dworkowa 3, Poland.

[2] Author for correspondence.

A23187 was measured in an aggregometer (Payton Associates) by the method of Born (1962). Test compounds were added to the platelet suspension in the aggregometer 3 min before the addition of the aggregating agent.

NO synthase assay

Washed platelets ($7–8 \times 10^{10}$) in 3 ml Tyrode solution were sonicated twice for 5 s and the homogenate centrifuged at 150,000*g* for 30 min at 4°C. The supernatant was then depleted of endogenous L-arginine by incubation with 500 mg AG50X8 (Dowex) cation exchange resin for 10 min at 4°C. Following incubation, the supernatant was removed and used as the source of cytosolic NO synthase. The activity of NO synthase was determined, following incubation for 20 min in the presence of NADPH (300 μM) and L-arginine (300 μM), by measuring the formation of cyclic GMP as described previously (Knowles *et al.*, 1989; Radomski *et al.*, 1990).

Reagents

Arachidonic acid sodium salt, ADP, L(D)-arginine, L-canavanine, N^G-nitro-L-arginine methyl ester (L-NAME, Sigma), A23187 (Calbiochem), M&B 22948 (May & Baker), L-NMMA, D-NMMA, L-NIO, prostacyclin sodium salt (Wellcome) and thrombin (Ortho Diagnostics) were obtained as indicated.

Statistics

All values are means ± s.e.mean of *n* experiments. Values were compared by use of Student's *t* test for paired or unpaired samples as appropriate and $P < 0.05$ was considered as statistically significant.

Results

Platelet aggregation

Platelet aggregation induced by ADP (10 μM) or arachidonic acid (3 μM) was inhibited in a concentration-dependent manner by L-arginine (Table 1) but not D-arginine (100 μM, $n = 3$). In contrast, platelet aggregation induced by thrombin (30 mu ml^{-1}) or A23187 (10 nM) was not inhibited by L-arginine (30 μM). Preincubation of platelets with a subthreshold concentration of prostacyclin (0.1 nM) enhanced the activity of L-arginine such that the IC_{50} against arachidonic acid and ADP was significantly reduced and its action against thrombin- or A23187-induced aggregation was evident (Table 1). The inhibition by L-arginine of platelet aggregation induced by thrombin or A23187 was also evident in the presence of M&B 22948 (Table 1).

Table 1 The effect of L-arginine on platelet aggregation induced by ADP, arachidonic acid, thrombin and A23187

	L-Arginine IC_{50} (μM, $n = 3–5$)		
	L-Arginine	*L-Arginine + PGI_2*	*L-Arginine + M&B 22948*
ADP	9.2 ± 1.4	3.8 ± 1.2	n.a.
Arachidonic acid	7.8 ± 1.6	2.6 ± 0.7	n.a.
Thrombin*	>100	16.5 ± 3.2	27.2 ± 5.2
A23187**	>100	28.3 ± 4.0	34.2 ± 5.8

* 22 ± 4% inhibition at 100 μM.
** 14 ± 3% inhibition at 100 μM.
n.a. not assayed. PGI_2 = prostacyclin

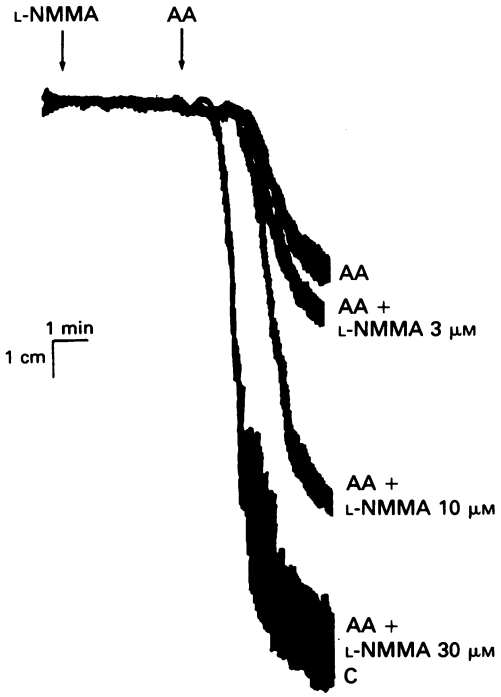

Figure 1 Enhancement of arachidonic acid-induced platelet aggregation by N^G-monomethyl-L-arginine (L-NMMA). Aggregation was induced by a submaximal concentration of arachidonic acid (AA, 1 μM). L-NMMA dose-dependently enhanced the effect of AA with a maximal effect at 30 μM. The maximal aggregation (C) was obtained with 10 μM AA. Representative tracings of 5 similar experiments.

Platelet aggregation induced by arachidonic acid was enhanced in a concentration-dependent manner by L-NMMA (Figure 1). L-NAME and L-NIO (both at 30 μM) were equipotent at enhancing platelet aggregation ($n = 3–5$). The maximum enhancement of arachidonic acid-induced aggregation by all these compounds was also similar (Figure 2). L-NMMA, L-NAME and L-NIO (all at 30 μM) enhanced platelet aggregation induced by ADP and thrombin to a similar

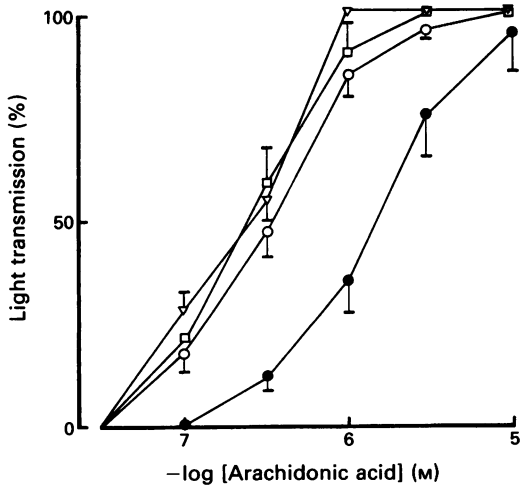

Figure 2 Enhancement of arachidonic acid-induced platelet aggregation by N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine methyl ester (L-NAME) or N-iminoethyl-L-ornithine (L-NIO). Platelet aggregation induced by arachidonic acid (●) was maximally enhanced to the similar extent by L-NMMA (○), L-NAME (□) or L-NIO (▽) all at 30 μM. The results are mean of 3–5 experiments; vertical lines show s.e.mean.

Table 2 The effect of N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine methyl ester (L-NAME) and N-iminoethyl-L-orthinine (L-NIO) on platelet aggregation

	Aggregating agent		
	ADP (μM)	*Thrombin* (mu ml^{-1})	*A23187* (nM)
Control	3.5 ± 0.5	16.0 ± 3.0	4.5 ± 1.0
L-NMMA	1.4 ± 0.3	9.0 ± 2.0	4.3 ± 1.2
L-NAME	1.0 ± 0.2	7.0 ± 1.0	3.9 ± 0.9
L-NIO	1.2 ± 0.2	8.0 ± 2.0	4.6 ± 1.1

Data shown are mean EC_{50} values ± s.e.mean (n = 3–5).

extent (n = 3–5), but did not have an effect on the aggregation induced by A23187 (Table 2). In this last respect there were no significant differences between the compounds. L-Canavanine, D-NMMA, D-NAME and D-NIO (100 μM, n = 3) did not affect platelet aggregation induced by ADP, arachidonic acid, thrombin or A23187 (n = 3).

The effect of L-NMMA, L-NAME and L-NIO on platelet NO synthase

Platelet NO synthase activity in the presence of L-arginine and NADPH (both at 300 μM) was inhibited in a concentration-dependent manner by L-NMMA, L-NAME and L-NIO (Figure 3). L-NIO was significantly more potent than either L-NMMA or L-NAME, which were not significantly different from each other (Figure 3). D-NMMA, D-NAME, D-NIO and L-canavanine (all at 300 μM, n = 3) did not affect the activity of platelet NO synthase.

Discussion

We have previously demonstrated that platelets contain an NO synthase which, when activated during platelet aggregation induced by collagen, synthesizes NO from L-arginine (Radomski *et al.*, 1990). This NO stimulates the soluble guanylate cyclase, increases cyclic GMP and down-regulates platelet aggregation. We have now demonstrated that other aggregating agents, such as ADP and arachidonic acid, activate the synthesis of NO since platelet aggregation induced by these agents was also inhibited by L-, but not D-arginine. Moreover, the inhibitors of NO synthase L-NMMA, L-NAME and L-NIO (Palmer & Moncada, 1989; Palacios *et al.*, 1989; Knowles *et al.*, 1990) enhanced aggregation induced by these agents.

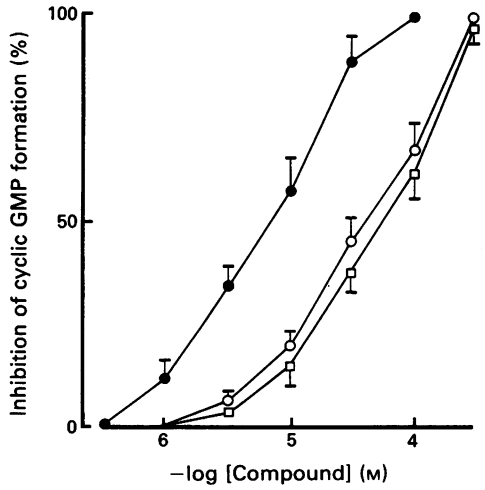

Figure 3 Inhibition of nitric oxide (NO) synthase activity in platelet cytosol by N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine methyl ester (L-NAME) or N-iminoethyl-L-ornithine (L-NIO). NO synthase activity in the presence of L-arginine and NADPH, both at 300 μM, was inhibited by L-NIO (●) L-NMMA (○) and L-NAME (□). L-NIO was the most potent inhibitor and L-NMMA and L-NAME were not significantly different from each other. The results are mean of 3–5 experiments; vertical lines show s.e.mean.

Interestingly, although L-arginine also inhibited platelet aggregation induced by thrombin and A23187, this was only evident under conditions in which the effect of NO is potentiated, such as in the presence of M&B 22948 or a subthreshold concentration of prostacyclin (Radomski *et al.*, 1987b). Furthermore, the inhibitors of NO synthase were similarly effective in potentiating aggregation induced by arachidonic acid and ADP, but were less so for thrombin and ineffective against aggregation induced by A23187. This profile of modulation of platelet aggregation by endogenous NO correlates well with that observed for the effect of exogenous NO on the aggregating action of these agents, for the order of potency of NO against these agents is arachidonic acid = ADP > thrombin > A23187 (Radomski *et al.*, 1987b).

These results show that aggregating agents stimulate a common pathway which leads to the synthesis of NO from L-arginine. The stimulation of platelets with aggregating agents results in an approximately 100 fold increase in the resting (100 nM) $[Ca^{2+}]_i$ (Ware *et al.*, 1986). Since the platelet NO synthase is Ca^{2+}-dependent (Radomski *et al.*, 1990), it is likely that this sharp increase in $[Ca^{2+}]_i$ activates the enzyme and triggers the synthesis of NO. The formation of NO in platelets is accompanied by stimulation of soluble guanylate cyclase and an increase in intra-platelet cyclic GMP levels (Radomski *et al.*, 1990). The mechanism by which elevated cyclic GMP levels attenuate platelet aggregation is not known. However, the increases in cyclic GMP cause Ca^{2+} sequestration (Busse *et al.*, 1987), leaving less free Ca^{2+} available for aggregation. The net biological effect after stimulation of the platelets therefore will depend on the amounts of free Ca^{2+} available for aggregation, for it appears that even when NO synthase is fully active, agents which are more powerful elevators of $[Ca^{2+}]_i$, such as A23187, overcome the inhibitory effect of intra-platelet NO.

The L-arginine analogues L-NMMA, L-NAME and L-NIO potentiated platelet aggregation to similar extents and with similar potency. However, L-NIO was approximately 8 times more potent than L-NMMA and L-NAME as an inhibitor of NO synthase in platelet cytosol. This is similar to the order of potency of these compounds against endothelial NO synthase (Moncada & Palmer, 1990), although a different rank order was observed in intact endothelial cells (Rees *et al.*, 1990). The reason for the difference in rank order of these compounds in whole platelets and platelet cytosol is not known, but may indicate differences in the uptake, distribution or metabolism of these compounds in the platelets. Similar differences between the activity of these compounds on neutrophils and neutrophil NO synthase have also been observed (McCall *et al.*, 1990). The activity of these inhibitors and the finding that L-canavanine does not affect platelet aggregation support our proposal that the platelet NO synthase is of the brain/endothelial type (Radomski *et al.*, 1990).

Platelet aggregation *in vivo* is likely to be regulated by intraplatelet NO, as well as by NO and prostacyclin released from vascular endothelium. The combined action of these two mediators could result in an enhanced inhibition of $[Ca^{2+}]_i$ elevation and subsequent inhibition of platelet aggregation (Radomski *et al.*, 1987a). The results of these experiments reinforce our original suggestions that the combined use of agents which increase activity of the adenylate and guanylate cyclase offers an interesting strategy for development of effective anti-platelet drugs (Radomski *et al.*, 1987a; Moncada *et al.*, 1988). They also point to the need for examining the pharmacological action of dietary L-arginine in respect to its anti-platelet potential.

References

AISAKA, K., GROSS, S.S., GRIFFITH, O.W. & LEVI, R. (1989). N^G-methylarginine, an inhibitor of endothelium-derived nitric oxide synthesis is a potent pressor agent in the guinea-pig: does nitric oxide regulate blood pressure in vivo? *Biochem. Biophys. Res. Commun.*, **160**, 881–886.

BORN, G.V.R. (1962). Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature*, **194**, 927–929.

BREDT, P.S. & SNYDER, S.A. (1989). Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 9030–9033.

BUSSE, R., LUCKHOFF, A. & BASSENGE, E. (1987). Endothelium-derived relaxant factor inhibits platelet activation. *Naunyn-Schmeidebergs Arch. Pharmacol.*, **336**, 566–571.

DUARTE, I.D.G., LORENZETTI, B.B. & FERREIRA, S.H. (1990). Acetylcholine induces peripheral analgesia by the release of nitric oxide. In *Nitric Oxide from L-Arginine: a Bioregulatory System*, ed. Moncada, S. & Higgs, E.A. pp. 159–164. North Holland: Elsevier.

GARTHWAITE, J., GARTHWAITE, G., PALMER, R.M.J. & MONCADA, S. (1989). NMDA receptor activation induces nitric oxide synthesis from arginine in rat brain slices. *Eur. J. Pharmacol.*, **172**, 413–416.

GIBSON, A., MIRZAZADEH, S., HOBBS, A.J. & MOORE, P.K. (1990). L-N^G-monomethyl arginine and L-N^G-nitroarginine inhibit non-adrenergic, non-cholinergic relaxation of the mouse anococcygeus muscle. *Br. J. Pharmacol.*, **99**, 602–606.

GILLESPIE, J.S., LIU, X. & MARTIN, W. (1989). The effects of L-arginine and N^G-monomethyl L-arginine on the response of the rat anococcygeus muscle to NANC nerve stimulation. *Br. J. Pharmacol.*, **98**, 1080–1082.

HIBBS, J.B. Jr, TAINTOR, R.R., VAVRIN, Z. & RACHLIN, E.M. (1988). Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.*, **157**, 87–94.

HIBBS, J.B. Jr, TAINTOR, R.R., VAVRIN, Z., GRANGER, D.L., DRAPIER, J.C., AMBER, I.J. & LANCASTER, J.R. (1990). Synthesis of nitric oxide from a terminal guanidino nitrogen atom of L-arginine: a molecular mechanism regulating cellular proliferation that targets intracellular iron. In *Nitric Oxide From L-Arginine: a Bioregulatory System*, ed. Moncada, S. & Higgs, E.A. pp. 183–216. North Holland: Elsevier.

HIBBS, J.B. Jr, VAVRIN, Z. & TAINTOR, R.R. (1987). L-Arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *J. Immunol.*, **138**, 550–565.

KNOWLES, R.G., PALACIOS, M., PALMER, R.M.J. & MONCADA, S. (1989). Formation of nitric oxide from L-arginine: a transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 5159–5162.

KNOWLES, R.G., PALACIOS, M., PALMER, R.M.J. & MONCADA, S. (1990). Kinetic characteristics of nitric oxide synthase from rat brain. *Biochem. J.*, **269**, 207–210.

KWON, N.S., NATHAN, C.F. & STUEHR, D.J. (1989). Reduced biopterin as a cofactor in the generation of nitrogen oxides by murine macrophages. *J. Biol. Chem.*, **264**, 20496–20501.

LUGNIER, C., SCHOEFFTER, P., LE BEC, A., STROUTHOU, E. & STOCLET, J.C. (1986). Selective inhibition of cyclic nucleotide phosphodiesterases of human, bovine and rat aorta. *Biochem. Pharmacol.*, **35**, 1743–1751.

MARLETTA, M.A., YOON, P.S., IYENGAR, R., LEAF, C.D. & WISHNOK, J.S. (1988). Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry*, **27**, 8706–8711.

McCALL, T.B., BOUGHTON-SMITH, N.K., PALMER, R.M.J., WHITTLE, B.J.R. & MONCADA, S. (1989). Synthesis of nitric oxide from L-arginine by neutrophils. Release and interaction with superoxide anion. *Biochem. J.*, **261**, 293–296.

McCALL, T.B., PALMER, R.M.J., BOUGHTON-SMITH, N.K., WHITTLE, B.J.R. & MONCADA, S. (1990). The L-arginine:nitric oxide pathway in neutrophils. In *Nitric Oxide From L-Arginine: A Bioregulatory System*, ed. Moncada, S. & Higgs, E.A. pp. 249–257. North Holland: Elsevier.

MELLION, B.T., IGNARRO, L.J., OHLSTEIN, E.H., PONTECORVO, E.G., HYMAN, A.L. & KADOWITZ, P.J. (1981). Evidence for the inhibitory role of guanosine 3',5'-monophosphate in ADP-induced human aggregation in the presence of nitric oxide and related vasodilators. *Blood*, **57**, 946–955.

MEYER, B., SCHMIDT, K., HUMBERT, R. & BOHME, E. (1989). Biosynthesis of endothelium-derived relaxing factor, a cytosolic enzyme in porcine aortic endothelial cells Ca^{2+}-dependently converts L-arginine into an activator of guanylate cyclase. *Biochem. Biophys. Res. Commun.*, **164**, 678–685.

MONCADA, S. & PALMER, R.M.J. (1990). The L-arginine:nitric oxide pathway in the vessel wall. In *Nitric Oxide from L-Arginine: A Bioregulatory System*, ed. Moncada, S. & Higgs, E.A. pp. 17–31. North Holland: Elsevier.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1989). Biosynthesis of nitric oxide from L-arginine: a pathway for the regulation of cell function and communication. *Biochem. Pharmacol.*, **38**, 1709–1715.

MONCADA, S., RADOMSKI, M.W. & PALMER, R.M.J. (1988). Endothelium-derived relaxing factor: identification as nitric oxide and role in the control of vascular tone and platelet function. *Biochem. Pharmacol.*, **37**, 2495–2501.

MOORE, P.K., AL-SWAYEH, O.A., CHONG, N.W.S., EVANS, R.A. & GIBSON, A. (1990). L-N^G-nitro-arginine (L-NOARG), a novel, L-arginine reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br. J. Pharmacol.*, **99**, 408–412.

MULSCH, A. & BUSSE, R., (1990). N^G-nitro-L-arginine (N^G-[imino-(nitroamino)methyl]-L-ornithine) impairs endothelium-dependent dilatations by inhibitory cytosolic nitric oxide synthesis from L-arginine. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **341**, 143–147.

PALACIOS, M., KNOWLES, R.G., PALMER, R.M.J. & MONCADA, S. (1989). Nitric oxide from L-arginine stimulates the soluble guanylate cyclase in adrenal glands. *Biochem. Biophys. Res. Commun.*, **165**, 802–809.

PALMER, R.M.J. & MONCADA, S. (1989). A novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. *Biochem. Biophys. Res. Commun.*, **158**, 348–352.

PALMER, R.M.J., ASHTON, D.S. & MONCADA, S. (1988a). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664–666.

PALMER, R.M.J., REES, D.D., ASHTON, D.S. & MONCADA, S. (1988b). L-Arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem. Biophys. Res. Commun.*, **153**, 1251–1256.

RADOMSKI, M.W. & MONCADA, S. (1983). An improved method for washing platelets with prostacyclin. *Thromb. Res.*, **30**, 383–389.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1987a). The anti-aggregating properties of vascular endothelium. Interactions between prostacyclin and nitric oxide. *Br. J. Pharmacol.*, **92**, 629–636.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1987b). Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets. *Br. J. Pharmacol.*, **92**, 181–187.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1990). An L-arginine to nitric oxide pathway in human platelets regulates aggregation. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 5193–5197.

REES, D.D., PALMER, R.M.J. & MONCADA, S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 3375–3378.

REES, D.D., SCHULZ, R., HODSON, H.F., PALMER, R.M.J. & MONCADA, S. (1990). Identification of some novel inhibitors of the vascular nitric oxide synthase *in vivo* and *in vitro*. In *Nitric Oxide from L-Arginine: A Bioregulatory System*, ed. Moncada, S. & Higgs, E.A. pp. 473–475. North Holland: Elsevier.

SALVEMINI, D., DE NUCCI, G., GRYGLEWSKI, R.J. & VANE, J.R. (1989). Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 6328–6332.

WARE, J.A., JOHNSON, P.C., SMITH, M. & SALZMAN, E.W. (1986). The effect of common agonists on cytoplasmic ionized calcium concentration in platelets: measurement with quin2 and aequorin. *J. Clin. Invest.*, **77**, 878–886.

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Reserpine induces vascular α_2-adrenergic supersensitivity and platelet α_2-adrenoceptor up-regulation in dog

[1]Luis Estan, Jean-Michel Senard, Marie-Antoinette Tran, Jean-Louis Montastruc & [2]Michel Berlan

Laboratoire de Pharmacologie Médicale et Clinique, INSERM U317, Faculté de Médecine 37, allées J. Guesde, 31073 Toulouse Cedex, France

1 The aim of the present study was to investigate the influence of catecholamine levels on the regulation of α_2-adrenoceptor sensitivity in dogs.

2 Blood pressure and heart rate values at rest, plasma catecholamine levels, platelet and adipocyte α_2-adrenoceptors as well as the α_2-mediated cardiovascular responses to clonidine ($10\,\mu g\,kg^{-1}$ i.v., after α_1-, β-adrenoceptor plus muscarinic blockade) or noradrenaline (0.5, 1, 2 and $4\,\mu g\,kg^{-1}$ i.v. after α_1- and β-adrenoceptor blockade) were measured before and after reserpine treatment ($0.1\,mg\,kg^{-1}\,day^{-1}$ s.c. over 15 days).

3 Reserpine induced a significant decrease in resting systolic and diastolic blood pressures ($213 \pm 2/87 \pm 6$ mmHg before vs $158 \pm 5/59 \pm 3$ mmHg after treatment) as well as in heart rate (91 ± 2 beats min^{-1} before vs 76 ± 3 beats min^{-1} after treatment).

4 A 5 min tilt test performed under chloralose anesthesia, failed to modify blood pressure before treatment whereas it induced a significant fall in the same animals after the 15 day treatment. Plasma levels of noradrenaline significantly decreased (262 ± 58 vs $66 \pm 31\,pg\,ml^{-1}$) whereas plasma adrenaline levels were unchanged.

5 The α_2-mediated pressor responses to noradrenaline were significantly increased after reserpine. Clonidine induced a marked pressor effect (+72 and +45% in systolic and diastolic blood pressures respectively) after reserpine treatment. This effect was suppressed by administration of RX-821002, a new specific α_2-adrenoceptor antagonist.

6 Reserpine treatment significantly increased platelet α_2-adrenoceptor number (identified with [^{3}H]-yohimbine or [^{3}H]-RX821002) with no change in K_d values. α_2-Adrenoceptor number remained unchanged in adipocytes (identified with [^{3}H]-RX821002).

7 These results show that a 15 day treatment with reserpine induces a vascular α_2-adrenergic supersensitivity and an up-regulation in platelet α_2-adrenoceptors. In contrast, this phenomenon does not involve all the tissues since adipocyte α_2-adrenoceptors escape the effect of reserpine. We suggest that the levels of plasma noradrenaline play an important role in the regulation of the platelet and vascular α_2-adrenoceptors. In contrast, adipocyte α_2-adrenoceptors are not affected by changes in plasma noradrenaline levels.

Introduction

Adrenoceptors and receptor-mediated functions are reduced by chronic exposure to catecholamines and chronic administration of adrenoceptor agonists may be associated with a progressive decrease in the response of the effectors due to a down-regulation of the adrenoceptors (Brodde, 1983; Stiles *et al.*, 1984; Mahan *et al.*, 1987).

The consequences of depletion of endogenous stores of noradrenaline on adrenoceptor sensitivity have been largely investigated (for review see Fleming & Westfall, 1988). It has been demonstrated by Roscher *et al.* (1981) and Hawthorn & Broadley (1984) that the depletion of noradrenaline in cardiac tissue causes a supersensitivity to inotropic and chronotropic effects of β-adrenoceptor agonists in cardiac muscle from various species. Several reports have suggested that the supersensitivity phenomenon is specific, i.e. demonstrable only with β-adrenoceptor agonists (Broadley & Lumley, 1977; Hawthorn & Broadley, 1984). Indeed, several studies have demonstrated increased β-adrenoceptor density after chronic reserpine treatment (Tenner *et al.*, 1982; Latifpour & McNeill, 1984; Chess-Williams *et al.*, 1987).

In contrast, the existence of an α-adrenoceptor up-regulation is still under discussion; there are species and tissue differences in this regard (Latifpour & McNeill, 1984). Chess-Williams *et al.* (1987) failed to observe differences in the number of cardiac α-adrenoceptors after reserpine and Nasseri *et al.* (1985) found supersensitivity in vas deferens after reserpine with no change in α_2-adrenoceptors. In contrast, Watanabe *et al.* (1982), Bylund & Martinez (1980) and Fortin & Sundaresan (1989) observed a significantly increased number of α_2-adrenoceptors after reserpine in rat vas deferens, salivary glands and kidney cortex respectively. Fortin & Sundaresan (1989) also described an increased number of β- and α_1-adrenoceptors (but not angiotensin II receptors) in kidney cortex after reserpine treatment. Indeed, a clinical study from our group demonstrated the existence of vascular α-adrenergic supersensitivity to noradrenaline and up-regulation of platelet α_2-adrenoceptors in patients suffering from neurogenic orthostatic hypotension with low plasma levels of noradrenaline (Valet *et al.*, 1989).

These conflicting results led us to reinvestigate the consequences of such a sustained low plasma catecholamine level on the regulation of α_2-adrenergic sensitivity in dogs using reserpine as a catecholamine depleting agent. We have also tested whether the changes in platelet α_2-adrenoceptor numbers observed with reserpine are associated with increased vascular α_2-receptor-mediated function.

[1] Present address: Departamento de Farmacologia y Farmacotecnica. Universidad de Valencia Avda. Blasco Ibanez 15. 46010 Valencia, Spain.
[2] Author for correspondence.

Methods

Animals and general procedure

Eight adult male beagle dogs (12–15 kg) received subcutaneous reserpine at the dose of 0.1 mg kg^{-1} daily for 15 days. This dose was selected according to preliminary experiments showing that it induces a marked decrease in plasma catecholamines without major behavioral disturbances nor weight loss (not shown). The duration of reserpine treatment (15 days) was chosen since (1) reserpine causes a complete emptying of adrenergic fibres after 24 h (Häggendahl & Dahlström, 1971) and (2) the turn-over of α_2-adrenoceptors is around 36 h (Taouis *et al.*, 1986; Paris *et al.*, 1987). α_2-Adrenergic sensitivity was assessed through physiological (blood pressure, heart rate, plasma catecholamine levels and head up tilt) and pharmacological (blood platelet [^{3}H]-yohimbine binding sites, adipocyte [^{3}H]-RX 821002 binding sites, blood pressure responses to clonidine and noradrenaline) tests performed at days 0 (controls: before reserpine) and 15 (treated: 24 h after the last injection of reserpine: end of treatment). Moreover, plasma catecholamine levels and platelet [^{3}H]-yohimbine binding sites were measured in conscious dogs on day 30 and 45, i.e. 15 and 30 days after the end of reserpine treatment. All the experiments and blood samplings were done at 08 h 00 min on 24 h-fasted animals.

Blood pressure and heart rate measurements in conscious dogs

Several days before any experiment, the dogs were trained to stand still for 1 to 2 h on a Pavlov table and accustomed to intravenous infusion, intra-arterial recording and venous blood sampling. Blood pressure was recorded by means of a catheter introduced into the abdominal aorta via the left femoral artery under local anaesthesia (with xylocaine) and connected to a Gould P23ID transducer on a Honeywell recorder. The heart rate was obtained by use of a heart period meter triggered by blood pressure.

α_2-Adrenoceptor-mediated blood pressure response

Before and 15 days after reserpine treatment, the effect of an acute intravenous injection of clonidine (10 μg kg^{-1}) was recorded 15 min after prazosin (0.1 mg kg^{-1}, i.v.) plus propranolol (1 mg kg^{-1}, i.v.) plus atropine (1 mg kg^{-1}, i.v.) in conscious dogs. Systolic and diastolic blood pressures and heart rate were measured 1, 5, 10 and 15 min after clonidine in conscious dogs. Under the same conditions, the effect of clonidine was studied 5 min after acute administration of RX-821002 (0.25 mg kg^{-1}, i.v.). In other experiments, before and 15 days after reserpine treatment, the acute pressor responses to various doses of noradrenaline (0.5, 1, 2 and 4 μg kg^{-1}) were studied 15 min after propranolol (1 mg kg^{-1}, i.v.) plus prazosin (0.1 mg kg^{-1}, i.v.). Each subsequent dose of noradrenaline was given at least 1 min after the return of blood pressure and heart rate to normal resting values.

Head up tilt (anaesthetized dogs)

The day after the noradrenaline test, each animal was placed on a tilting table, anaesthetized with α-chloralose (80 mg kg^{-1}, i.v.), intubated with a cuffed tracheal tube and spontaneously respired. It was supported under the shoulders in the supine position by straps. After measurement of resting blood pressures, heart rate and plasma catecholamines in this position, the tilting table was positioned at 80° for 5 min: blood pressure and heart rate were recorded every 30 s and plasma sampling for catecholamines taken at 1 and 5 min. A second head up tilt was performed under the same conditions (chloralose anaesthesia) at the end of the reserpine treatment (day 15).

Blood sampling and catecholamine assays

Fresh blood was collected (from a catheter introduced into the jugular vein 30 min beforehand in order to prevent any stress) on lithium heparin with 10 nM sodium metabisulphite, centrifuged at 2000 *g* for 10 min at 0°C. Plasma was stored at −80°C. Catecholamines were selectively isolated from the sample at 0°C, in darkness, by adsorption to activated alumina, then eluted with 0.1 M perchloric acid. Dihydroxybenzylamine was used as an internal standard to monitor recovery from this extraction step. Noradrenaline and adrenaline were assayed by a Waters high performance liquid chromatography apparatus using electrochemical (amperometric) detection: the working electrode potential was set at 0.65 V against a Ag/AgCl reference electrode. Catecholamines were separated on a C18 column (3.9 × 150 mm) at a constant flow rate of 1 ml min^{-1}. The electrochemical detector response was linear for concentrations ranging from 25 pg ml^{-1} to 100 ng ml^{-1}. Under these conditions the detection limit was 50 pg ml^{-1} (Valet *et al.*, 1988).

Platelet and adipocyte membrane preparation and binding procedure

The platelet membranes were prepared as follows: 20 ml of venous blood was collected over 2 ml of 0.16 M sodium citrate solution and immediately centrifuged at 160 *g* for 10 min at room temperature. The platelet rich plasma (PRP) was collected and the platelet membranes were prepared as previously described (Villeneuve *et al.*, 1985). The adipocyte membranes were prepared as follows: fat biopsies (omental adipose tissue) were taken immediately after the induction of general anaesthesia by pentobarbitone and isolated adipocytes prepared (according to Berlan & Lafontan, 1985, with minor modifications). Krebs-Ringer bicarbonate buffer containing bovine serum albumin (3.5%) (KRBA) and glucose (6 nM), pH 7.4 was used as previously described (Berlan & Lafontan, 1985). After collagenase digestion (1 mg ml^{-1} KRBA buffer), isolated fat cells were washed three times and the packed cells were washed four times in a hypotonic lysing medium to elicit total cell breakage and recover all fat cells ghosts.

The membranes from platelet or adipocytes were used for adrenoceptor determination by a radioligand binding technique as previously described by Berlan & Lafontan (1980). Total binding of [^{3}H]-yohimbine or [^{3}H]-RX-821002 was determined by incubating 100 μl aliquots of the resuspended membrane preparation (100–200 μg protein assayed by the method of Lowry) with the radioligand in a total volume of 400 μl or binding buffer. Specific binding was defined as the difference between total and non-specific binding determined in parallel with an excess (10 μM) of adrenaline. Incubations were carried out for 20 min at 25°C under constant shaking at 100 cycles per min. The samples were filtered through fibreglass filters (Whatman GF/C) placed on a Millipore manifold sampling unit. The filters were washed twice with 10 ml of cold binding buffer and the radioactivity trapped on the filter was counted in a Packard spectrometer with an efficiency of 35%. The number of sites (B_{max}) and K_d values were calculated with a computer-assisted analysis of binding at saturation and according to Scatchard plots.

Data analysis

Each experiment was performed on the eight animals. Values are means ± s.e.mean. Statistical analysis was made using paired *t* test and differences considered significant when P was less than 0.05.

Drugs and chemicals

[^{3}H]-yohimbine (90 Ci mmol^{-1}) was obtained from Amersham (Amersham, U.K.) and [^{3}H]-RX 821002 (2-(2-methoxy,

1,4 benzodioxan-2yl)-2-imidazoline) was a gift from Reckitt and Colman Labs (Kingston-upon-Hull, U.K.). (−)-Noradrenaline bitartrate (Levophed, sterile commercial ampoule) came from Winthrop (Clichy, France), clonidine (Catapressan, sterile commercial ampoule) came from Boehringer Ingelheim (F.R.G.), reserpine (Serpasil, sterile commercial ampoule, reserpine dissolved in acetic acid, propylene glycol and water) and phentolamine mesylate from Ciba Geigy (Basel, Switzerland), prazosin (dissolved in 0.9% NaCl) from Pfizer (Orsay, France), (±)-propranolol (Avlocardyl, sterile commercial ampoule, propranolol dissolved in 0.9% NaCl) from Sigma (France).

Results

Effect of chronic reserpine treatment on resting blood pressure, heart rate, plasma catecholamine levels

Blood pressure, heart rate and plasma catecholamine levels were measured in conscious animals before and after 15 days with reserpine treatment (0.1 mg kg^{-1} daily, s.c.). The treatment induced significant decreases in plasma noradrenaline (75%) (without any modification in plasma adrenaline levels), in systolic (26%) and diastolic (32%) blood pressures and heart rate (16%) (Table 1). It should be mentioned that before reserpine the mean value of systolic (but not diastolic) blood pressure was slightly elevated although the animals were quiet (see the values of adrenaline). We have no explanation for this unexpected observation.

Effect of chronic reserpine treatment on cardiovascular and plasma catecholamine responses to tilting

A 5 min tilt was performed under chloralose anaesthesia before and after treatment. Before reserpine, tilting failed to modify systolic and diastolic blood pressures and induced a significant increase (25% after 1 and 5 min) in heart rate (Figure 1). Fifteen days after reserpine treatment, resting blood pressures and heart rate (measured in lying position in anaesthetized animals) were significantly ($P < 0.05$) lower than those before reserpine (as in conscious animals). Under these conditions, 1 min tilting significantly ($P < 0.05$) decreased systolic (39%) and diastolic (37%) blood pressures and increased heart rate (56%). Similar variations were observed after 5 min, i.e. at the end of the test.

Before reserpine, tilting induced an early significant ($P < 0.05$) increase in plasma noradrenaline (98% after 1 min) as well as adrenaline (210% after 1 min) levels which was sustained during the whole test (133% and 638% after 2 min for noradrenaline and adrenaline respectively). In treated animals, tilting failed to modify plasma noradrenaline levels. However, a significant ($P < 0.05$) increase (315%) in plasma adrenaline level was observed at the end of the test (5 min).

Table 1 Effect of a 15-day reserpine treatment (0.1 mg kg^{-1} daily) on cardiovascular parameters and plasma catecholamine levels in 8 conscious dogs

	Controls	*Treated*
Systolic blood pressure (mmHg)	213 ± 2	158 ± 5*
Diastolic blood pressure (mmHg)	87 ± 6	59 ± 2*
Heart rate (beats min^{-1})	91 ± 2	76 ± 3*
Plasma noradrenaline (pg ml^{-1})	262 ± 58	66 ± 31*
Plasma adrenaline (pg ml^{-1})	133 ± 24	163 ± 32

The measurements were made the day before the beginning of reserpine treatment (controls) and the day after the last injection of reserpine (treated). Values are the mean ± s.e.mean.
* Significantly different ($P < 0.05$) compared to the values measured in controls.

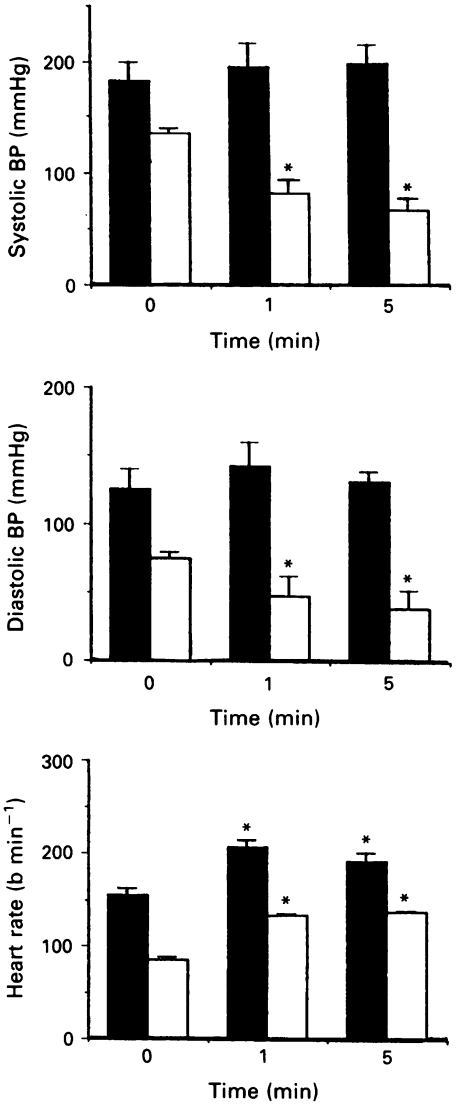

Figure 1 Effect of a 5 min head up tilt on systolic and diastolic blood pressures (BP in mmHg) and heart rate (beats min^{-1}) in 8 chloralose-anaesthetized dogs before (solid columns) and after 15-day treatment with reserpine (0.1 mg kg^{-1}, daily) (open columns). The values were measured in the lying position (time 0) and 1 and 5 min after the beginning of the tilt test. Statistical evaluations were made using Student's t test for paired comparison when the values at times 1 and 5 min (i.e. in standing position) were compared with corresponding values at time 0 (i.e. in lying position). * $P < 0.05$. Mean values are given with s.e.mean shown by vertical bars.

It is noticeable that plasma noradrenaline (but not adrenaline) levels measured in the lying position in reserpinized anaesthetized animals were much lower than those measured in the same reserpinized unanaesthetized dogs (15 ± 5 and 66 ± 31 respectively, $P < 0.05$).

Effect of chronic reserpine treatment on the cardiovascular responses to clonidine and noradrenaline

In order to explore the consequences of chronic reserpine treatment on vascular α_2-adrenoceptor-mediated responses, the effect of intravenous administration of clonidine (10 μg kg^{-1}) on systolic and diastolic blood pressures were studied after α_1- (prazosin) plus β- (propranolol) adrenoceptor plus muscarinic (atropine) receptor blockade. Blood pressure (and heart rate: not shown) values were similar in control and reserpine-treated dogs after this adrenoceptor plus muscarinic receptor blockade (Table 2). In control dogs, clonidine failed to modify blood pressure during the 15 min following its

Table 2 Influence of the α_2-adrenoceptor antagonist RX-821002 (0.25 mg kg^{-1}, i.v.) on the effect of clonidine (10 μg kg^{-1}, i.v.) on peak pressor effects (in mmHg) in control and 15-day reserpine treated conscious dogs (0.1 mg kg^{-1}, daily)

	Control		*Treated*	
	Systolic	*Diastolic*	*Systolic*	*Diastolic*
A	216 ± 8	77 ± 3	156 ± 8	56 ± 6
B	127 ± 15[a]	80 ± 1	144 ± 6	90 ± 6
C	153 ± 15	100 ± 7	248 ± 21[b]	145 ± 9[b]
D	144 ± 9	87 ± 8	166 ± 12	97 ± 17
E	150 ± 5	95 ± 6	170 ± 13	110 ± 8

In each animal ($n = 8$) blood pressures (mmHg) were measured in the following order: (A) Basal blood pressure (after 30 min rest); (B) 15 min after propranolol (1 mg kg^{-1}, i.v.) + prazosin (0.2 mg kg^{-1}, i.v.) + atropine (1 mg kg^{-1}, i.v.); (C) 1 min after clonidine; (D) 15 min after RX-821002; (E) 1 min after clonidine. Values are the mean ± s.e.mean.
[a] Significantly different ($P < 0.02$) compared with A values.
[b] Significantly different ($P < 0.02$) compared with B values.

administration although a slight (but not significant) tendency to increase and then a slight (but not significant) decrease were observed during the first 5 min and at the 15th min respectively (Figure 2). In reserpine-treated animals, clonidine induced a significant pressor response (+72% for systolic blood pressure and +61% for diastolic blood pressure) (Figure 2) without any modification in heart rate. The pressor effect of clonidine was abolished by previous administration of 0.25 mg kg^{-1} i.v. RX 821002, an α_2-adrenoceptor antagonist (Table 2).

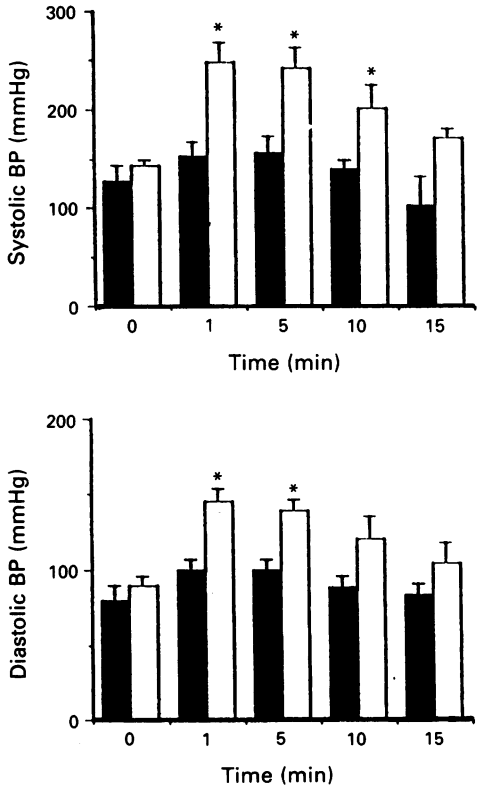

Figure 2 Effect of clonidine (10 μg kg^{-1}, i.v. as a bolus) on systolic and diastolic blood pressures (mmHg) in 8 conscious dogs before (solid columns) and after (open columns) reserpine treatment (0.1 mg kg^{-1}, daily for 15 days). The values were measured after prazosin (0.1 mg kg^{-1}, i.v.) plus propranolol (1 mg kg^{-1}, i.v.) plus atropine (1 mg kg^{-1}, i.v.). Mean values are given with s.e.mean shown by vertical bars. * $P < 0.05$ when values obtained after reserpine are compared with those measured before reserpine in the same animals.

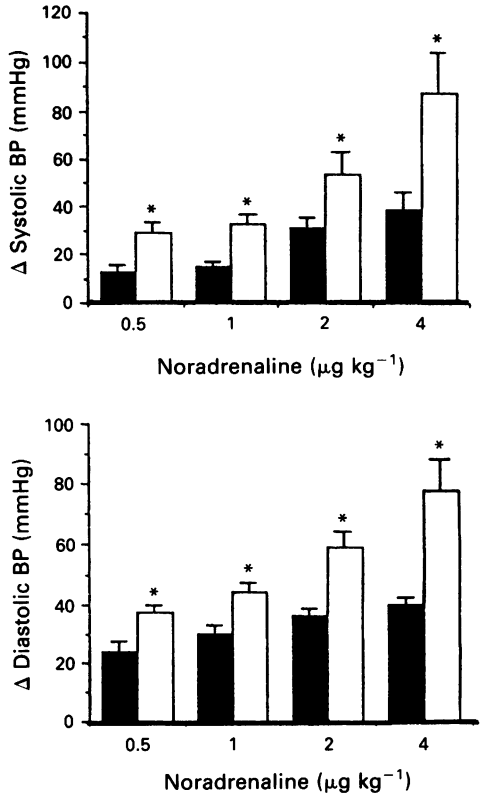

Figure 3 Pressor responses to noradrenaline (0.5, 1, 2 and 4 μg kg^{-1}, i.v. as bolus) in 4 conscious dogs before (solid columns) and after (open columns) reserpine treatment (0.1 mg kg^{-1} daily for 15 days). The values represent the maximal increase in systolic and diastolic blood pressures (BP in mmHg) after noradrenaline injection measured after prazosin (0.1 mg kg^{-1}, i.v.) plus propranolol (1 mg kg^{-1}, i.v.). Mean values are given with s.e.mean shown by vertical bars. * $P < 0.05$ when values obtained after reserpine are compared with those measured before reserpine in the same animals.

The effect of noradrenaline (0.5, 1, 2 and 4 μg kg^{-1}) was studied 15 min after β- and α_1-blockade in conscious animals. Under these conditions, resting values of systolic and diastolic blood pressures and heart rate were respectively 163 ± 2, 70 ± 6 mmHg and 98 ± 3 beats min^{-1} before reserpine and 131 ± 6 ($P < 0.01$), 48 ± 2 ($P < 0.02$) mmHg and 82 ± 10 beats min^{-1} (NS) after reserpine treatment. Noradrenaline induced a dose-dependent increase in systolic and diastolic blood pressure before as well as after reserpine (Figure 3). However, the magnitude of this pressor response was significantly ($P < 0.05$) higher in reserpinized animals.

Effect of chronic reserpine treatment on platelet and adipocyte α_2-adrenoceptors

Reserpine treatment significantly ($P < 0.02$) increased the number of platelet α_2-adrenoceptors labelled with [^{3}H]-RX821002 or with [^{3}H]-yohimbine (153 ± 21 fmol mg^{-1} protein after the 15-day treatment vs 101 ± 14 fmol mg^{-1} protein before reserpine, $n = 4$, without any significant modification in K_D values: 2.06 ± 0.43 vs 1.97 ± 0.20) (Table 3 and Figure 4).

For labelling α-adrenoceptors in adipocytes, [^{3}H]-RX 821002 was used since this compound is a better radioligand for α_2-adrenoceptor sites in this tissue when compared with other classical α_2-adrenoceptor radioligands (Carpene *et al.*, 1989), especially when the α_2-binding sites are low, which is the case in normal non-obese dogs (Taouis *et al.*, 1987) as in this study. Competition studies by yohimbine, (−)- and (+)-adrenaline and prazosin against [^{3}H]-RX 821002 on adipocyte membranes were made in order to validate the

Table 3 Effect of a 15-day reserpine treatment (0.1 mg kg^{-1} daily) on platelet and adipose tissue α_2-adrenoceptors obtained from 8 dogs

	Controls		*Treated*	
	B_{max} (fmol mg^{-1} protein)	K_D (nmol l^{-1})	B_{max} (fmol mg^{-1} protein)	K_D (nmol l^{-1})
Platelet	136 ± 16	3.80 ± 1.03	314 ± 40**	2.43 ± 0.74
Adipocyte	98 ± 29	1.62 ± 0.20	128 ± 15	1.71 ± 0.52

The maximal number (B_{max}) of [^{3}H]-yohimbine (platelet) or [^{3}H]-RX 821002 (adipose tissue) binding sites was determined by Scatchard analysis. K_D is the equilibrium dissociation constant (−1/slope of Scatchard plot). Values are the mean ± s.e.mean.
** Significantly different ($P < 0.02$) compared to the values measured in controls.

α_2-adrenoceptor selectivity of the radioligand. The order of potency was: yohimbine > (−)-adrenaline ≫ (+)-adrenaline = prazosin (Table 4 and Figure 5). Reserpine treatment did not modify [^{3}H]-RX 821002 binding on fat cell membranes (Table 3). Fifteen days after the end of reserpine treatment, platelet [^{3}H]-yohimbine binding site number remained significantly elevated (and plasma noradrenaline levels low). Normal values were measured 30 days after the end of reserpine treatment (plasma noradrenaline was in the normal range) (Figure 6). In contrast, there was no change in plasma adrenaline levels

Table 4 Inhibition of [^{3}H]-RX 821002 binding by yohimbine, (−)- or (+)-adrenaline and prazosin on adipocyte membranes from control untreated dogs

Competing drug	EC_{50} (nmol l^{-1})	K_i (nmol l^{-1})	*n Hill*
Yohimbine	6.88 ± 0.87	1.71 ± 0.22	0.93 ± 0.02
(−)-Adrenaline	977 ± 308	243 ± 77	0.53 ± 0.03
(+)-Adrenaline	9669 ± 2871	2409 ± 751	0.43 ± 0.02
Prazosin	10122 ± 767	2524 ± 372	0.94 ± 0.12

K_i: equilibrium dissociation constant (apparent dissociation constant was calculated from the equation: $EC_{50}/(1 + ([^3H]\text{ ligand})/K_D \text{ ligand})$. EC_{50}: concentration of competitive agent causing 50% inhibition of specific [^{3}H]-RX 821002 binding. K_D ligand: K_D values obtained by Scatchard analysis derived from saturation curves. Fat cell membranes were incubated with 4 nM of [^{3}H]-RX 821002. Each value is the mean ± s.e.mean of 3 separate experiments performed in duplicate.

Table 5 Inhibition of [^{3}H]-yohimbine binding by UK-14,304 on platelet or adipocyte membranes from control and 15 day reserpine-treated dogs (0.1 mg kg^{-1}, daily)

	K_{iH} (nmol l^{-1})	K_{iL} (nmol l^{-1})	% *RH*
		Platelets	
Control	1.77 ± 0.32	78.1 ± 3.4	44 ± 3.4
Treated	1.97 ± 0.81	270 ± 59.5*	54 ± 2.4
		Adipocytes	
Control	2.51 ± 1.30	2179 ± 880	59.2 ± 8.1
Treated	0.78 ± 0.60	2453 ± 1468	50.5 ± 3.2

Data were analysed by probit transformation and the Hill coefficients determined. The inhibition curves were fitted by two-site inhibition models and values were obtained using TWOSITEINHIB (Barlow, 1983). K_{iH} and K_{iL} which represent the inhibition constants at the high and low affinity state receptors respectively were calculated from the equation: $K_i = EC_{50}/(1 + ([^3H\text{-yohimbine}]/K_d)$, where EC_{50} is the concentration of UK-14,304 displacing 50% of bound radioligand and K_d is the dissociation constant of the radioligand determined by Scatchard analysis. The proportion of receptors in the high affinity state (RH) is given as percentage of total binding sites. Values are the mean ± s.e.mean.
* Significantly different ($P < 0.05$) compared to the values measured in controls.

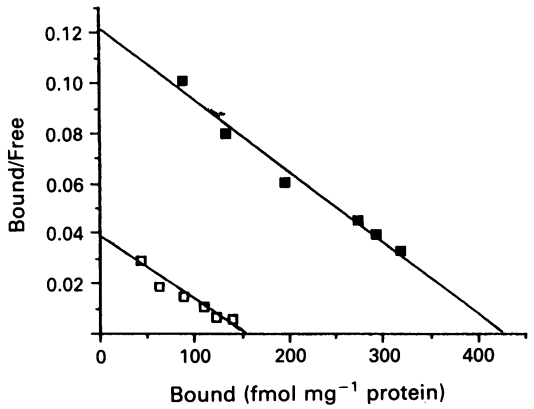

Figure 4 An example of [^{3}H]-yohimbine binding to dog platelet membrane in the same dog before (□) and after (■) reserpine treatment (0.1 mg kg^{-1} daily for 15 days). The figure represents the Scatchard plot derived from the saturation binding curve. The equilibrium dissociation constant ($K_D = -1/\text{slope}$) and the maximal number of binding sites (B_{max} = intersection of the curve with the abscissa scale) were calculated by regression analysis.

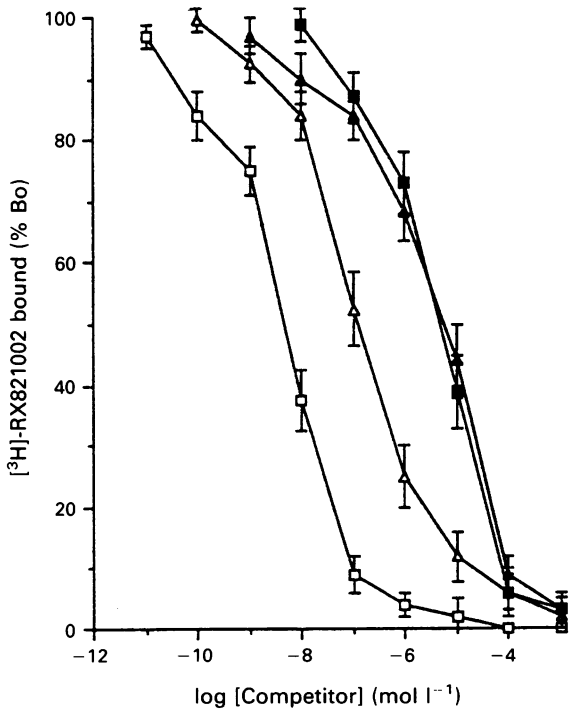

Figure 5 Inhibition of [^{3}H]-RX 821002 binding to dog fat cell membranes by various concentrations of (□) yohimbine, (△) (−)-adrenaline, (▲) (+)-adrenaline and (■) prazosin. The data are expressed as a percentage of the specific binding in absence of competitor (B_0). Each point represents the mean of three separate experiments performed in duplicate; s.e.mean shown by vertical bars. The inhibition-curves were fitted by a one-site model. The parameters obtained from the analysis of the data are presented in Table 5.

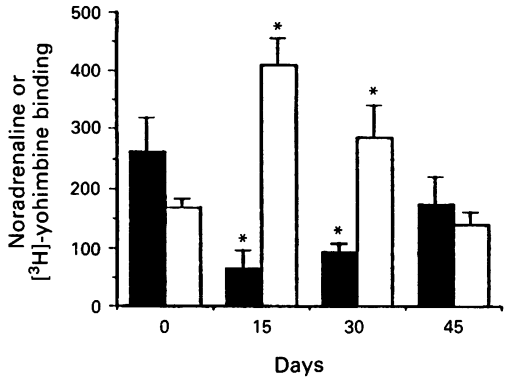

Figure 6 Time course of plasma noradrenaline levels (pg ml^{-1}; solid columns) and platelet [^{3}H]-yohimbine binding sites (fmol mg^{-1} protein; open columns) in 8 reserpine-treated dogs (0.1 mg kg^{-1} daily for 15 days, i.e. from day 1 to day 15). The values were measured before (day 0), at the end (day 15) and 15 (day 30) and 30 (day 45) days after the end of reserpine treatment. Mean values are given with s.e.mean shown by vertical bars. * $P < 0.05$ when compared with pretreatment values (day 0).

(133 ± 24, 163 ± 32, 140 ± 18 and 153 ± 46 pg ml^{-1} plasma before reserpine, at the end of reserpine treatment, 15 and 30 days after the end of reserpine treatment respectively).

α_2-Adrenoceptors exist in two affinity states in the membrane, the high affinity state (RH) coupled to adenylate cyclase, and the low affinity state (RL) uncoupled. The two affinity states can be delineated on membranes by the competition of UK-14304 (an α_2-adrenoceptor agonist) against [^{3}H]-yohimbine. The computer-aided non-linear regression analysis of the competition curves (using the 'twositeinhib' model as described by Barlow, 1983) showed that the proportion of the α_2-adrenoceptors in the high affinity state is the same before and after reserpine treatment in platelet or in adipocyte membranes (Table 5).

Discussion

The purpose of the present study was to determine whether administration of reserpine (recognized as an effective agent chronically to interrupt or reduce adrenergic neurotransmission) results in a modification of α_2-adrenergic sensitivity in the dog; i.e. if noradrenaline depletion from sympathetic nerve endings for a prolonged period of time produces an up-regulation of platelet α_2-adrenoceptors and α_2-adrenergic supersensitivity in vascular tissue. We selected a schedule of treatment with reserpine leading to a marked reduction in plasma noradrenaline levels and resting blood pressure or heart rate (Table 1).

The development of vascular supersensitivity to noradrenaline after pretreatment with reserpine has been previously demonstrated in the cat's cardiovascular system by Fleming & Trendelenburg (1961). In fact, it was never clearly shown that this supersensitivity was linked to changes in number (or affinity) of vascular α-adrenoceptors (Fleming & Westfall, 1988). Both α_2- and α_1-adrenoceptors are present on certain blood vessels (Timmermans & Van Zwieten, 1981). The present results in dogs demonstrate the existence of a supersensitivity of vascular α_2-adrenoceptors after reserpine since: (1) the pressor response to noradrenaline (after α_1- plus β-adrenoceptor blockade) was increased in reserpine-treated dogs, and (2) clonidine, which failed to change blood pressure significantly in untreated animals (probably because it was administered after α_1, β-adrenoceptor plus muscarinic blockade) elicited under the same conditions, a marked pressor response after reserpine. These results suggest that a low level of plasma noradrenaline may be responsible for the vascular α_2-supersensitivity. Since direct measurement of α_2-adrenoceptors by binding on vessels remains difficult and has led to controversial data (Watanabe *et al.*, 1982; Nasseri *et al.*, 1985), we investigated α_2-adrenoceptors on more accessible tissue (blood platelets and adipose tissue) for which the binding to α_2-adrenoceptors is well established.

Platelet α_2-adrenoceptors showed a significant increase after a 15 day reserpine treatment. The adaptive up-regulation of platelet α_2-adrenoceptors appears to be a long-lasting phenomenon, reversible and closely related to the plasma noradrenaline level (Figure 6). So, the low plasma noradrenaline level seems to be responsible for the platelet α_2-adrenoceptor up-regulation. A direct effect of reserpine on platelet α_2-adrenoceptors can be discounted since we verified that it failed to compete with [^{3}H]-yohimbine binding on platelet membranes (not shown). Fortin & Sundaresan (1989) also suggested that the α_2-adrenoceptor up-regulation that they observed in rat kidney cortex after reserpine treatment is linked to the low level of circulating catecholamines since surgical renal denervation only failed to modify the α_2-adrenoceptor number. Moreover, Langer (1981) suggested that in the dog, vascular α_2-adrenoceptors (which are mainly extrasynaptic as the platelet α_2-adrenoceptors) are stimulated by circulating noradrenaline but not by the endogenously released neurotransmitter and Bobik & Anderson (1983) demonstrated that surgical denervation of arteries in the dog was not followed by any appreciable rise in [^{3}H]-yohimbine binding sites. Taken together, these results could reasonably suggest that in dogs, vascular α_2-adrenoceptors are regulated by circulating noradrenaline.

The numbers of both platelet α_2-adrenoceptors in low and high affinity states were increased after development of up regulation (the proportion remained constant: Table 5) and the absolute increment of the receptors in the high affinity state (effective receptors in the transduction of the neurotransmitter message) can explain the increased response of the tissues to α_2-agonists.

It has long been maintained that changes in receptors after reserpine are not the sole mechanism underlying supersensitivity in smooth muscle (Fleming *et al.*, 1973). In fact, we cannot exclude the occurrence of a non-specific phenomenon in the vascular responses to noradrenaline (or clonidine) although the number of α_2-adrenoceptors increased in most of the studies with different tissues (Bylund & Martinez, 1980; Watanabe *et al.*, 1982; Fortin & Sundaresan, 1989), including the present one.

The increase in platelet α_2-adrenoceptors after reserpine treatment also agrees with the data from Bylund & Martinez (1980). Watanabe *et al.* (1982) and Fortin & Sundaresan (1989). However, these authors only investigated one tissue each in rat (salivary glands, vas deferens and kidney cortex respectively) and, as far as we know, no information is available concerning different tissues from the same animals after reserpine. Thus, it was of interest to compare the evolution of α_2-adrenoceptors located on two different tissues i.e. platelets and adipose tissue (Taouis *et al.*, 1987).

Surprisingly, we observed no modification in adipocyte α_2-adrenoceptor number after reserpine. This discrepancy cannot be attributed to the nature of the ligand used for labelling the α_2-adrenoceptors in this tissue since [^{3}H]-RX 821002 is considered to be a better radioligand than [^{3}H]-yohimbine for labelling this population of α-adrenoceptors (Langin *et al.*, 1989), especially in rat and hamster adipose tissue (Carpene *et al.*, 1989; Saulnier-Blache *et al.*, 1989): the non-specific binding for [^{3}H]-RX 821002 is very low (10%) when compared with [^{3}H]-yohimbine (40–60%), thus allowing a better identification of α_2-adrenoceptors when their number is low. Moreover, the total number of α_2-adrenoceptors identified by [^{3}H]-RX 821002 on fat cells is quite similar to that previously obtained with [^{3}H]-yohimbine (Taouis *et al.*, 1989). This compound also exhibits specific α_2-selectivity in dog adipose tissue (Table 4 and Figure 5). Moreover, we also found that

reserpine treatment failed to change the percentage of α_2-adrenoceptors in high and low affinity states.

We can propose one explanation for this lack of up-regulation of fat cell α_2-adrenoceptors: adrenaline (when compared to noradrenaline) is the major stimulant of the adipocyte α_2-adrenoceptors (Lafontan & Berlan, 1982). In fact, adrenaline levels did not change after reserpine. Moreover, such a difference in regulation of α_2-adrenoceptors by agonists (noradrenaline or adrenaline) between tissues has been previously described in rabbits by Deighton *et al.* (1988a,b). Recently, Crampes *et al.* (1989) also found that platelet α_2-adrenoceptors increased after moderate hypocaloric diet in obese humans without any modification in α_2-adrenoceptor responsiveness in adipose tissue. So, it seems that fat cell α_2-adrenoceptor number is not regulated by endogenous noradrenaline but rather by other factors such as the development of fat cell size and fat deposit (Carpene *et al.*, 1983; Marcus *et al.*, 1987; Taouis *et al.*, 1987). Although, according to Deighton *et al.* (1988a,b), extrapolations of platelet binding data to other tissues containing similar populations of α_2-adrenoceptors should be exercised with caution, the present data suggest that platelet (and not adipocyte) α_2-adrenoceptors can be a suitable index for investigating the vascular α_2-adrenergic sensitivity. This hypothesis is also supported by recent data from Daly *et al.* (1988) suggesting a functional subdivision in α_2-adrenoceptors. These authors, using various substituted benzazepines, suggest that platelet α_2-adrenoceptors are similar to those in brain and saphenous vein but different from those located at the presynaptic level on sympathetic nerve endings. This conclusion also agrees with our observations in Parkinsonian patients suffering from orthostatic hypotension who exhibit low noradrenaline (but not adrenaline) plasma levels, increased numbers of platelet α_2-adrenoceptors and vascular supersensitive responses to noradrenaline (Valet *et al.*, 1989). Thus, we suggest that circulating noradrenaline is the major regulatory agent of platelet α_2-adrenoceptor number and vascular α_2-adrenergic sensitivity.

Another point of discussion could be the difference between the results of Nasseri *et al.* (1985) and ours. These authors, using [^{3}H]-rauwolscine on rat caudal artery, established that there were no measurable changes in ligand binding to α_2-adrenoceptors after reserpine administration. They concluded that changes in α-adrenoceptors are not involved in post-junctional supersensitivity of smooth muscle caused by chronic reserpine treatment. Our results fit better with those of Watanabe *et al.* (1982) who observed an increment in rat vas deferens postsynaptic α_2-adrenoceptors after reserpine. These conflicting data are probably related to the sensitivity of the methods available for quantifying α_2-adrenoceptor binding sites in vascular tissue.

Other results deserve some comment. Tilting is a well-established method for evaluating the mechanisms of adaptation to orthostatism (Linde & Hjemdahl, 1982; Schatz, 1986). In control dogs, the adaptation of the sympathetic nervous system was clearly shown during tilting by the increase in plasma noradrenaline and adrenaline levels. After reserpine treatment, the response pattern seen during tilting bears striking differences: tilting failed to increase plasma noradrenaline levels and arterial blood pressure fell during the whole manoeuvre (Figure 1). Plasma adrenaline levels were not reduced by reserpine treatment confirming the fact that the adrenal medulla is more resistant than sympathetic nerve endings to catecholamine depletion by reserpine (see Rand & Jurevics, 1977). However, during tilting, plasma adrenaline levels only increased after 5 min (in contrast to control dogs) suggesting that reserpine alters the orthostatism-induced release of adrenaline from the adrenal medulla. The tilting manoeuvre is therefore an accurate procedure with which to validate the noradrenergic depleting effect of reserpine. Finally, it can be concluded that reserpinized dogs are appropriate models of orthostatic hypotension.

In conclusion, the present data demonstrate that reserpine treatment induces a platelet α_2-adrenoceptor up-regulation and a vascular α_2-adrenergic supersensitivity to administered noradrenaline (or clonidine) in dog. However, this α_2-adrenoceptor regulation does not involve all the tissues investigated in this study since in contrast to platelets, adipocytes escape the effect of reserpine.

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References

BARLOW, R.B. (1983). *Biodata: Handling with Microcomputers.* Amsterdam: Elsevier. Biosolft.

BERLAN, M. & LAFONTAN, M. (1980). Identification of alpha 2-adrenergic receptors in human fat cell membranes by 3H-clonidine binding. *Eur. J. Pharmacol.*, **67**, 481–484.

BERLAN, M. & LAFONTAN, M. (1985). Evidence that adrenaline acts preferentially as an antilipolytic agent in abdominal human subcutaneous fat cells: assessment by analysis of beta and alpha 2 adrenoceptors properties. *Eur. J. Clin. Invest.*, **15**, 341–348.

BOBIK, A. & ANDERSON, P. (1983). Influence of sympathectomy on alpha2 adrenergic receptor binding sites in canine blood vessels. *Life Sciences*, **33**, 331–336.

BROADLEY, K.J. & LUMLEY, P. (1977). Selective reserpine-induced supersensitivity of the positive inotropic and chronotropic responses to isoprenaline and salbutamol in guinea-pig isolated atria. *Br. J. Pharmacol.*, **59**, 51–60.

BRODDE, O.E. (1983). Endogenous and exogenous regulation of human alpha and beta adrenergic receptors. *J. Recept. Res.*, **3**, 151–162.

BYLUND, D.B. & MARTINEZ, J.R. (1980). Alpha 2 adrenergic receptors appear in rat salivary glands after reserpine treatment. *Nature*, **285**, 229–230.

CARPENE, C., BERLAN, M. & LAFONTAN, M. (1983). Influence of development and reduction of fat stores on the antilipolytic alpha 2-adrenoceptor in hamster adipocytes: comparison with adenosine and beta-adrenergic lipolytic responses. *J. Lipid Res.*, **24**, 766–774.

CARPENE, C., GALITZKY, J. & LAFONTAN, M. (1989). A new tool for the study of rat fat cell alpha 2 adrenergic receptor regulation: the alpha 2 antagonist RX 821002. *Int. J. Obesity*, **13**, S1, 188.

CHESS-WILLIAMS, R.G., GRASSBY, P.F., BROADLEY, K.J. & SHERIDAN, D.J. (1987). Cardiac alpha- and beta-adrenergic receptor sensitivity and binding characteristics after chronic reserpine pretreatment. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **336**, 646–651.

CRAMPES, F., MARCERON, M., BEAUVILLE, M., RIVIERE, D., GARRIGUES, M., BERLAN, M. & LAFONTAN, M. (1989). Platelet alpha2-adrenoceptors and adrenergic adipose tissue responsiveness after moderate hypocaloric diet in obese subjects. *Int. J. Obesity*, **13**, 99–110.

DALY, R.N., SULPIZIO, A.C., LEVITT, B., DEMARINIS, R.M., REGAN, J.W., RUFFOLO, R.R. & HIEBLE, J.P. (1988). Evidence for heterogeneity between pre- and postjunctional alpha-2 adrenoceptors using 9-substituted 3-benzazepines. *J. Pharmacol. Exp. Ther.*, **247**, 122–128.

DEIGHTON, N.M., BROWN, A.D., HAMILTON, C.A. & REID, J.L. (1988a). Regulation of adrenergic receptor number following chronic noradrenaline infusion in the rabbit. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **338**, 517–522.

DEIGHTON, N.M., HAMILTON, C.A., RONES, C.R. & REID, J.L. (1988b). The effects of chronic administration of adrenaline on the function and number of adrenoceptors in the rabbit. *J. Cardiovasc. Pharmacol.*, **12**, 332–337.

FLEMING, W.W., MCPHILLIPS, J.J. & WESTFALL, D.P. (1973). Postjunctional supersensitivity and subsensitivity of excitable tissues to drugs. *Ergeb. Physiol.*, **68**, 55–119.

FLEMING, W.W. & TRENDELENBURG, U. (1961). The development of supersensitivity to noradrenaline after pretreatment with reserpine. *J. Pharmacol. Exp. Ther.*, **133**, 41–51.

FLEMING, W.W. & WESTFALL, D.P. (1988). Adaptive supersensitivity. In *Handb. Exp. Pharmacol.*, Vol. 90, *Catecholamines I.* ed. Trendelenburg, U. & Weiner, N., pp. 509–559. Berlin: Springer Verlag.

FORTIN, T.L. & SUNDARESAN, P.R. (1989). Reserpine but not surgical denervation regulates rat renal beta-adrenergic receptors. *Am. J. Physiol.*, **256** (Renal Fluid Electrolyte Physiol. 25), F532–F539.

HÄGGENDAL, J. & DAHLSTRÖM, A. (1971). The recovery of noradrenaline in adrenergic nerve terminals of the rat after reserpine treatment. *J. Pharm. Pharmacol.*, **23**, 81–89.

HAWTHORN, M.H. & BROADLEY, K.J. (1984). Reserpine-induced supersensitivity occurs for beta-adrenoceptor-mediated responses of heart and trachea but not of the uterus and lung. *Eur. J. Pharmacol.*, **105**, 245–255.

LAFONTAN, M. & BERLAN, M. (1982). Characterization of physiological agonist selectivity of human fat cell alpha 2 adrenoceptors: adrenaline is the major stimulant of alpha 2 adrenoceptors. *Eur. J. Pharmacol.*, **82**, 107–111.

LANGER, S.Z. (1981). Presynaptic regulation of the release of catecholamines. *Pharmacol. Rev.*, **32**, 337–362.

LATIFPOUR, J. & MACNEILL, J.H. (1984). Reserpine-induced changes in cardiac adrenergic receptors. *Can. J. Physiol. Pharmacol.*, **62**, 23–26.

LANGIN, D., LAFONTAN, M., STILLINGS, M. & PARIS, H. (1989). [3H] RX 821002, a new tool to label alpha2-adrenoceptors. *Eur. J. Pharmacol.*, **167**, 95–104.

LINDE, B. & HJEMDAHL, P. (1982). Effect of tilting on adipose tissue vascular resistance and sympathetic activity in humans. *Am. J. Physiol.*, **242**, H161–H167.

MAHAN, L.C., McKERNAN, R.M. & INSEL, P.A. (1987). Metabolism of alpha and beta adrenergic receptors in vitro and in vivo. *Ann. Rev. Pharmacol. Toxicol.*, **27**, 215–235.

MARCUS, C., KARPE, B., BOLME, P., SONNENFELD, T. & ARNER, P. (1987). Changes in catecholamine-induced lipolysis in isolated human fat cells during the first year of life. *J. Clin. Invest.*, **79**, 1812–1818.

NASSERI, A., BARAKEH, J.F., ABEL, P.W. & MINNEMAN, K.P. (1985). Reserpine-induced postjunctional supersensitivity in rat vas deferens and caudal artery without changes in alpha adrenergic receptors. *J. Pharmacol. Exp. Ther.*, **234**, 350–357.

PARIS, H., TAOUIS, M. & GALITZKI, J. (1987). In vitro study of alpha2-adrenoceptor turnover and metabolism using the adrenocarcinoma cell line HT29. *Mol. Pharmacol.*, **242**, 1041–1049.

RAND, M.J. & JUREVICS, H. (1977). The pharmacology of Rauwolfia alkaloïds. In *Handb. Exp. Pharmacol.* Vol. 39, *Antihypertensive Agents*, ed. Gross, F. pp. 77–159. Berlin: Springer Verlag.

ROSCHER, A.A., WIESMANN, U.N. & HONEGGER, U.E. (1981). Changes in beta adrenergic receptors in submaxillary glands of chronically reserpine- or isoproterenol-treated rats. *J. Pharmacol. Exp. Ther.*, **216**, 419–424.

SAULNIER-BLACHE, J.S., CARPENE, C., LANGIN, D. & LAFONTAN, M. (1989). Imidazolinic radioligand for the identification of hamster adipocyte alpha2-adrenoceptors. *Eur. J. Pharmacol.*, **171**, 145–157.

SCHATZ, I.J. (1986). *Orthostatic Hypotension.* Philadelphia: F.A. Davis Company.

STILES, G.L., CARON, M.G. & LEFKOWITZ, R.J. (1984). Beta-adrenergic receptors: biochemical mechanisms of physiological regulation. *Physiol. Rev.*, **64**, 661–743.

TAOUIS, M., BERLAN, M. & LAFONTAN, M. (1986). Alpha2-adrenergic receptor turnover in adipose tissue and kidney: irreversible blockade of alpha2-adrenergic receptors by benextramine. *Mol. Pharmacol.*, **31**, 89–96.

TAOUIS, M., BERLAN, M., MONTASTRUC, P. & LAFONTAN, M. (1987). Characterization of dog fat cell adrenoceptors: variations in alpha-2 and beta adrenergic receptors distribution according to the extent of the fat deposits and the anatomical location. *J. Pharmacol. Exp. Ther.*, **242**, 1041–1049.

TENNER, T.E., MUKERJEE, A. & HESTER, R.K. (1982). Reserpine-induced supersensitivity and the proliferation of cardiac beta-adrenoceptors. *Eur. J. Pharmacol.*, **77**, 61–65.

TIMMERMANS, P.B.M.W.M. & VAN ZWIETEN, P.A. (1981). The postsynaptic alpha2 adrenergic receptor. *J. Auton. Pharmacol.*, **1**, 171–183.

VALET, P., TRAN, M.A., DAMASE-MICHEL, C., DE SAINT BLANQUAT, G., DANG-TRAN, L., ANGLADE, F., KOENIG-BERARD, F. & MONTASTRUC, J.L. (1988). Rilmenidine (S 3341) and sympathoadrenal system: adrenoceptors, plasma and adrenal catecholamines in dogs. *J. Auton. Pharmacol.*, **8**, 319–326.

VALET, P., SENARD, J.M., DURRIEU, G., BERLAN, M., TRAN, M.A., MONTASTRUC, J.L. & MONTASTRUC, P. (1989). Alpha adrenergic supersensitivity in Parkinsonians with orthostatic hypertension. *Br. J. Clin. Pharmacol.*, **27**, 682P.

VILLENEUVE, A., BERLAN, M., LAFONTAN, M. & MONTASTRUC, J.L. (1985). Characterization of dog platelet alpha adrenergic receptor: lack of in vivo down regulation by adrenergic agonist treatment. *Comp. Biochem. Physiol.*, **81**, 181–187.

WATANABE, Y., LAI, R.T., MAEDA, H. & YOSHIDA, H. (1982). Reserpine and sympathetic denervation cause increase of postsynaptic alpha2-adrenoceptors. *Eur. J. Pharmacol.*, **80**, 105–108.

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Factors influencing the onset of ouabain inhibition of Na,K-ATPase from guinea-pig myocardium

F. Ebner

Institut für Pharmakologie und Toxikologie der Technischen Universität München, Biedersteinerstr. 29, D-8000 München 40, Federal Republic of Germany

1 The onset of ouabain inhibition was quantified by analysis with an integrated rate equation from experiments in which the activity of Na,K-ATPase from guinea-pig myocardium had been altered with adenosine 5'-triphosphate (ATP, 0.3–9 mmol l^{-1}) in the absence and presence of a detergent.

2 Under control conditions with increasing ouabain (0.1–100 μmol l^{-1}) and ATP (0.3–1 mmol l^{-1}) concentrations, inhibition developed faster. The acceleration by ouabain became less effective at saturating concentrations leading to a non-linear relationship between pseudo-first-order rate constants of inhibition and ouabain concentration. With a rise of ATP to 3 and 9 mmol l^{-1}, i.e., near total Mg concentration (5 mmol l^{-1}), inhibition was retarded presumably because the free concentrations of Mg and uncomplexed ATP changed. Varying the ATP concentration had little effect on ouabain potency at steady state; Hill coefficients were < 1.

3 The detergent alamethicin (23 μg ml^{-1}) neither interfered with Na,K-ATPase activity nor with inhibition at steady state but accelerated its onset. This supports a role for a lipid barrier in the development of inhibition.

4 While the reaction of low concentrations of ouabain with the receptors seemed to govern inhibition rate, with an increase in steroid concentration in the presence of alamethicin, ATP-dependent enzyme activity interfered with the onset of inhibition. The transition of the enzyme between ouabain-sensitive and ATP-hydrolytic conformations consequently causes the non-linear concentration-dependence of pseudo-first-order rate constants. As the Hill coefficient was < 1, a reaction of ouabain with two receptors also could have contributed to the special concentration-dependence of inhibition rates.

Introduction

Ouabain binding to Na,K-ATPase has been shown to depend on phosphorylation (Matsui & Schwartz, 1968; Post *et al.*, 1969) and to be altered by interventions that modulate enzyme activity, e.g., different cation concentrations or hormones (Clausen & Hansen, 1977; Joiner & Lauf, 1978). From these findings a role of enzyme activity in the onset of inhibition by ouabain may be inferred. In view of the different conformations which hydrolyze adenosine 5'-triphosphate (ATP) and bind ouabain (for review see Skou, 1988) a delay in the onset of inhibition should reflect both the concentration and generation of the ATP-hydrolyzing conformation and its transition to the ouabain-sensitive conformation. One might suppose, if all enzyme molecules are not immediately accessible to ouabain because a fraction is in a ouabain-insensitive conformational state, that the rates of formation and breakdown of ouabain-sensitive and ATP-hydrolyzing conformations may interfere with the rate of inhibition. With an increase in ATP concentration, the activation of hydrolysis via low- and high-affinity ATP binding sites (Karlish *et al.*, 1978) could enhance the rate of appearance of the ouabain-sensitive conformation. Superimposed on the reaction of ouabain with its receptors, especially at high concentrations when the steroid instantaneously reacts with all available receptors, the conformational transitions of the enzyme could govern inhibition rate.

In contrast to binding kinetics (for review see Hansen, 1984) inhibition of Na,K-ATPase by cardioactive steroids with special reference to the pre-steady state has been given little attention, as there are comparably few studies on the on- or offset of the steroid effect (e.g., Albers *et al.*, 1968; Skou *et al.*, 1971; Yoda, 1973; Yoda *et al.*, 1973; Yoda & Yoda, 1973; Clark *et al.*, 1975). However, knowledge of the factors which interfere with the pre-steady state of the reaction of the steroids with the receptors seems to be desirable, with regard to the mechanism of enzyme action as well as the validity of parameters estimated for the reaction of the steroid with receptors. Since fast reactions are inaccessible to the discontinuous sampling protocol in binding experiments, the continuous measurement of the pre-steady-state kinetics of inhibition would be preferable.

Continuous recordings of the onset of inhibition over a broad range of inhibitor concentrations, at the different rates of hydrolysis associated with different ATP concentrations, seem to be missing, in particular under those conditions when enzyme activity is proposed to be limiting. In order to quantify the onset of ouabain inhibition of Na,K-ATPase, a special integrated rate equation was developed that enables the estimation of the pertinent parameters independent of enzyme decay during the experiment.

Methods

Preparation of Na,K-ATPase

The enzyme was prepared as described previously with minor modifications (Halbach *et al.*, 1981; Ebner & Schönsteiner, 1985). In brief, immediately after excision, the hearts of five guinea-pigs (150–200 g) were cooled in ice-cold solution of 0.75 mol l^{-1} KCl and 5 mmol l^{-1} histidine. The connective tissue was removed and the ventricles (ca. 3 g) were minced in 40 ml KCl-histidine buffer with a Waring Blender 8011 C at full speed (15 s), homogenized (Potter Elvehjem S, 3 strokes at 800 r.p.m.) and passed through four layers of gauze. The filtrate was made up to 80 ml with KCl-histidine buffer, centrifuged at 170*g* for 4 min to remove the large fragments and at 1000*g* for 20 min. The pellet was suspended in 30 ml of 10 mmol l^{-1} histidine, 0.25 mol l^{-1} sucrose, homogenized with 3 strokes and further diluted to 80 ml. After centrifugation at 1000*g* for 20 min the pellet was resuspended in 10 ml of 40% (w/w) sucrose, 10 mmol l^{-1} histidine, made up to 80 ml with 10 mmol l^{-1} histidine, and sedimented at 1000*g* for 20 min. To extract the contractile proteins in the presence of 5 ml of 10 mmol l^{-1} histidine, with 0.4 mol l^{-1} LiBr, a concentration of 2 mg protein ml^{-1} LiBr-histidine solution was prepared and the suspension stirred for 1 h. After the addition of 40 ml

histidine (10 mmol l^{-1}), the cell fragments were spun down (1000*g*, 25 min), washed twice, at first in 20 ml histidine solution, then in 9 ml, at 1000*g*. The preparation was kept for use in 1 ml of 10 mmol l^{-1} histidine. pH of all solutions was 7.4; temperature was 0°C. Protein was determined according to Lowry *et al.* (1951).

Na,K-ATPase assay

Na,K-ATPase activity was determined with the coupled enzyme test (pyruvate kinase-lactate dehydrogenase, PK/LDH). In the cuvette the solution was vigorously stirred and kept at 35°C; pH was 7.4. The reaction medium contained in a final volume of 1.3 ml (in mmol l^{-1}): KCl 5.9, NaCl 140, $MgCl_2$ 5, NaN_3 5 and EGTA 0.5 (to block mitochondrial and Ca-ATPases, respectively), Tris 50, Tris-ATP 3, phosphoenolpyruvate 0.5, NADH 0.24, $(NH_4)_2SO_4$ 16.8, pyruvate kinase 7 u and lactate dehydrogenase 10 u as PK/LDH mixture, i.e., in several thousand fold excess over ATPase activity; four fold increase of PK/LDH concentration did not affect the activity. After 15 min preincubation, Na,K-ATPase was added (5–25 μg protein in 50 μl histidine, 10 mmol l^{-1}); enzyme activity depended linearly on protein concentration. After a further 10 min, water (control) or ouabain was injected in a volume of 5 μl and the time course was followed for 15–30 min. Mixing was complete within less than 1 s. NADH absorbance was monitored at 334 nm. Absorbance values were transmitted to the computer at 1 or 2 s intervals via an RS 232 interface.

[^{3}H]-ouabain binding to Na,K-ATPase

[^{3}H]-ouabain binding was determined under the conditions of the enzyme assay at 35°C with ATP-regenerating enzymes in an incubation volume of 2.6 ml; additionally 1.66 μCi [^{3}H]-ouabain (corresponding to a concentration of 28.8 nmol l^{-1}), and unlabelled ouabain were injected. Before the addition of [^{3}H]-ouabain, 0.166 μCi, [^{14}C]-sorbitol was applied for the estimation of non-specific ouabain binding. [^{14}C]-sorbitol space was identical to [^{3}H]-ouabain space in the presence of 100 μmol l^{-1} ouabain. Protein concentration was between 0.22 and 0.33 mg per 2.6 ml incubation volume. After different incubation times aliquots (100 μl) were drawn, rapidly filtered over Whatman GF/C glass fibre filters and washed with 1 ml of ice-cold incubation medium. The double-label technique did not necessitate complete wash-out of non-specifically bound ouabain. Partial reduction was sufficient to calculate non-specific [^{3}H]-ouabain binding via the [^{14}C]-sorbitol content of each sample (see below). The samples were counted by liquid scintillation, 6 to 8 h after 10 ml of scintillation cocktail HP had been added. Medium samples (50 μl) were also counted in HP.

An integrated rate equation of enzyme inhibition

During the assay, Na,K-ATPase activity deteriorated by 5–15% during 15 min incubation. In the presence of 0.1 mmol l^{-1} lactate or of 0.1 mmol l^{-1} Na_2HPO_4 the activity was not different from the control, therefore product inhibition can be excluded. A comparison with the activity of aliquots which had been incubated under similar conditions for different periods but without substrates (no ATP, phosphoenolpyruvate, NADH) showed that 'ageing' of Na,K-ATPase was responsible for the reduction of enzyme activity during the assay. If only steady-state values of inhibition were compared after constant incubation the decay of activity was negligible. However, for the pre-steady state of inhibition it was found that the baseline change of activity interfered with and even precluded its quantification. Therefore it was desirable to estimate the parameters of pre-steady-state and steady-state inhibition independently from enzyme decay.

In the control situation the turn-over of substrate S i.e. $(dS/dt)_c$ follows (constant k) enzyme concentration E. If E decays with some function f(t) which for instance could be monoexponential, we obtain

$$-(dS/dt)_c = kEf(t) \quad (1)$$

To approximate the time course of inhibition with a pseudo-first-order rate constant (k_1), the time course of the inactive enzyme EI_t generated by the reaction with inhibitor I is set to

$$EI_t = EI_\infty(1 - \exp(-k_1 t)) \quad (2)$$

where EI_∞ is the equilibrium value of EI_t. In the presence of the inhibitor the active enzyme ($E - EI_t$) now decays with f(t). The rate equation of substrate turnover $(dS/dt)_i$ corresponding to equation 1 then reads

$$-(dS/dt)_i = k(E - EI_\infty(1 - \exp(-k_1 t)))f(t) \quad (3)$$

Integration of equation 3 after a function f(t) had been chosen the integrated rate equation and fitting to the time courses of substrate in the absence and presence of the inhibitor would provide the pertinent rate constants. This procedure, however, requires the appropriate function f(t) and long duration of the experiment to obtain information on the decay. Lack of substrate or product inhibition precludes this approach. However, selection of an explicit function f(t) and estimation of the pertinent parameters can be avoided when we consider the properties of equations 1 and 3. Their combination gives

$$(dS/dt)_i = (1 - EI_\infty/E(1 - \exp(-k_1 t)))(dS/dt)_c \quad (4)$$

Equation 4 transforms the slope of any control curve to that in the presence of the inhibitor during the onset of its effect independent of enzyme decay. When we can describe the slope of the control accurately enough without mechanistic meaning, the parameters of inhibition can be estimated.

The flexibility of polynomials allows their adjustment to a large number of functions. Actually cubic polynomials

$$S = S_0 + bt + ct^2 + dt^3 \quad (5)$$

could be fitted with considerable precision to the control curves ($S_0 = S$ at $t = 0$). A quadratic expression would have served equally well. Substitution of $(dS/dt)_c$ in equation 4 with the differentiated equation 5 and integration provides the integrated rate equation which determines the time course of S during the onset of inhibition independent of enzyme decay

$$\begin{aligned} S = S_0 &+ (1 - EI_\infty/E)(bt + ct^2 + dt^3) \\ &- \exp(-k_1 t)EI_\infty/E \\ &\times (b/k_1 + 2c/k_1^2(k_1 t + 1) \\ &+ 3d(t^2/k_1 + 2t/k_1^2 + 2/k_1^3)) \\ &+ EI_\infty/E(b/k_1 + 2c/k_1^2 + 6d/k_1^3) \end{aligned} \quad (6)$$

Equation 6 will automatically be reduced to the linear case by setting c and d = 0 when enzyme decay is absent.

Evaluation of experiments

The fitting of equation 6 to experimental data (Gauss-Newton method) provided estimates of pseudo-first-order rate constant k_1 and fractional inhibition $EI\,E^{-1}$. Enzyme activity was determined by differentiation of a cubic polynomial fitted to the time course of absorbance (equation 5; for $t = 0$ $dS/dt = b$). Half-maximal inhibitory concentrations (IC_{50}) and slope P′ (= Hill coefficient) of concentration-inhibition curves at steady state were calculated by fitting a Hill-type equation

$$(EI_\infty E^{-1} = MI^{P'}(I^{P'} + IC_{50}^{P'})^{-1} \quad (7)$$

to experimental data; M = maximal inhibition, I = inhibitor concentration. Confidence limits of estimates were calculated from standard deviation × t value. Linear regressions were computed by the method of least squares.

Tissue:medium ratios of specific [^{3}H]-ouabain binding were obtained from (d.p.m.$_{\text{spec. ouabain filter}}$ mg^{-1} protein of sample) (d.p.m.$_{\text{ouabain medium}}$ μl^{-1} medium sample)$^{-1}$ after the total amount of [^{3}H]-ouabain in the filter had been corrected for that amount of ouabain corresponding to [^{14}C]-sorbitol space; d.p.m.$_{\text{spec. ouabain filter}}$ = d.p.m.$_{\text{total ouabain filter}}$ − d.p.m.$_{\text{sorbitol filter}}$ (d.p.m.$_{\text{ouabain medium}}$/d.p.m.$_{\text{sorbitol medium}}$); d.p.m. disintegrations per min. The data from single experiments or arithmetic means ± s.e.mean, if not stated otherwise, are shown.

Materials

The suppliers of chemicals were: NaN_3, Tris-(hydroxymethyl)aminomethane, L-histidine, Na_2-ATP (Merck, Darmstadt, Germany); NADH (disodium salt), phosphoenolpyruvate (tricyclohexylammonium salt), ouabain (Boehringer, Mannheim, Germany); lactate dehydrogenase/pyruvate kinase mixture in 2.2 mol l^{-1} ammonium sulphate, ethylene glycol bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), alamethicin (Sigma, München, Germany); Dowex 50W × 8 200–400 mesh (H^+), Na-dodecylsulphate (Serva, Heidelberg, Germany); [^{3}H]-ouabain (specific activity 22.1 Ci mmol^{-1}) in ethanol:benzene = 9:1; [^{14}C]-sorbitol (specific activity 346 mCi mmol^{-1}) in ethanol:water = 9:1 (New England Nuclear, Dreieich, Germany); scintillation cocktail HP (Beckman, München, Germany).

Tris-ATP was prepared from Na_2-ATP with Dowex 50 × 8.

Results

Ouabain inhibition of Na,K-ATPase at different ATP concentrations

Original data for Na,K-ATPase activity under control conditions and after the addition of 100 μmol l^{-1} ouabain using the superimposed fit of equation 6 demonstrated the inhibitory effect of the cardioactive steroid (Figure 1). The initial curvature of the recording with ouabain reflects the onset of inhibition before the effect approaches its steady state. In comparable experiments at different ouabain and ATP concentrations, the onset of inhibition in terms of a pseudo-first-order rate constant and the fractional inhibition at steady state were quantified. The hydrolytic activity in the controls varied between 0.26, 0.37, 0.41 and 0.29 μmol mg^{-1} protein min^{-1} at 0.3, 1, 3 and 9 mmol l^{-1} ATP, respectively. Although the heart enzyme was one hundred fold less purified than a preparation from renal outer medulla (Fuchs & Ebner, 1989) similar findings with the enzyme of high specific activity preclude a role of enzyme purity.

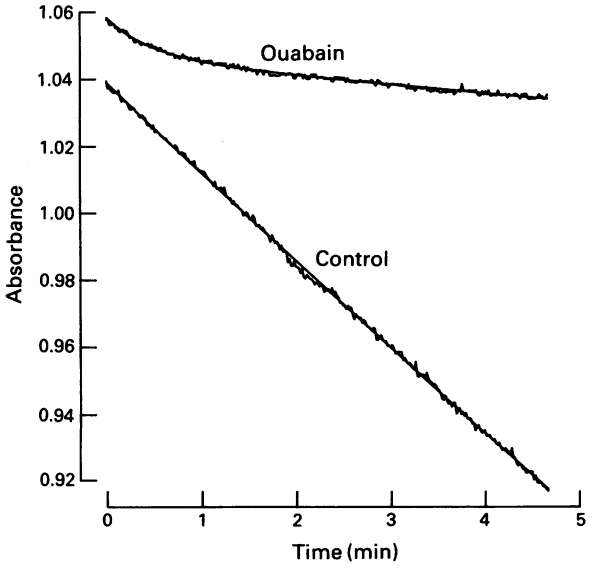


Figure 1 The time course of Na,K-ATPase inhibition by ouabain. The onset of Na,K-ATPase inhibition by 100 μmol l^{-1} ouabain is shown in comparison with the control (original recordings). The ATP concentration was 3 mmol l^{-1}, temperature 35°C. The curves obtained by fitting equation 6 to data are superimposed. Parameter estimates of fit: k_1 2.0315, $EI_\infty E^{-1}$ 0.8981, S_0 (ouabain) 1.0584, S_0 (control) 1.0390, b −0.0271, c 0.0003, d <0.0001. Abscissa scale: time after the injection of water (control) or ouabain. Ordinate scale: NADH absorbance at 334 nm.

At 0.3 mmol l^{-1} ATP the onset of inhibition accelerated with the lower ouabain concentrations more than they did near the maximum of inhibition (Figure 2), so that the resulting relationship was nonlinear. The nonlinear dependence of rate constants on inhibitor concentration was preserved when inhibition accelerated parallel to the higher hydrolysis rate at 1 mmol l^{-1} ATP ouabain. A further increase of ATP to 3 and 9 mmol l^{-1} i.e., close to total Mg concentration (5 mmol l^{-1}), must reduce free Mg and increase uncomplexed ATP concentration (cf. Skou *et al.*, 1971). Either effect consequently could have retarded inhibition and made its rate independent of ouabain concentration at 9 mmol l^{-1} ATP. The variation of ATP concentration in these experiments altered fractional inhibition of ATP hydrolysis at steady state by only a small amount (Figure 3). Half-maximal inhibitory concentrations of ouabain (IC_{50}) and the maximal fractional inhibition were only slightly different, although they tended towards lower values with an increase in ATP concentration. Estimates of IC_{50} ± 95% confidence limits with equation 7 were 0.59 ± 0.09, 0.59 ± 0.20, 0.37 ± 0.057, 0.35 ± 0.24 μmol l^{-1} at 0.3, 1, 3, 9 mmol l^{-1} ATP, respectively. These IC_{50} values are one order of magnitude lower than the dissociation constants from the ratio of apparent dissociation over association rates derived from the relationship between rate constants and low ouabain concentrations in Figure 2. The corresponding maximal fractional inhibition data were 0.89 ± 0.026, 0.92 ± 0.05, 0.85 ± 0.02, and 0.75 ± 0.09. With the exception of 9 mmol l^{-1} ATP the Hill coefficient of inhibition was significantly <1, as observed previously (Ebner & Schönsteiner, 1985; 0.78 ± 0.09, 0.73 ± 0.18, 0.86 ± 0.10, 0.88 ± 0.47).

Alamethicin accelerates Na,K-ATPase inhibition by ouabain

Limited access of reactants to enzyme particles could interfere with ATP hydrolysis or inhibition. An effect of an unstirred layer of membrane particles on the onset of inhibition was

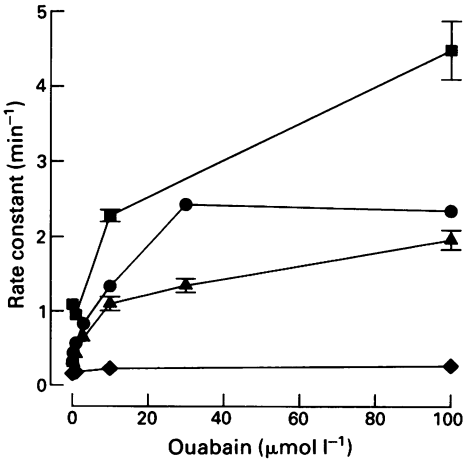


Figure 2 The onset of Na,K-ATPase inhibition by ouabain. With pseudo-first order rate constants (in min^{-1}, ordinate scale) the onset of inhibition was quantified at different ouabain (abscissa scale, linear) and ATP concentrations (ATP, 0.3 (▲), 1.0 (■), 3.0 (●) and 9.0 (◆) mmol l^{-1}). Arithmetic means with some s.e.mean (vertical lines) of 3–7 experiments are shown. At ouabain concentrations < 10 μmol l^{-1} and at 9 mmol l^{-1} ATP s.e.mean were smaller than symbols.

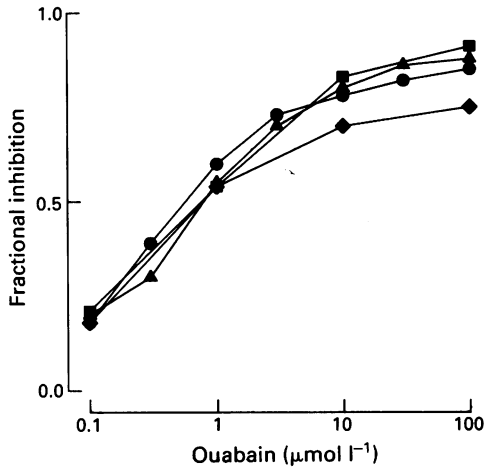


Figure 3 The inhibition of Na,K-ATPase by ouabain at steady state. Ouabain inhibition at steady state is shown as fractional inhibition (ordinate scale) of total ATP hydrolysis at different ATP concentrations ((▲) 0.3, (■) 1.0, (●) 3.0 and (◆) 9.0 mmol l^{-1}). Ouabain concentration is indicated on abscissa scale (log scale). Arithmetic means are shown; s.e.mean were smaller than symbols.

excluded by the ineffectiveness of raising viscosity with 50 mmol l^{-1} sucrose in two preparations (k_1 2.39 and 2.2 min^{-1} vs 2.35 ± 0.12, $n = 7$, of control at 100 μmol l^{-1} ouabain, 3 mmol l^{-1} ATP). Also lipids could impede the access if the reactants have to permeate membrane vesicles to become effective. This idea was tested with detergents which facilitate the permeation of lipids. The detergent-like peptide alamethicin opens vesicles of enzymes (Jones *et al.*, 1980) and extracts lipids from cell membranes (Bonnafous *et al.*, 1979). At a concentration of 23 μg ml^{-1} it neither interfered with ATP hydrolysis (Figure 4) nor influenced either decay rate or fractional inhibition. At the various ouabain (0.1, 1, 10, 100 μmol l^{-1}) and ATP concentrations, inhibition at steady state after alamethicin treatment was identical to the controls (regression coefficient 1.016 ± 0.037, $n = 16$; condition for identity = 1) as were the parameters of the ouabain-inhibition curves (the tendency for IC_{50} values to be lower with an increase in ATP concentration and Hill coefficients < 1 persisted). In contrast to its ineffectiveness at steady state, alamethicin accelerated the onset of ouabain inhibition 2.3 fold but preserved the typical concentration-dependence with respect to ouabain and ATP (Figure 5). Comparable tests with 0.2 mg ml^{-1} Na-dodecylsulphate at 35°C at first failed, because the enzyme was rapidly inactivated within 2–3 min. However, when incubated for 30 min at room temperature before the test, the accelerating effect of Na-dodecylsulphate (0.1 mg ml^{-1}) was comparable to that of alamethicin without loss of activity (data not shown).

In the presence of alamethicin the rate constants of inhibition clearly correlated with ATPase activity, as determined by the different ATP concentrations (Figure 6) whereby the influence of enzyme activity on inhibition rate increased progres-

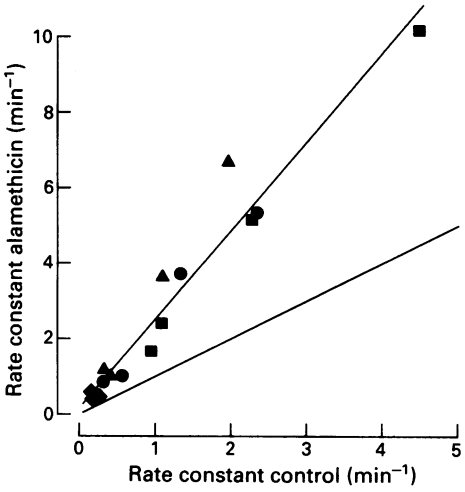


Figure 5 Alamethicin accelerates the onset of ouabain inhibition. Arithmetic means of pseudo-first-order rate constants of the onset of inhibition in min^{-1} under control conditions (abscissa scale, data from Figure 2) and in the presence of alamethicin (23 μg ml^{-1}; $n = 3$–7; ordinate scale) were correlated at the different ouabain (0.1, 1, 10, 100 μmol l^{-1}) and ATP concentrations ((▲) 0.3, (■) 1.0, (●) 3.0 and (◆) 9.0 mmol l^{-1}). The parameters of the correlation of means were: $r = 0.97$, $P < 0.01$; y = (0.15 ± 0.23) + (2.34 ± 0.15)x. The flatter straight line stands for the identity of data (slope = 1).

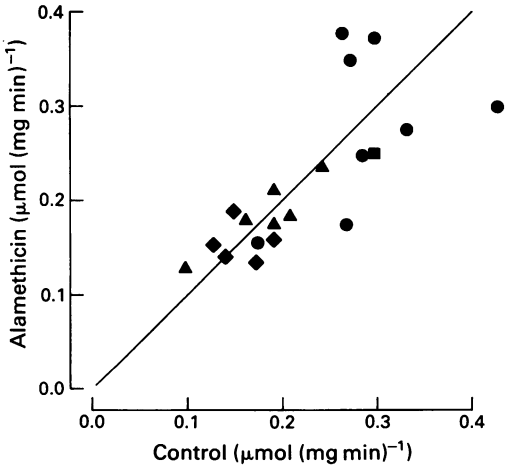


Figure 4 Na,K-ATPase activity in the presence of alamethicin. In the same preparations the ATP-hydrolytic activity (in μmol (mg protein $min)^{-1}$) was determined under control conditions (abscissa scale) and in the presence of alamethicin (ordinate scale). Control data represent the initial activity at t = 0; to obtain the data with alamethicin the control activity was multiplied with fractional inhibition or activation under steady-state conditions. Data from individual experiments are shown. The straight line with slope = 1 corresponds to the condition of identity. The various symbols represent the different ATP concentrations ((▲) 0.3, (■) 1.0, (●) 3.0 and (◆) 9.0 mmol l^{-1}).

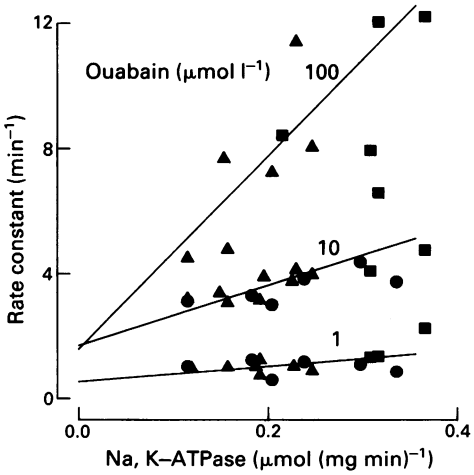


Figure 6 Na,K-ATPase activity interferes with the onset of ouabain inhibition. The data from individual preparations of Na,K-ATPase activity (μmol (mg protein $min)^{-1}$, abscissa scale) obtained at different ATP concentrations ((▲) 0.3, (■) 1.0, (●) 3.0 and (◆) 9.0 mmol l^{-1}), were correlated with pseudo-first-order rate constants at different ouabain concentrations (min^{-1}, ordinate scale; experiments of Figure 5). Regressions: ouabain 1 μmol l^{-1}, $r = 0.52$, $P < 0.05$, y = (0.55 ± 0.27) + (2.57 ± 1.12)x; ouabain 10 μmol l^{-1}, $r = 0.77$, $P < 0.001$, y = (1.72 ± 0.50) + (9.63 ± 2.13)x; ouabain 100 μmol l^{-1}, $r = 0.88$, $P < 0.001$, y = (1.61 ± 1.42) ± (30.82 ± 5.91)x.

sively from 1 to 100 μmol l^{-1} ouabain. All data obtained with 9 mmol l^{-1} ATP and 3 mmol l^{-1} ATP, 100 μmol l^{-1} ouabain, were omitted from the plot. With these high ATP concentrations inhibition rate was retarded to a greater degree than would be expected relative to enzyme activity.

[^3H]-ouabain binding to Na,K-ATPase

Under the conditions of the preceding experiments (35°C, 145 mmol l^{-1} Na, 5.9 mmol l^{-1} K, ATP-regenerating enzymes) the time course of [^{3}H]-ouabain binding (0.03 μmol l^{-1}) to Na,K-ATPase was evaluated in the presence of 0, 0.3, and 3 μmol l^{-1} unlabelled ouabain. From these experiments tissue: medium ratios (T:M) of specific [^{3}H]-ouabain binding, i.e., corrected for that amount of ouabain corresponding to [^{14}C]-sorbitol space, were calculated. T:M = 1 corresponds to 0.03, 0.3 and 3 μmol ouabain mg^{-1} protein at the respective ouabain concentration in μmol l^{-1}. With an increase in ouabain concentration, saturation of binding became evident. Accordingly, steady-state T:M values (estimated by fitting a monoexponential time curve to data) declined from 60.7 $\pm$ 0.7 to 35.5 $\pm$ 0.3 and 6.2 μl mg^{-1} at 0.03, 0.3, and 3 μmol l^{-1} ouabain, respectively. At 3 μmol l^{-1} ouabain, specific binding was therefore nearly saturated, being close to [^{14}C]-sorbitol space. With pseudo-first-order rate constants of 0.21 $\pm$ 0.02 and 0.29 $\pm$ 0.02 min^{-1} ($\pm$95% confidence intervals), at 0.03 and 0.3 μmol l^{-1} ouabain, binding developed at a rate similar to enzyme inhibition.

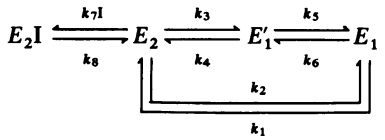
Discussion

Subsequent to the occupation of a single class of receptors with immediate onset of inhibition the effect should develop monoexponentially with linear dependence of pseudo-first-order rate constant k on ouabain concentration I (cf. also Wallick & Schwartz, 1974), where $k = k_{+1}I + k_{-1}$; k_{+1} and k_{-1} are the rate constants of association and dissociation, respectively. At low ouabain concentrations the time course of inhibition actually correlated with [^{3}H]-ouabain binding and accelerated with an increase in ouabain concentration. However, the difference between IC_{50} values and k_{-1} k_{+1}^{-1} and the reduced acceleration of inhibition rate with high ouabain concentrations remain at variance.

Limited access of reactants to the enzyme could have contributed to the nonlinear relationship between inhibition rates and ouabain concentration. Alamethicin actually accelerated inhibition but left enzyme activity and steady-state inhibition unaltered. This suggests that although ouabain had to permeate lipids to gain access to the receptor, its steady-state concentration at the receptor was not limited. Moreover, the nonlinear relationship between inhibition rate and ouabain concentration was preserved in the presence of the detergent. Since the aggregation of ouabain molecules at high concentrations is also unlikely, because of concentration-independent diffusion of ouabain in glass fibre strips (Ebner & Siegl, 1988; unpublished results), different mechanisms seem to be relevant to inhibition rate.

In view of the Hill coefficient being < 1, two receptors may participate in ouabain inhibition in accordance with previous results (e.g., guinea-pig heart, Ebner & Schönsteiner, 1985; rat heart, Wallick & Schwartz, 1988) and with their demonstration in binding experiments (e.g., ox brain, Taniguchi & Iida, 1972, guinea-pig heart, Godfraind *et al.*, 1980). The reaction of ouabain with two independent receptors implies a linear relationship between the rate constants of two exponentials and ouabain concentration. This relationship is governed by the solutions of quadratic equations in the case of interdependent receptors. That is, in both cases the concentration-dependence of pseudo-first-order rate constants is at least qualitatively similar to that of Figure 2. Although isozymes of Na,K-ATPase in guinea-pig myocardium have not been demonstrated definitely (Ng & Akera, 1987; Sweadner, 1989) in contrast to other tissues or species (e.g., Sweadner, 1979; Ng & Akera, 1987; Maixent *et al.*, 1987), isozymes and interdependent ouabain receptors could be relevant to the present observations. Different populations of ouabain receptors, however, do not suffice to explain all the experimental findings; in particular the relationship between rate and enzyme activity Figure 6) must be based on a different mechanism.

According to Sen *et al.* (1969) ouabain reacts with the K-sensitive phosphoprotein E_2-P which is transformed to a ouabain-containing dephosphoenzyme in a precursor-product relationship. Studies on phosphorylation (Matsui & Schwartz, 1968; Post *et al.*, 1969) have also suggested its relevance to ouabain binding. While steady-state inhibition (Figure 3) was only marginally influenced by enzyme activity, presumably because the activity could not be varied widely enough (inferred from similar experiments on a highly active enzyme from guinea-pig renal outer medulla, Fuchs & Ebner, 1989), ATP hydrolysis clearly interfered with inhibition rate; i.e., the reaction of ouabain with a definite conformation of the enzyme, E_2-P, did not immediately become evident. With a simplified model of Na,K-ATPase activity some of the pertinent features of pre-steady-state inhibition could be simulated. The access of the reactants to the enzyme was assumed to be unrestricted, conforming to the experimental situation in the presence of alamethicin. By disregarding various intermediate or phosphorylated states, the inhibitor I is supposed to react with E_2 while ATP is hydrolyzed at E_1,



E'_1 was added to the model to interfere at various steps of the reaction sequence. The concentration of E_1 determines ATP hydrolysis by $-dATP/dt = k_1E_1$, where k_1 also contains ATP concentration. The backward reaction was omitted as ADP concentration is kept low by ATP-regenerating enzymes. The time course of E_1 accordingly reflects the onset of inhibition, which is accelerated by the concentration of inhibitor until the transition between enzyme conformations becomes rate-limiting (Figure 7a). The value for the maximal rate constant of inhibition (0.65) was below that of the individual rate constants (1) presumably because E_1 declines less rapidly than it would do in a single reaction. At a given inhibitor concentration (Figure 7b) enzyme activity (k_1E_1) interferes with inhibition rate. Basically this effect does not depend on the mechanism which alters enzyme activity, i.e,. variation of the various rate constants, but an increase in the inhibitor concentration augments its impact.

In the presence of Tris in Na-rich media, Na,K-ATPase is kept in a Na-like (E_1, ADP-sensitive) conformation (Jørgensen, 1975; Jørgensen & Petersen, 1982) while the E_2 (K-sensitive) form is favoured by K (Jørgensen, 1975) in the absence of Tris (Skou & Esmann, 1980). At a given Na:K ratio increasing the ATP concentration also favours E_1 (Skou, 1979). At 100 mmol l^{-1} Na, 37.7 mmol l^{-1} K was necessary for half-maximal reversal of the Na-induced shift of E_2 to E_1 (Skou & Esmann, 1983), therefore E_1 is likely to predominate in our experiments. Hence, delayed onset of inhibition would be conceivable because of the protracted decline of the ATP-hydrolytic conformations after occupation of the ouabain-sensitive conformation by the steroid. This effect becomes increasingly relevant when, with high ouabain concentrations, the conformational transitions of the enzyme cycle interfere with inhibition rate (cf. Figure 7b vs Figure 6). However, as compared with maximal inhibition rates (half-times of approximately 5 s at 1 mmol l^{-1} ATP) the Na-induced conformational transition of Na,K-ATPase with half-times of ms (Karlish & Yates, 1978; Skou & Esmann, 1983) is fast. Although K-induced transition of E_2 to E_1 with half-times of a few s is slower (Karlish & Yates, 1978; Skou & Esmann, 1983), submillimolar ATP concentrations accelerate this reac-

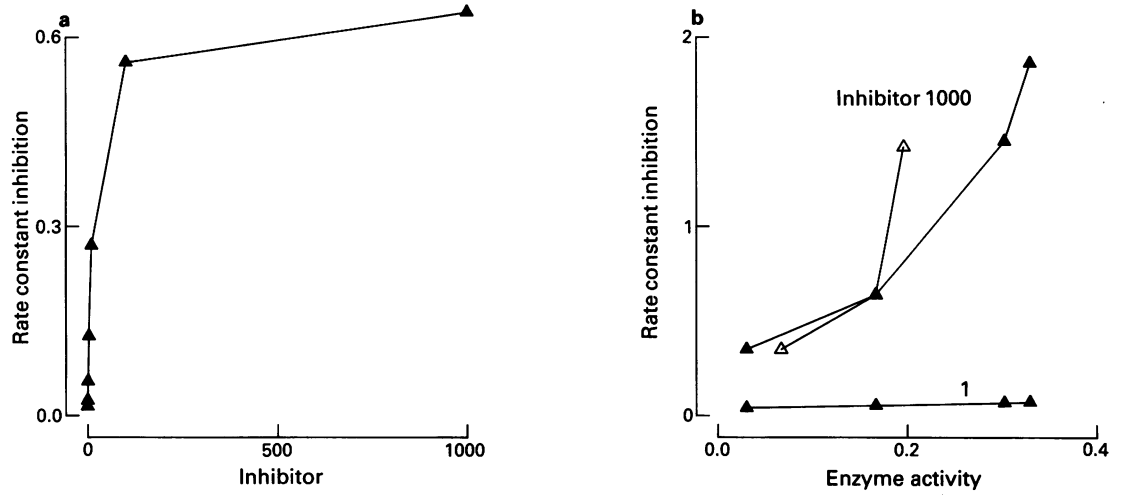


Figure 7 Model calculations for the relationship between Na,K-ATPase activity and the onset of ouabain inhibition. (a) The dependence of the onset of inhibition on inhibitor concentration and the effect of enzyme activity. For arbitrarily chosen parameters ($k_1 = 1$, $k_2 = 0$, $k_{3-6} = 1$, $k_7 = 0.1$, $k_8 = 0.01$) and arbitrary units half-times of the reduction of E_1 after the addition of different inhibitor concentrations (abscissa scale) were calculated in numerical solutions of the model and transformed to pseudo-first-order rate constants (ordinate scale). (b) The relationship between enzyme activity and the onset of inhibition. For arbitrarily chosen parameters (same as in (a)) and arbitrary units enzyme activity (E_1k_1, abscissa scale) was altered by varying k_1 (0.1, 1, 10, 100, ▲) and pseudo-first-order rate constants of the reduction of E_1 (ordinate scale) were calculated after the addition of a high (1000) or low (1) inhibitor concentration. For (△) enzyme activity was altered by varying k_3 and k_4 (both 0.1, 1, 10, $k_1 = 1$) at an inhibitor concentration = 1000.

tion by nearly two orders of magnitude. The difference between transition and maximal inhibition rates therefore indicates an interference with, but not the complete control of, inhibition rate by the conformational transitions of the enzyme.

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References

ALBERS, R.W., KOVAL, G.J. & SIEGEL, G.J. (1968). Studies on the interaction of ouabain and other cardioactive steroids with sodium-potassium-activated adenosine triphosphatase. *Mol. Pharmacol.*, **4**, 324–336.

BONNAFOUS, J.-C., DORNAND, J. & MANI, J.C. (1979). Detergent-like effects of alamethicin on lymphocyte plasma membranes. *Biochem. Biophys. Res. Commun.*, **86**, 536–543.

CLARK, A.F., SWANSON, P.D. & STAHL, W.L. (1975). Increase in dissociation rate constants of cardiotonic steroid-brain ($Na^+ + K^+$)-ATPase complexes by reduction of the unsaturated lactone. *J. Biol. Chem.*, **250**, 9355–9359.

CLAUSEN, T. & HANSEN, O. (1977). Active Na-K transport and the rate of ouabain binding. The effect of insulin and other stimuli on skeletal muscle and adipocytes. *J. Physiol.*, **270**, 415–430.

EBNER, F. & SCHÖNSTEINER, G. (1985). Inhibition of sarcolemmal Na, K, Mg ATPase from the guinea pig heart is not compatible with a homogeneous population of non-interacting ouabain receptors. *Experientia*, **41**, 1147–1149.

EBNER, F. & SIEGL, H. (1988). Diffusion characteristics dissociate ouabain binding from inotropic effect in guinea-pig myocardium. *Br. J. Pharmacol.*, **95**, 399–404.

FUCHS, K. & EBNER, F. (1989). Na,K-ATPase activity determines the apparent affinity of ouabain to the enzyme. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **340**, R50.

GODFRAIND, Th., DE POVER, A. & TONA LUTETE, D.-N. (1980). Identification with potassium and vanadate of two classes of specific ouabain binding sites in a ($Na^+ + K^+$) ATPase preparation from the guinea-pig heart. *Biochem. Pharmacol.*, **29**, 1195–1199.

HALBACH, S., SCHÖNSTEINER, G., EBNER, F. & REITER, M. (1981). The effects of p-chloromercuriphenylsulfonic acid (PCMBS) on force of contraction of mammalian myocardium and on ATP hydrolysis by sarcolemmal ATPase. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **318**, 121–129.

HANSEN, O. (1984). Interaction of cardiac glycosides with ($Na^+ + K^+$)-activated ATPase. A biochemical link to digitalis-induced inotropy. *Pharmacol. Rev.*, **36**, 143–163.

JØRGENSEN, P.L. (1975). Purification and characterization of (Na^+, K^+)-ATPase V. Conformational changes in the enzyme. Transitions between the Na-form and the K-form studied with tryptic digestion as a tool. *Biochim. Biophys. Acta*, **401**, 399–415.

JØRGENSEN, P.L. & PETERSEN, J. (1982). High-affinity ^{86}Rb-binding and structural changes in the α-subunit of Na^+, K^+-ATPase as detected by tryptic digestion and fluorescence analysis. *Biochim. Biophys. Acta*, **705**, 38–47.

JOINER, C.H. & LAUF, P.K. (1978). Modulation of ouabain binding and potassium pump fluxes by cellular sodium and potassium in human and sheep erythrocytes. *J. Physiol.*, **283**, 177–196.

JONES, L.R., MADDOCK, S.W. & BESCH, H.R. (1980). Unmasking effect of alamethicin on the (Na^+,K^+)-ATPase, β-adrenergic receptor-coupled adenylate cyclase, and cAMP-dependent protein kinase activities of cardiac sarcolemmal vesicles. *J. Biol. Chem.*, **255**, 9971–9980.

KARLISH, S.J.D. & YATES, D.W. (1978). Tryptophan fluorescence of ($Na^+ + K^+$)-ATPase as a tool for study of the enzyme mechanism. *Biochim. Biophys. Acta*, **527**, 115–130.

KARLISH, S.J.D., YATES, D.W. & GLYNN, I.M. (1978). Conformational transitions between Na^+-bound and K^+-bound forms of ($Na^+ + K^+$)-ATPase, studied with formycin nucleotides. *Biochim. Biophys. Acta*, **525**, 252–264.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

MAIXENT, J.M., CHARLEMAGNE, D., DE LA CHAPELLE, B. & LELIEVRE, L.G. (1987). Two Na, K-ATPase isoenzymes in canine cardiac myocytes. Molecular basis of inotropic and toxic effects of digitalis. *J. Biol. Chem.*, **262**, 6842–6848.

MATSUI, H. & SCHWARTZ, A. (1968). Mechanism of cardiac glycoside inhibition of the (Na^+-K^+)-dependent ATPase from cardiac tissue. *Biochim. Biophys. Acta*, **151**, 655–663.

NG, Y.-C. & AKERA, T. (1987). Two classes of ouabain binding sites in ferret heart and two forms of Na^+-K^+-ATPase. *Am. J. Physiol.*, **252**, H1016–H1022.

POST, R.L., KUME, S., TOBIN, T., ORCUTT, B. & SEN, A.K. (1969). Flexibility of an active center in sodium-plus-potassium adenosine triphosphatase. *J. Gen. Physiol.*, **54**, 306s–326s.

SEN, A.K., TOBIN, T. & POST, R.L. (1969). A cycle for ouabain inhibi-

tion of sodium- and potassium-dependent adenosine triphosphatase. *J. Biol. Chem.*, **244**, 6596–6604.

SKOU, J.CH. (1979). Effects of ATP on the intermediary steps of the reaction of the $(Na^+ + K^+)$-ATPase. IV. Effect of ATP on $K_{0.5}$ for Na^+ and on hydrolysis at different pH and temperature. *Biochim. Biophys. Acta*, **567**, 421–435.

SKOU, J.CH. (1988). Overview: The Na,K-pump. In *Methods in Enzymology, Biomembranes*, part P, *ATP-Driven Pumps and Related Transport: The Na,K-pump*. Vol. 156. ed. Fleischer, S. & Fleischer, B. pp. 1–25. San Diego, New York, Berkeley, Boston, London, Sydney, Tokyo, Toronto: Academic Press.

SKOU, J.C., BUTLER, K.W., & HANSEN, O. (1971). The effect of magnesium, ATP, P_i, and sodium on the inhibition of the $(Na^+ + K^+)$-activated enzyme system by g-strophanthin. *Biochim. Biophys. Acta*, **241**, 443–461.

SKOU, J.C. & ESMANN, M. (1980). Effects of ATP and protons on the Na:K selectivity of the $(Na^+ + K^+)$-ATPase studied by ligand effects on intrinsic and extrinsic fluorescence. *Biochim. Biophys. Acta*, **601**, 386–402.

SKOU, J.C. & ESMANN, M. (1983). The effects of Na^+ and K^+ on the conformational transitions of $(Na^+ + K^+)$-ATPase. *Biochim. Biophys. Acta*, **746**, 101–113.

SWEADNER, K.J. (1979). Two molecular forms of $(Na^+ + K^+)$-stimulated ATPase in brain. Separation, and difference in affinity for strophanthidin. *B. Biol. Chem.*, **254**, 6060–6067.

SWEADNER, K.J. (1989). Isozymes of the Na^+/K^+-ATPase. *Biochim. Biophys. Acta*, **988**, 185–220.

TANIGUCHI, K. & IIDA, S. (1972). The role of phospholipids in the binding of ouabain to sodium- and potassium-dependent adenosine triphosphatase. *Mol. Pharmacol.*, **9**, 350–359.

WALLICK, E.T. & SCHWARTZ, A. (1974). Thermodynamics of the rate of binding of ouabain to the sodium, potassium-adenosine triphosphatase. *J. Biol. Chem.*, **249**, 5141–5147.

WALLICK, E.T. & SCHWARTZ, A. (1988). Interaction of cardiac glycosides with Na^+, K^+-ATPase. In *Methods in Enzymology*, Vol. 156, *Biomembranes*, Part P, *ATP-Driven Pumps and Related Transport: The Na,K-Pump*. ed. Fleischer, S. & Fleischer, B. San Diego, New York, Berkeley, Boston, London, Sydney, Tokyo, Toronto: Academic Press.

YODA, A. (1973). Structure-activity relationships of cardiotonic steroids for the inhibition of sodium- and potassium-dependent adenosine triphosphatase. I. Dissociation rate constants of various enzyme-cardiac glycoside complexes formed in the presence of magnesium and phosphate. *Mol. Pharmacol.*, **9**, 51–60.

YODA, A. & YODA, S. (1973). Structure-activity relationships of cardiotonic steroids for the inhibition of sodium- and potassium-dependent adenosine triphosphatase. III. Dissociation rate constants of various enzyme-cardiac glycoside complexes formed in the presence of sodium, magnesium, and adenosine triphosphatase. *Mol. Pharmacol.*, **10**, 494–500.

YODA, A., YODA, S. & SARRIF, A.M. (1973). Structure-activity relationships of cardiotonic steroids for the inhibition of sodium- and potassium-dependent adenosine triphosphatase. II. Association rate constants of various enzyme-cardiac glycoside complexes. *Mol. Pharmacol.*, **9**, 766–773.

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Bradykinin-induced vasoconstriction of rat mesenteric arteries precontracted with noradrenaline

[1]Juan C. Fasciolo, Leonor Vargas, M. Cristina Lama & Héctor Nolly

Fisiología Patológica, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Mendoza, Argentina

1 Administration of bradykinin caused dose-dependent vasoconstriction in rat isolated perfused mesenteric arteries precontracted with noradrenaline.

2 The vasoconstrictor response was not mediated by BK_1-bradykinin receptors.

3 Inhibition of cyclo-oxygenase with indomethacin, aspirin or meclofenamate abolished the vasoconstrictor effect of bradykinin, showing that a member of the arachidonic acid cascade may be involved.

4 Inhibitors of thromboxane synthesis (imidazole and UK 38485) did not affect or only reduced the bradykinin-induced vasoconstriction.

5 The endoperoxide H_2/thromboxane A_2 receptor antagonist SQ 29548 significantly reduced the vasoconstrictor effect of bradykinin, but did not affect the vasoconstrictor response to noradrenaline, adrenaline, vasopressin, 5-hydroxytryptamine or prostaglandins.

6 The eicosanoid(s) that mediate bradykinin-induced vasoconstriction appear to be synthesized outside the arterial endothelium.

7 The data suggest that the vasoconstrictor effect of bradykinin in the rat isolated mesenteric artery is mediated by vasoconstrictor arachidonic acid metabolites including the cyclic endoperoxides and/or the thromboxanes.

Introduction

Bradykinin is a nonapeptide with potent vascular actions mediating systemic vasodilatation and a fall in blood pressure. A number of studies, however, have shown that bradykinin may also increase arterial pressure or cause a biphasic response. In most arterial vessels bradykinin induces relaxation (see Regoli, 1984). In the rat mesenteric vascular bed bradykinin relaxes the arteries and constricts the veins (Northover & Northover, 1970). In rabbit isolated kidney and ear arteries bradykinin induces vasoconstriction (Barabe *et al.*, 1979). Despite the very well known vasorelaxant effects of kinins, the nature and role of their vasoconstrictor effect in the regulation of vascular tone is far from clear.

In the present study, in the rat isolated perfused mesenteric arterial preparation precontracted with noradrenaline and perfused with a modified Tyrode solution, we found that bradykinin induces vasoconstriction. The bradykinin-induced vasoconstriction was abolished by use of cyclo-oxygenase inhibitors. Because the metabolism of arachidonic acid via the cyclo-oxygenase pathway results in the formation of various eicosanoids that are able to cause vasoconstriction, these are candidates for mediating the vasoconstrictor effect of bradykinin. To test this hypothesis we examined the vasoconstrictor effect of bradykinin in the isolated, precontracted mesenteric vessels of the rat in the presence and in the absence of cyclo-oxygenase inhibitors, thromboxane synthesis inhibitors and a thromboxane A_2/endoperoxide H_2 receptor antagonist.

Methods

The vascular bed of the superior mesenteric artery of the rat was perfused with a modified Tyrode solution by a peristaltic pump as described by McGregor (1965); the mesentery was cut near the intestinal border. Since the output of the pump was constant, changes in perfusion pressure reflect changes in the vascular tone of these vessels. The output of the pump was kept at 3–4 ml per min to maintain a perfusion pressure of about 12 mmHg. The perfusion pressure was measured with a Statham transducer (P2 3AC) and an electronic recorder.

[1] Author for correspondence at: San Lorenzo 722-5° piso, Mendoza (5500), Argentina.

The composition of the perfusing solutions was as follows in mM: NaCl 134, KCl 5, $CaCl_2$ 0.6, Na_2HPO_4 5, NaH_2PO_4 0.5 and glucose 5. Osmolarity was 285 mOsmol l^{-1} and pH 7.45. The perfusing solution was gassed with oxygen and maintained at 38°C with a thermostatic circulating bath and the vascular preparation was placed on a heated table.

Experiments were carried out after the vessels had been perfused for one h to allow stabilization of the responses. The agonists were dissolved in the solution used for perfusion and a 50 μl bolus injected rapidly with a microsyringe through the cannula inserted in the artery. The injection of 50 μl of the saline solution produced a volume artifact not followed by any vasomotor response. The sensitivity of preparations from different rats differed widely, but in the same preparation sensitivity to any given agonist was usually maintained within fairly constant limits, although after 4 or 5 h of perfusion the reactivity increased. Noradrenaline (0.25 ml min^{-1} of a 3×10^{-5} M solution) was added to the perfusing solution with a Harvard pump.

Drugs used were: (−)-noradrenaline D-bitartrate (NA) (Sterling-Winthrop); (−)-adrenaline D-bitartrate (Ad) (Sigma); 5-hydroxytryptamine creatinine sulphate (5-HT) (Sigma); arginine vasopressin (Sigma); bradykinin triacetate (BK) (Sigma); prostaglandins E_1, E_2, $F_{2\alpha}$ (Sigma); imidazole (Sigma); indomethacin (Sigma); sodium meclofenamate (Parke-Davis); UK 38485 (3-1H-imidazol-lyl-methyl)-2-methyl-1H-indole-1-propanoic acid, Pfizer); Aspirin (Bayer); SQ 29548 (1S-[1α . 2β(5Z),3β,4α)]-7-[3-[2-[(phenylamino)-carbonyl]hydrazinomethyl] - 7 - oxabicyclo(2.2.1)hept - 2 - yl] - 5 - heptenoic acid, Squibb). The reagents used for preparing the solutions were of analytical grade. The bradykinin BK_1-receptor antagonist was kindly provided by Dr Regoli.

Analysis of variance and test of multiple comparison were done according to Scheffe (1963).

Results

Bradykinin (10^{-6}–10^{-4} M), injected in the cannula inserted in the mesenteric artery was without vascular effects when the preparation was perfused with the modified Tyrode solution. After addition of noradrenaline (10^{-7} M) to the Tyrode solution, which increased the perfusion pressure from 12–15 mmHg to 40–80 mmHg, bradykinin induced

vasoconstrictor responses that were dose-dependent from 10^{-6} M to 10^{-4} M (Figure 1). Although the vasoconstrictor responses were always present, the magnitude of the response varied with different preparations. Successive injections usually produced decreasing responses. The perfusion pressure increased slowly to reach a peak about 2 min after the injection and remained elevated for 4 to 6 min (Figure 1). Vasoconstrictor responses were usually preceded by a small vasodilatation.

The vasoconstriction did not seem to be mediated through BK_1-receptors since the BK_1-receptor antagonist [Leu^8], des Arg-bradykinin, did not affect the bradykinin-induced vasoconstriction. In five experiments bradykinin 10^{-5} M increased the perfusion pressure of the mesenteric arteries by 13.2 ± 2.1 mmHg, while after injection of the BK_1-receptor antagonist in doses as high as 200 μg ml^{-1} the increase was 13.8 ± 4.1 mmHg.

Inhibition of cyclo-oxygenase

The delay and the slow rise of the vasoconstrictor response to bradykinin suggested that a locally generated mediator might be involved. Bradykinin is known to release prostaglandins in blood vessels, so we tested the effects of cyclo-oxygenase inhibitors on the response to bradykinin. After the mesenteric arteries had been precontracted with noradrenaline, bradykinin and noradrenaline were injected before and after the vessels had been perfused with indomethacin (10^{-4} M) over 15 min.

Figure 2 shows that indomethacin abolished the bradykinin-induced vasoconstriction without a significant reduction in the noradrenaline-induced vasoconstriction, thus indicating that vascular reactivity had been preserved. No recovery of the bradykinin-induced vasoconstriction was observed 1 h after the indomethacin perfusion had ceased. Meclofenamate (10^{-6} M) perfused over 15 min was also able to inhibit bradykinin-induced vasoconstriction without affecting the vasoconstriction to noradrenaline (Figure 2). Aspirin (3×10^{-3} M) was also able to inhibit bradykinin-induced vasoconstriction. The responses partially recovered one h after the aspirin perfusion had finished.

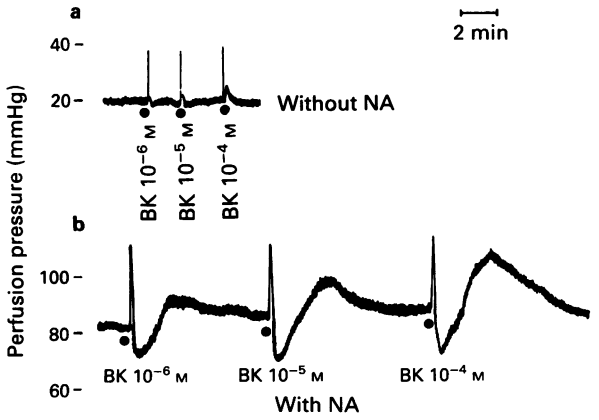

Figure 1 Effect of noradrenaline (NA) on bradykinin (BK)-induced vasoconstriction. (a) Mesenteric arteries perfused with Tyrode solution. Vessels were relaxed and the perfusion pressure was 19 mmHg. The spikes were artifacts caused by the volume of fluid injected. (b) Effect of BK after the addition of NA to the perfusing solution (increases the perfusion pressure to 80 mmHg).

Role of endothelium

To find out whether the endothelium was responsible for the release of the vasoconstrictor eicosanoid, the endothelial lining of the mesenteric artery was removed either by using distilled water or alcohol. Distilled water was perfused through the mesenteric arteries for five min and the vasodilator effect of acetylcholine (10^{-4} M) compared before and after treatment. The disruption of the endothelium was considered complete when acetylcholine no longer induced vasodilatation and the vasoconstrictor responses to noradrenaline and other agonists were maintained without significant reductions (Figure 3). Under these conditions bradykinin-induced vasoconstriction was not significantly reduced. Similar results were obtained after the endothelial layer had been destroyed by one or two injections of 250 μl of 96% alcohol into the cannula inserted in the mesenteric artery (Figure 3).

Effects of different eicosanoids

To see whether products of the arachidonic acid cascade could induce vasoconstrictor responses in the mesenteric vessels, bolus doses of prostaglandin E_1 (PGE_1), PGE_2 and $PGF_{2\alpha}$ were injected in concentrations ranging from 10^{-7} M to 10^{-4} M. No vasoconstrictor responses were elicited when the mesenteric vessels were perfused with Tyrode solution, but

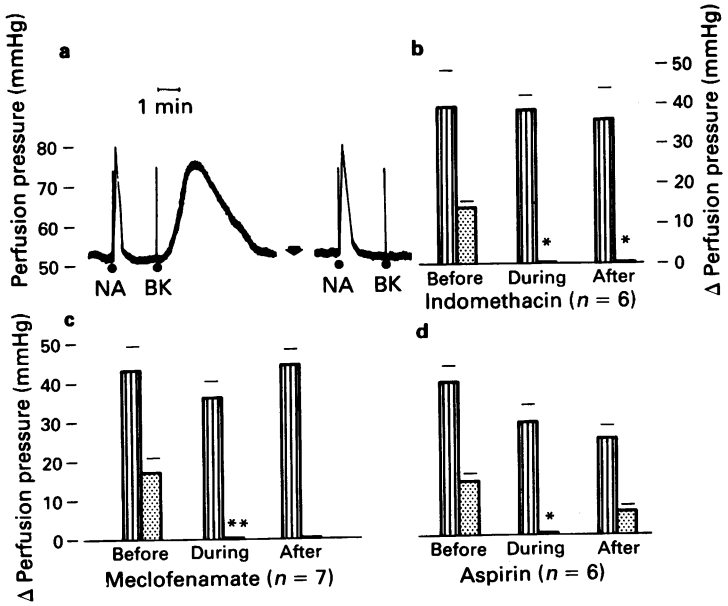

Figure 2 Inhibition of the bradykinin (BK)-induced vasoconstriction by indomethacin (b), meclofenamate (c) and aspirin (d). (a) A record of one experiment showing that bradykinin vasoconstriction was abolished by indomethacin while that to noradrenaline (NA) was unaffected. Shaded columns, responses to NA; stippled columns, responses to BK. ** $P < 0.001$, * $P < 0.01$.

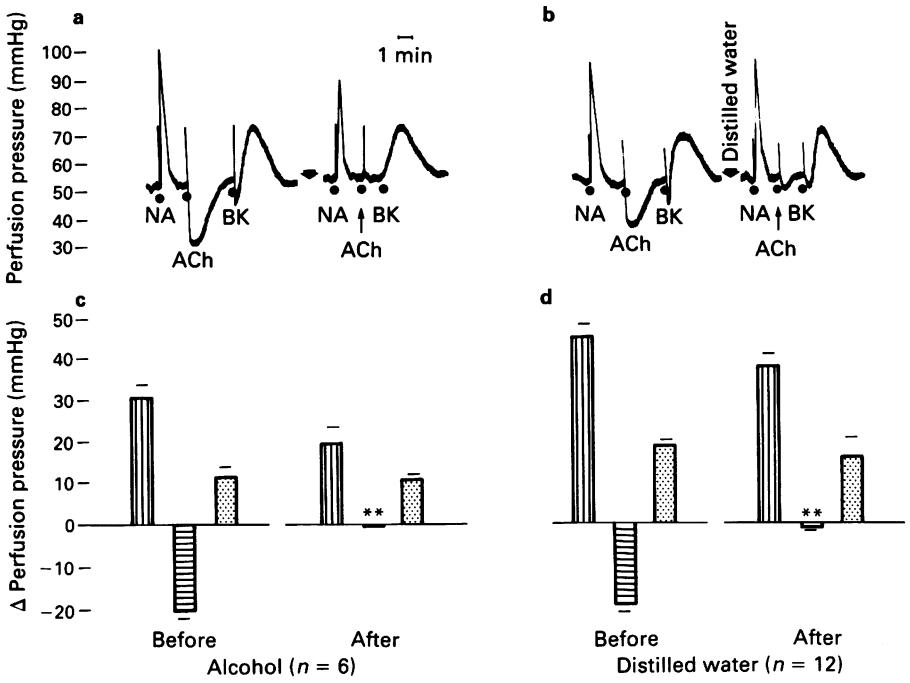

Figure 3 Effect of disruption of the endothelial layer on responses of mesenteric arteries to various substances. After the injection of alcohol (a and c) acetylcholine vasodilation was abolished while the response to bradykinin was little affected. Similar results after distilled water (b and d). ACh = acetylcholine 10^{-4} M, BK = bradykinin 4×10^{-5} M and NA = noradrenaline. Shaded columns, responses to NA; hatched columns, responses to ACh; stippled columns, responses to BK. ** $P < 0.001$.

after the addition of noradrenaline to the perfusion fluid, these agents induced a dose-dependent vasoconstriction (Figure 4).

Effect of thromboxane A_2/endoperoxide H_2 receptor blockade

To test whether thromboxane A_2 and/or endoperoxide H_2 were involved in the vasoconstriction indcued by bradykinin, SQ 29548 a potent blocker of their receptors was tested in 15 experiments (Figure 5). SQ 29548 (0.4 mg 100 ml^{-1}) significantly ($P < 0.001$) reduced bradykinin-induced vasoconstriction. As previously shown (Figure 4) PGE_1, PGE_2 and $PGF_{2\alpha}$ were able to constrict the mesenteric arteries precontracted with noradrenaline, thus it was possible that they were mediators of bradykinin vasoconstriction. To examine this, the effect of SQ 29548 was tested. As shown in Figure 6 a bolus dose of 0.04 mg 100 ml^{-1} that abolished bradykinin-induced vasoconstriction did not reduce the vasoconstriction induced by these prostaglandins, showing that different receptors are involved.

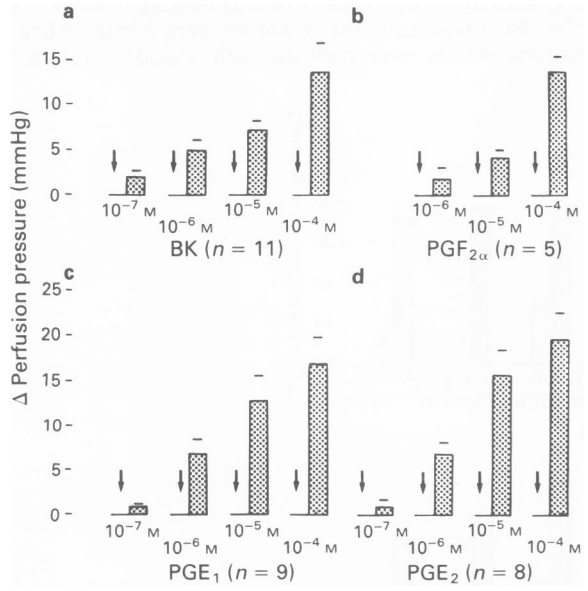

Figure 4 Vasoconstriction induced by (a) bradykinin (BK) and (b, c and d) prostaglandins. These drugs were without a vasoconstrictor effect before the mesenteric arteries were perfused with noradrenaline (NA). When the vessels were precontracted with NA dose-dependent vasoconstriction was induced by prostaglandin E_1 (PGE_1), PGE_2, $PGF_{2\alpha}$ and bradykinin. Arrows show lack of response before the addition of NA and stippled columns, responses after the addition of NA to the perfusing solution.

Effect of thromboxane A_2 synthesis inhibitors

Figure 7 shows the effect on bradykinin vasoconstriction of UK 38485 and imidazole, two well known inhibitors of thromboxane A_2 (TXA_2) synthesis. A solution of UK 38485

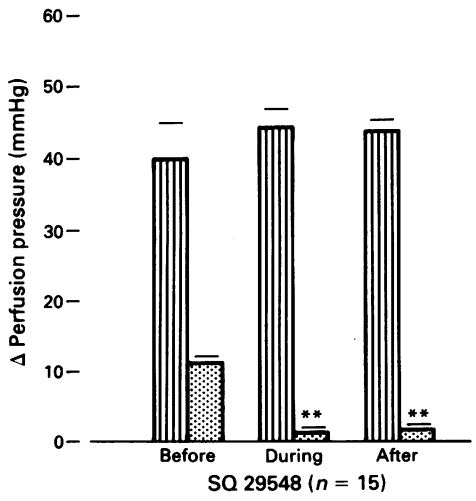

Figure 5 Effect of thromboxane A_2/endoperoxide H_2 receptor blockade. SQ 29548 significantly reduced bradykinin-induced vasoconstriction (stippled columns) while that to noradrenaline (shaded columns) was unaffected. ** $P < 0.001$.

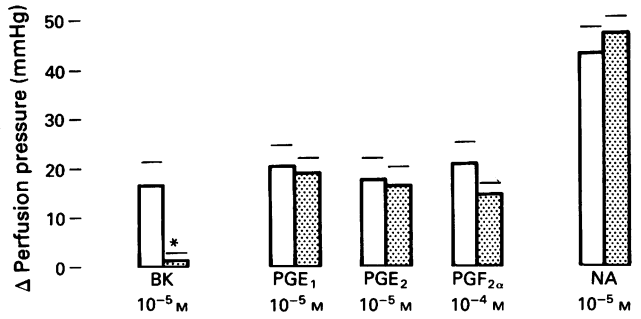

Figure 6 Effect of thromboxane A_2/endoperoxide H_2 receptor blockade on prostaglandin vasoconstruction. By perfusing with SQ 29548 solution, bradykinin (BK) vasoconstriction was almost completely abolished, while noradrenaline (NA); prostaglandin E_1 (PGE_1)-, PGE_2- and $PGF_{2\alpha}$-induced vasoconstrictor responses were not significantly reduced. Open columns, responses before the addition of SQ 29548; stippled columns, responses after the addition of SQ 29548; $n = 6$. * $P < 0.01$.

(0.8 mg 100 ml^{-1}) perfused through the mesenteric vessels for 15 min reduced bradykinin vasoconstriction, which partially recovered 30 min after the administration of the drug had ceased. On the other hand, imidazole (5×10^{-4} M) after 1 h of perfusion did not significantly reduce bradykinin-induced vasoconstriction.

Discussion

In the present study we investigated the mechanism of the vasoconstrictor effect of bradykinin on the mesenteric arteries of the rat precontracted with noradrenaline. Bradykinin is known to dilate most arteries (Regoli, 1984), although in rabbit isolated kidney and ear arteries it causes vasoconstriction (Barabé *et al.*, 1979).

The mechanism by which bradykinin induces vasodilatation or vasoconstriction on different vascular beds is unknown. The first step in the action of bradykinin involves the binding of bradykinin to specific receptors. Two classes of bradykinin receptors have been identified. BK_1-receptors mediate contraction of large arteries and veins and relaxation of peripheral resistance vessels. BK_2-receptors mediate relaxing effects on isolated arteries of the dog and others species, and contraction of veins and resistance arteries (Regoli & Barabé, 1980). In our experiments the use of a BK_1-receptor antagonist did not change the bradykinin-induced vasoconstriction in the mesenteric arteries, suggesting that BK_1-receptors are not involved.

Bradykinin is known to release prostaglandins in various tissues including veins and arteries. Bradykinin has been shown to release arachidonic acid in isolated tissues from

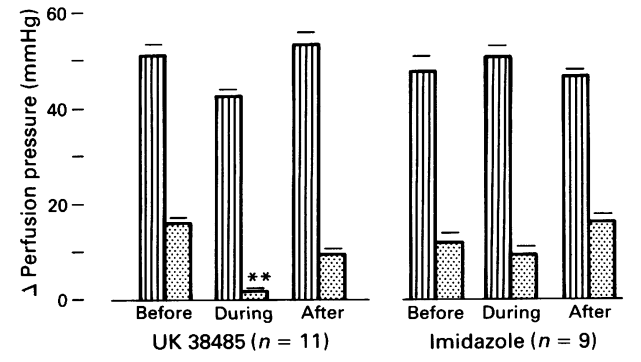

Figure 7 Effect of thromboxane A_2 synthesis inhibitors. (a) UK 38485 significantly reduced bradykinin (BK)-induced vasoconstriction. (b) Imidazole did not change significantly bradykinin-induced vasoconstriction. Shaded columns, responses to noradrenaline; stippled columns, responses to BK. ** $P < 0.001$.

rabbit (Needleman *et al.*, 1978). The possibility that bradykinin-induced vasoconstriction was mediated by products of the arachidonic cascade was investigated here. Inhibition of cyclo-oxygenase by indomethacin, meclofenamate and aspirin abolished bradykinin-induced vasoconstriction, strongly suggesting that it was mediated by prostaglandins.

The metabolism of arachidonic acid via the cyclo-oxygenase pathway results in the formation of various eicosanoids among them PGE_1, PGE_2, $PGF_{2\alpha}$, thromboxane A_2 and endoperoxide H_2 which cause vasoconstriction in various arteries (Toda & Miyazaki, 1978; van Holst *et al.*, 1982; Hayashi *et al.*, 1986; Lüscher & Vanhoutte, 1986).

In order to characterize the eicosanoid that mediates bradykinin-induced vasoconstriction we tested the possibility that PGE_1, PGE_2 and $PGF_{2\alpha}$, which produced vasoconstriction in our preparation, contributed to the bradykinin response. In one series of experiments we determined that SQ 29548, a potent antagonist of thromboxane A_2/endoperoxide H_2 receptors, greatly reduced the bradykinin-induced vasoconstriction while the pressor effect of PGE_1, PGE_2 and $PGF_{2\alpha}$ was only slightly affected. In other experiments, treatment with UK 38485 an inhibitor of thromboxane synthetase, reduced but did not abolish the bradykinin vasoconstriction and imidazole, another inhibitor of thromboxane synthesis, was without effect on bradykinin-induced vasoconstriction. One interpretation of these findings is that in mesenteric arteries the vasoconstrictor effect of bradykinin is mainly mediated by endoperoxides. The application of exogenous prostaglandin endoperoxides causes contraction of isolated vascular smooth muscle via an interaction with receptors which are shared with thromboxane A_2 (Coleman *et al.*, 1981; Saussy *et al.*, 1985). As suggested by the results of the present study, prostaglandin endoperoxides formed endogenously by bradykinin may stimulate the contraction of vascular smooth muscle. However, we cannot exclude the participation of thromboxane A_2 and other prostaglandins. Pertaining to this point, the results of a recent study suggest that prostaglandin endoperoxides contribute to the high blood pressure in rats made hypertensive by high salt intake and chronic infusion of angiotensin II (Mistry & Nasjletti, 1988).

In our experiments bradykinin was found to be without vascular effects on the relaxed branches of the mesentery artery, while it induced a dose-dependent vasoconstriction when these vessels were precontracted with noradrenaline. The question arises as to whether bradykinin releases the prostanoid only when the arteries are contracted with noradrenaline, or whether the prostanoid released is vasoconstrictor only in the presence of noradrenaline. Our data show that PGE_1, PGE_2 and $PGF_{2\alpha}$ constrict the mesenteric arteries only after they have been precontracted with noradrenaline, suggesting that the second alternative may be correct.

We tried to find out whether the synthesis of the eicosanoid mediating bradykinin-induced vasoconstriction occurs in the endothelium of these arteries. We compared the bradykinin-induced vasoconstriction in rat mesenteric arteries with intact endothelium to that in arteries after endothelium removal with distilled water or alcohol. The endothelium is not involved since bradykinin-induced vasoconstriction was not abolished after the endothelium was removed. Although we do not know in what cells synthesis of the vasoconstrictor prostanoids occurs, it seems that smooth muscle cells may be the target of bradykinin. In dog isolated arteries (coronary, coeliac, femoral, splenic, mesenteric, gastric) precontracted with noradrenaline, bradykinin induces a relaxation response not blocked by indomethacin which appears to be endothelium-mediated, while in cats and rabbits the relaxation does not depend on the endothelium and is blocked by cyclo-oxygenase inhibitors (Cherry *et al.*, 1982). On the other hand, in rabbit arteries, the relaxation induced by bradykinin does not depend on the endothelium and is blocked by cyclo-oxygenase inhibitors (Toda, 1974; Regoli *et al.*, 1982). This appears to indicate that in rabbit arteries the vasodilator prostanoid released by bradykinin may be formed outside the

endothelium, probably in smooth muscle. In cultured mesenteric smooth muscle cells bradykinin increases the synthesis of 6-keto PGE_1, a metabolite of PGI_2 and the effect is inhibited by acetylsalicylic acid (Takeuchi *et al.*, 1988).

In summary, this study demonstrates that bradykinin causes dose-dependent constriction of the rat mesenteric arteries precontracted with noradrenaline. The vasoconstrictor responses are not mediated by BK_1-receptors. Inhibition of cyclo-oxygenase abolishes the vasoconstrictor effect of bradykinin. The eicosanoid(s) responsible is formed outside the endothelium. We conclude that cyclic endoperoxides and/or thromboxanes appear to mediate bradykinin-induced vasoconstriction, although the participation of other eicosanoids cannot be excluded.

The authors thank Dr D. Regoli for the gift of (Leu^8), des Arg^9-BK a BK_1-receptor antagonist and Dr A. Nasjletti for supplying SQ 29548 and UK 38485. We also thank Dr L.M. Rinaldini for the assistance in the preparation of the manuscript. This work was supported by grants from CONICET, (PID N° 311160088 and PID N° 307400088).

References

BARABE, J., MARCEAU, F., THERIAULT, B., DROUIN, J.N. & REGOLI, D. (1979. Cardiovascular actions of kinins in the rabbit. *Can. J. Physiol. Pharmacol.*, **57**, 78–91.

CHERRY, P.P., FURCHGOTT, R.F., ZAWADZKI, J.V. & JOTHIANANDAS, D. (1982). Role of endothelial cells in relaxation of isolated arteries by bradykinin. *Physiological Sciences*, **79**, 2106–2110.

COLEMAN, R., HUMPHREY, P., KENNEDY, I., LEVY, G. & LUMLEY, P. (1981). Comparison of the actions of UK 6619, a prostaglandin H_2 analogue with those of prostaglandin H_2 and thromboxane H_2 on some isolated smooth muscle preparations. *Br. J. Pharmacol.*, **73**, 773–778.

HAYASHI, S., PARK, M.K. & KUEHL, J.J. (1986). Effect of prostaglandins and arachidonic acid on baboon cerebral and mesenteric arteries. *Prostaglandins*, **32**, 587–595.

LÜSCHER, T.T. & VANHOUTTE, P.M. (1986). Endothelium dependent contraction to acetylcholine in the aorta of spontaneously hypertensive rats. *Hypertension* **8**, 344–348.

MISTRY, M. & NASJLETTI, A. (1988). Role of pressor prostanoids in rats with angiotensin II-salt-induced hypertension. *Hypertension* **11**, 758–762.

McGREGOR, D.D. (1965). The effect of sympathetic nerve stimulation on vasoconstrictor responses in perfused mesenteric blood vessels of the rat. *J. Physiol.*, **177**, 21–30.

NEEDLEMAN, P., BRONSON, S.D., SYCHE, A., SIVAKOFF, M. & NICOLAU, K.C. (1978). Cardiac and renal prostaglandin I_2 biosynthesis and biological effect on isolated perfused rabbit tissue. *J. Clin. Invest.*, **61**, 839–849.

NORTHOVER, R.M. & NORTHOVER, B.J. (1970). The effect of vasoactive substances on rat mesenteric blood vessels. *J. Pathol.*, **101**, 99–108.

REGOLI, D. (1984). Neurohumoral regulation of precapillary vessels. The kallikrein-kinins systems. *J. Cardiovasc. Pharmacol.*, **6**, (Supp. 2), S401–S412.

REGOLI, D. & BARABE, J. (1980). Pharmacology of bradykinin and related kinins. *Pharmacol. Rev.*, **32**, 1–46.

REGOLI, D., MIZRAHI, J., D'ORLEANS-JUSTE, P. & CARANIKAS, S. (1982). Effects of kinins on isolated blood vessels. Role of endothelium. *Can. J. Physiol. Pharmacol.*, **60**, 1580–1583.

SAUSSY, D., MAIS, D., KNAPP, D. & HALUSHKA, P.V. (1985). Thromboxane A_2 and prostaglandin endoperoxide receptors in platelets and vascular smooth muscle. *Circulation*, **72**, 1202–1207.

SCHEFFE, H. (1963). The Analysis of Variance. New York: John Wiley.

TAKEUCHI, K., ABE, K., YASUJAMA, H., SATO, M., OMATA, K., KASAI, Y., KOMAZAHUA, M. & YOSHINAGA, K. (1988). The cellular action of bradykinin in vascular smooth muscle. *Am. J. Hypertens.*, **2**, 105A.

TODA, N. (1974). The action of vasodilating drugs on isolated basilar coronary arteries of the dog. *J. Pharmacol. Exp. Ther.*, **191**, 139–146.

TODA, N. & MIYAZAKI, M. (1978). Role of isolated dog cerebral and peripheral arteries to prostaglandins after application of aspirin and polyphoretin phosphate. *Stroke*, **9**, 490–497.

VAN HOLST, H., GRANSTROM, E., HAMARSTROM, S., SAMUELSON, B. & STEINER, L. (1982). Effect of Leucotrienes C_4D_4, prostaglandins and thromboxane A_2 on isolated human cerebral arteries. *Acta Neurochir.*, **62**, 177–185.

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Effects of organophosphorus anticholinesterases on nicotinic receptor ion channels at adult mouse muscle endplates

John E.H. Tattersall

Biology Division, Chemical Defence Establishment, Porton Down, Salisbury, Wiltshire SP4 0JQ

1 The effects of a range of organophosphorus anticholinesterases on the nicotinic acetylcholine receptor ion channel at the adult mouse muscle endplate were investigated by use of single-channel recording techniques. Diisopropylfluorophosphate (DFP), sarin and soman had no effect on open times at concentrations of up to 100 μM, but ecothiopate (Eco) and O-ethyl S-[2-(diisopropylamino)ethyl]methyl phosphonothiolate (VX) were found to have voltage- and concentration-dependent open channel-blocking actions at concentrations of 1–50 μM. In addition to its channel-blocking action, Eco (50 μM) had a weak agonist effect: it is suggested that this may be attributable to thiocholine produced by hydrolysis of Eco.

2 Rate constants for blockade by Eco and VX were determined according to a sequential model. The greater voltage-dependence of the block by Eco was due to a greater voltage sensitivity of the blocking rate constant compared to VX: the voltage-dependence of the unblocking rate constant was similar for both compounds.

3 In control recordings, the frequency of channel opening declined exponentially with time after formation of the gigaseal. Sarin and soman both increased the rate of this decline, indicating that they accelerated the rate of desensitization of the receptors. Eco and VX reduced the initial frequency of opening, which may have been due to enhancement of a fast phase of desensitization during gigaseal formation, or to blockade of closed channels.

4 It is concluded that the direct actions of organophosphates on nicotinic receptor ion channels are of little importance for their toxicity under normal conditions, since they occur only at much higher concentrations than those which cause inhibition of acetylcholinesterase. Such actions may become apparent, however, when therapies against the anticholinesterase effects of organophosphates increase their lethal dose sufficiently. These direct actions should also be taken into account when the effects of organophosphates on cholinergic transmission are studied.

Introduction

A wide range of compounds has been found to block non-competitively the flow of current through acetylcholine-activated ion channels at vertebrate neuromuscular junctions (Lambert *et al.*, 1983; Barrantes, 1988). These include local anaesthetics, tricyclic antidepressants, histrionicotoxin and its derivatives, the hallucinogenic agent phencyclidine (PCP), antimuscarinics, antinicotinics and anticholinesterases. Such channel blockers are believed to interact with one or more non-competitive inhibitory binding sites which become available when the channel opens (Adams, 1987).

The study described here used the patch clamp technique to investigate the direct interactions of a number of organophosphorus esters with the nicotinic cholinoceptor ion channel. These agents are irreversible inhibitors of cholinesterases, and intoxication with them can lead to a wide range of symptoms resulting from the accumulation of released acetylcholine at nicotinic and muscarinic synapses. The organophosphates soman, diisopropylfluorophospate (DFP) and O-ethyl S-[2-(diisopropylamino) ethyl]methylphosphonothiolate (VX), in addition to their well-known disruption of cholinergic transmission through the inhibition of acetylcholinesterase, have been reported to have direct effects on the nicotinic cholinoceptor in amphibian muscle (Kuba *et al.*, 1974; Albuquerque *et al.*, 1984; 1985; Rao *et al.*, 1987). These include channel blocking, desensitizing and agonist activities. Such additional actions of organophosphates may complicate studies of the effects of acetylcholinesterase inhibition on cholinergic transmission, and it has been suggested that they may also be significant for the development of therapies for the treatment of poisoning by these compounds (Albuquerque *et al.*, 1985).

The present study examined the effects of a range of organophosphates on single acetylcholine receptor ion channels at mammalian skeletal muscle endplates, in order to evaluate the possible contribution of such effects to the toxicity of these compounds. The preparation used was the dissociated flexor digitorum brevis muscle from the mouse, a species for which extensive toxicological information is available. A preliminary report of some of this work has been published previously in abstract form (Tattersall, 1989).

Methods

Flexor digitorum brevis muscles were dissected from the hind limbs of adult mice (1.5–3 months old). These were dissociated into single fibres by an enzymatic method previously described by Brehm & Kullberg (1987). Briefly, the muscles were incubated for 2 h at 37°C in Dulbecco's modification of Eagles medium (Flow Laboratories) containing 3 mg ml^{-1} collagenase (Sigma, type I). They were then washed and incubated for a further 15 min at 37°C in Ca^{2+}-free minimum essential medium (MEMS, Flow Laboratories) containing 0.5 mg ml^{-1} trypsin (Sigma, Type XI) and 0.2 mg ml^{-1} disodium edetate (EDTA). The muscles were dissociated by gentle trituration through a Pasteur pipette in saline which had the following composition (mM concentrations): NaCl 120, KCl 1, $CaCl_2$ 1, HEPES 10, pH 7.2 with NaOH. All experiments were performed at room temperature (20°C).

Conventional intracellular recordings were made in order to determine the physiological condition of dissociated fibres. Intracellular micropipettes were filled either with 3 M KCl or with 5 M potassium acetate, and had resistances of 10–20 MΩ. A bridge amplifier was used to inject current through the recording electrode.

Patch pipettes were pulled from borosilicate glass to a tip diameter of 1–2 μm and filled with physiological saline containing acetylcholine, either alone or in combination with an organophosphate. Single channel currents were recorded in cell-attached patches at or near the endplate region with an Axopatch 1-B amplifier (Axon Instruments, Burlingame, CA,

U.S.A.), sampled continuously at 44 kHz by a pulse code modulator and stored on videotape. The signals were played back through a low-pass filter (4-pole Bessel, 3 dB cutoff 5 kHz), and digitized at 20 Hz by a Labmaster interface (Scientific Solutions, Solon, OH, U.S.A.) for subsequent computer analysis by the pCLAMP, IPROC and LPROC programmes (Axon Instruments). Sections of data in which more than one channel was open were excluded from the analysis. All resolvable sojourns at the baseline level (duration greater than 100 μs) were treated as true closings. Histograms of open and closed durations were fitted with one or more exponential functions by the least squares method. At the end of each recording, the resting potential of the muscle fibre was measured by breaking the membrane patch to record in the whole cell mode. This value was later confirmed from the current-voltage curve for the channel, assuming the reversal potential to be 0 mV. Patches were only accepted for analysis if the two values agreed within 10 mV.

Burst analysis

Analysis of burst durations was performed in patches with relatively low open probabilities, i.e. those in which the mean interburst closed interval was greater than 100 ms. In these patches, the bursts of openings were well separated and readily distinguishable. The burst terminator value (the threshold level used to discriminate between intra- and interburst closed durations) used during analysis was at least 7 times the mean intraburst closed time determined from exponential fits of closed duration histograms (Alkondon & Albuquerque, 1989). Total open time per burst was calculated by multiplying the duration of each burst by the open probability during that burst. Histograms of burst durations and total open time per burst were fitted with exponential functions using a maximum likelihood method.

Model of channel block

A simple sequential model of channel block was used to interpret the data. This assumed that the blocking drug binds reversibly to the conducting state of the channel to form a species with little or no conductance (Adams, 1976; Adler *et al.*, 1978; Neher & Steinbach, 1978). The model may be represented by the following reaction scheme:

$$nA + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A_nR \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_nR^* \underset{D}{\overset{D\ G.c}{\rightleftharpoons}} A_nRD \quad (F)$$

where A is the agonist molecule, R is the nicotinic cholinoceptor ion channel, n is the number (usually considered to be two) of agonist molecules bound per nicotinic cholinoceptor, A_nR is the agonist-bound closed state, A_nR^* is the open state and A_nRD is the blocked state. The closed and blocked states are usually indistinguishable on the basis of conductance, but may be distinguished by their temporal characteristics.

According to this model, the mean open duration is equal to $1/(\alpha + G.c)$, where G is the forward rate constant for the blocking reaction and c is the concentration of the drug. Thus, a plot of the reciprocal of the open time versus the concentration of the blocking drug yields a straight line, the slope of which is equal to G and the intercept of which is equal to α. As the unblocking reaction is the only step leading away from the blocked state, the unblocking rate constant, F, may be estimated as the inverse of the mean duration of the blocked state (Neher & Steinbach, 1978).

Statistics

Data are expressed as mean $\pm$ s.d. Groups of data were compared by Student's t test. A difference between means was considered significant if the probability of it being due to random variation, P, was less than 0.05.

Drugs

Ecothiopate iodide (phospholine eyedrops) was purchased from Ayerst Laboratories (Andover, Hants., UK). Acetylcholine and diisopropylfluorophosphate were purchased from Sigma. Sarin, soman and VX (>98% pure) were synthesized at this Establishment and stored as 5 mg ml^{-1} solutions in isopropanol. They were diluted to final concentrations with physiological saline. Control experiments for these compounds were performed by use of acetylcholine dissolved in appropriate dilutions of isopropanol in saline. The mean open times were not changed significantly by the concentrations of isopropanol used (up to 0.2%). All drugs were applied through the interior of the patch pipette. Since all recordings were made in the cell-attached mode, only one drug concentration was applied to each patch.

Results

Resting potential

The resting potential of muscle fibres measured with intracellular microelectrodes was -51.2 ± 8.3 mV ($n = 41$). Intracellular current injection elicited overshooting action potentials, which were accompanied by contractions of the fibre. Measurements of resting potentials made during patch clamp experiments gave a value of -51.9 ± 13.0 mV ($n = 47$), which was not significantly different from that measured with intracellular electrodes. None of the organophosphates tested significantly altered the resting potential.

Acetylcholine-activated channel openings

Acetylcholine (300 nM) in the patch pipette induced channel openings which appeared as square wave pulses, in which the noise level in the open state remained similar to that during the closed state (Figure 1). The single channel amplitude increased linearly with membrane potential at all voltages tested (i.e. up to 10 pA). The single channel conductance, calculated from the current-voltage relationship, was 60.5 ± 13.0 pS ($n = 13$). A second type of channel with a much lower conductance was observed in about half of the patches (52 out of 93), but constituted less than 5% of the total numbers of openings in those patches. Due to the low frequency of these events, no attempt was made to analyse this channel.

Effects of organophosphates on ACh-activated channel openings

Of the compounds tested, only Eco and VX had any detectable effect on the open times of acetylcholine-activated channels. Sarin, soman and DFP had no discernible effect at concentrations up to 100 μM. In the presence of Eco, channel openings were chopped into bursts of much shorter openings by brief transitions to the closed channel current level (Figure 1). With VX, the channel openings were again reduced in duration, but the intraburst transitions to the closed channel current level were longer with Eco, resulting in bursts of widely-spaced short openings (Figure 1).

In control patches, the majority of open times of the 60 pS channel showed a single exponential distribution, but there was a slight excess of short openings, as described by Henderson *et al.* (1987) (Figure 2). The mean open time increased exponentially as a function of membrane voltage, with an e-fold increase per 73 ± 18 mV of hyperpolarization (see Figure 3). Closed duration histograms could be fitted by a single exponential (Figure 2), and there was no evidence of a second, faster exponential function in the closed time distribution. The mean closed time varied greatly between patches, from about 100 ms to several seconds. It also tended to increase with time during each recording, presumably due to desensitization of the receptors.

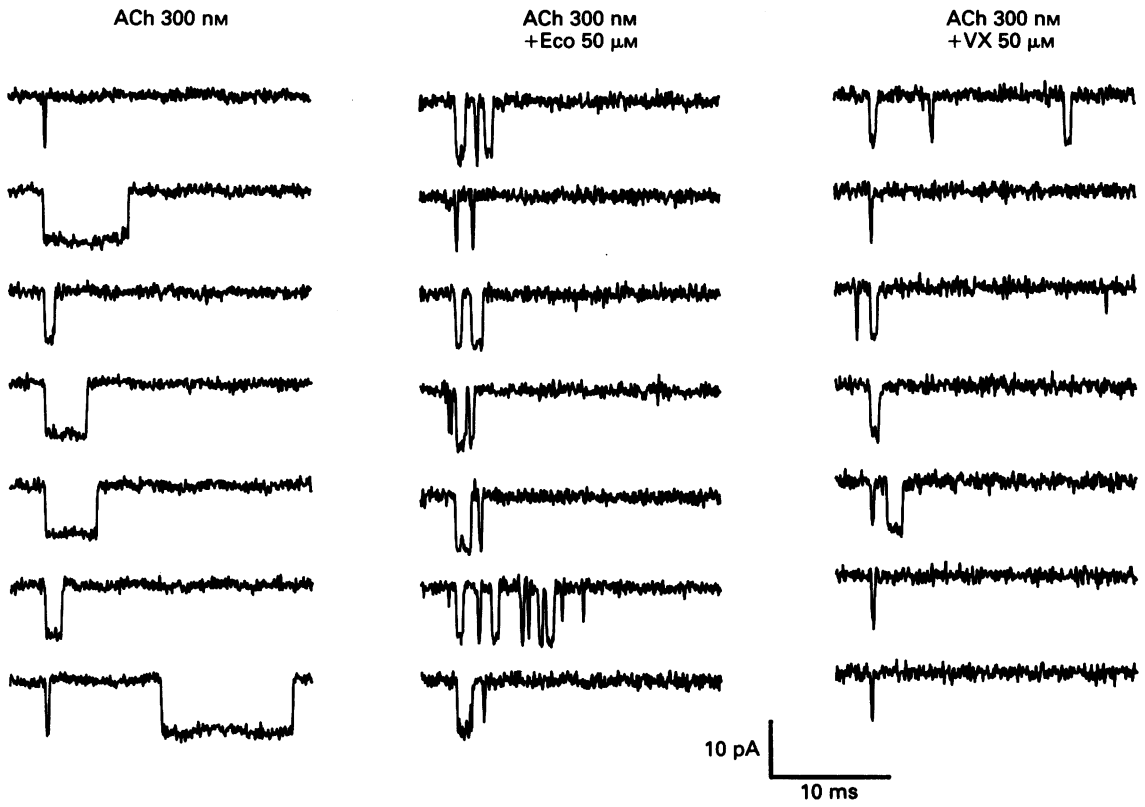

Figure 1 Samples of acetylcholine (ACh)-activated single channel currents in control patches (left) and in the presence of 50 μM ecothiopate (Eco, centre) or 50 μM O-ethyl S-[2-(diisopropylamino) ethyl]methyl phosphonothiolate (VX, right). The traces show consecutive openings captured by an event detector. The membrane potential was −100 mV in each patch.

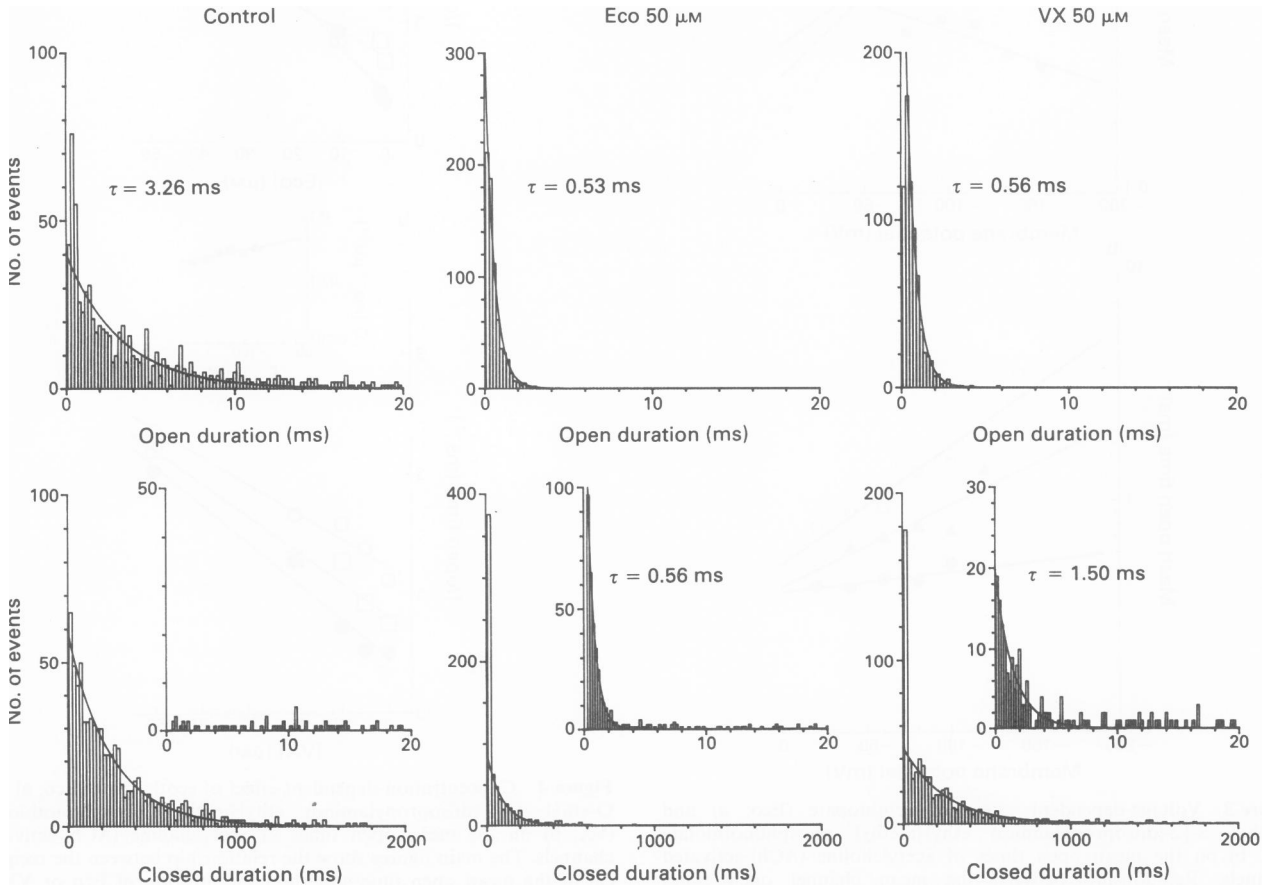

Figure 2 Open (top) and closed (bottom) duration histograms for single acetylcholine (ACh)-activated channels in control conditions (left) and in the presence of 50 μM ecothiopate (Eco, centre) or 50 μM O-ethyl S-[2-(diisopropylamino) ethyl]methyl phosphonothiolate (VX, right). The insets show the events in the first 20 ms bin of the closed duration histogram above on an expanded time scale. τ values are the time constants of the single exponential functions fitted by least squares regression analysis. The first bin in each histogram was excluded from the regression

In the presence of Eco or VX, the open time histograms could again be fitted by a single exponential function at all concentrations tested, but the mean open time was shorter than in control patches (Figure 2). With both compounds, the closed time distributions showed an additional fast exponential component compared with controls (Figure 2), which was related to the intraburst closures.

The effect of Eco and VX on the open time was concentration-dependent, such that increasing concentrations of either compound caused a progressive loss in the voltage-sensitivity of the mean channel open time (Figure 3). The effect was more marked with Eco, which at a concentration of 50 μM reversed the voltage-dependence of the open time. The reduction in open time produced by Eco was thus small at the normal resting potential of the fibres, but became more pronounced as the membrane was hyperpolarized. In contrast, VX caused a marked shortening of the mean open time at all membrane potentials tested.

As predicted by the simple sequential model, the inverse of the mean open time was linearly related to the concentration of Eco or VX, up to a concentration of 50 μM, and the slope of this relationship was used to determine the forward rate constant of the channel-blocking step, G (Figure 4). In the case of Eco, the slope increased as the membrane was hyperpolarized, indicating that G was markedly voltage-dependent. With VX, however, the slope changed little with increasing hyperpolarization, and G therefore showed much less voltage-dependence.

According to the model, the fast exponential which appeared in the closed time distribution in the presence of Eco or VX described the duration of the blocked state of the channel. Closed durations briefer than 20 ms were well fitted by single exponentials (see Figure 2), and the time constants of these fast exponentials were used to calculate F, the backward rate constant for the blocking reaction. This rate constant showed a similar voltage dependence for both Eco and VX, but was approximately 3.5 times faster for Eco than for VX at all membrane potentials (Figure 5).

Knowing the values of F and G, and their voltage-dependence, it was possible to calculate K_D, the dissociation

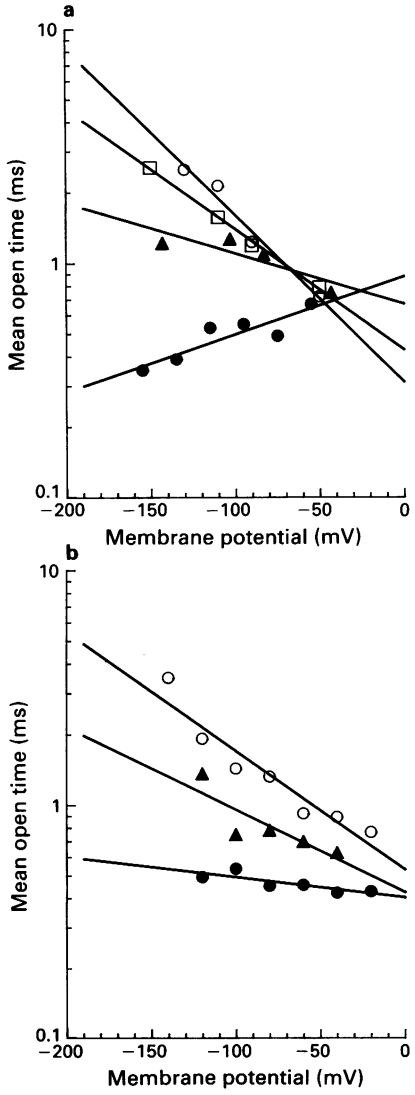

Figure 3 Voltage-dependent effect of ecothiopate (Eco, a) and O-ethyl S-[2-(diisopropylamino) ethyl]methyl phosphonothiolate (VX, b) on the mean open times of acetylcholine (ACh)-activated channels. Relationship between the mean channel open times (logarithmic scale) and membrane potentials from single channel recordings obtained with ACh (0.3 μM) either alone (○) or together with 1 (□), 10 (▲) or 50 (●) μM Eco or VX. Each point represents the mean of values from at least three patches. Lines were fitted by linear regression.

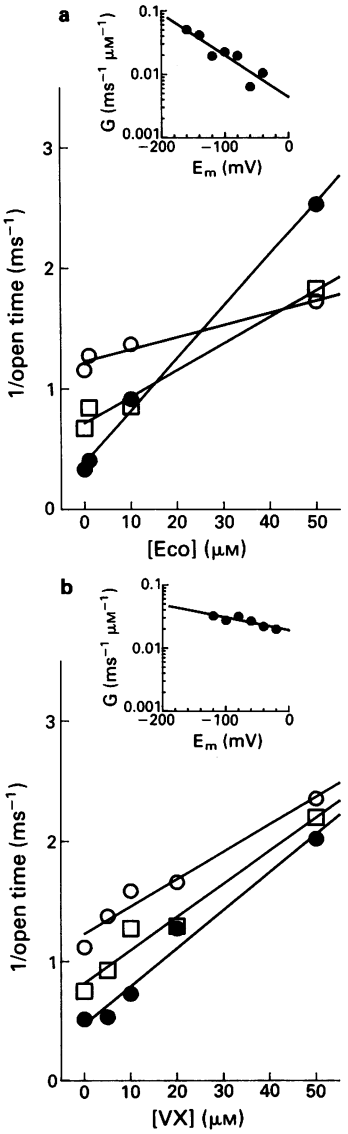

Figure 4 Concentration-dependent effect of ecothiopate (Eco, a) and O-ethyl S-[2-(diisopropylamino) ethyl]methyl phosphonothiolate (VX, b) on the mean open times of acetylcholine (ACh)-activated channels. The main figures show the relationship between the reciprocal of the mean open time and the concentration of Eco or VX at membrane potentials of −40 (○), −80 (□) and −120 (VX) or −140 (Eco) (●) mV. Each point represents the mean of values from at least three patches. Insets show the relationship between the blocking rate constant, G, (logarithmic scale) and membrane potential (E_m). Lines were fitted by linear regression.

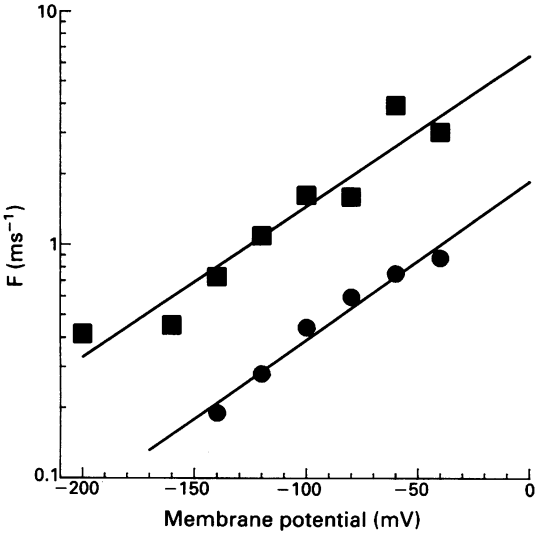

Figure 5 Relationship between the unblocking rate constant, F, (logarithmic scale) and membrane potential for ecothiopate (■) and O-ethyl S-[2-(diisopropylamino) ethyl]methyl phosphonothiolate (●). Lines were fitted by linear regression.

constant for the blocked state, and its voltage dependence. The voltage dependence of K_D can be described by a Boltzmann distribution (Woodhull, 1973; Adler *et al.*, 1978; Neher & Steinbach, 1978), in which the argument of the exponential is

$$-ze\delta V/kT,$$

where ze is the charge on the blocking drug molecule, δ is the fraction of the membrane potential sensed by the drug molecule as it reaches its binding site, k is Boltzmann's constant and T is the absolute temperature. The values obtained for F, G, K_D and δ are given in Table 1. The voltage dependence of K_D, and therefore the value of δ, for Eco was approximately 1.5 times that for VX. This was due solely to the greater voltage sensitivity of G for Eco, since the voltage-dependence of F was the same for both compounds.

Table 1 Constants for open channel-block by ecothiopate and O-ethyl S-[2-(diisopropylamino) ethyl]methyl phosphonothiolate (VX)

	Ecothiopate	*VX*
G, $ms^{-1}\,\mu M^{-1}$	$0.00446 \times e^{(mV/-66.3)}$	$0.0193 \times e^{(mV/-217.2)}$
F, ms^{-1}	$6.45 \pm e^{(mV/67.3)}$	$1.86 \times e^{(mV/64.3)}$
K_D (=F/G), μM	$1458 \times e^{(mV/33.4)}$	$96.5 \times e^{(mV/49.6)}$
δ	0.76	0.51

The dissociation constant of the blocked conformation, K_D, was calculated as F/G. The electrical distance, δ, was calculated from the voltage-dependence of K_D, using the Boltzmann equation described in the text.

Effect of organophosphates on burst duration

According to the simple sequential model, the mean burst duration should increase linearly with the concentration of the blocking drug, while the mean total open time per burst should remain constant, since the only way the channel can become unblocked is via the open state (Colquhoun & Hawkes, 1983; Adams, 1987). In order to test the model, therefore, burst durations and total intraburst open times were measured in the presence of various concentrations of Eco and VX. Histograms of burst duration and intraburst open time were fitted with single exponential functions, and the results are shown in Figure 6. At a membrane potential of −40 mV, the mean open time per burst remained constant as the concentration of Eco or VX was increased from 0 to 50 μM; however, the mean burst duration also remained constant as the concentration of either drug was increased. This deviation from the predictions of the model was more evident at a membrane potential of −120 mV, when both the mean burst duration and the mean total open time per burst actually decreased as the concentration of either Eco or VX increased. Such behaviour indicates that there is another, voltage-dependent, route by which the receptor can leave the blocked state, other than by going back through the open state.

Effect of organophosphates on channel opening frequency

In order to test whether any of the organophosphates had other actions which might affect the frequency of opening endplate channels, such as desensitization, bursts of channel openings were counted over 30 s periods. The initial frequency of opening, measured as the number of bursts in the first 30 s after formation of the gigaseal, was compared between treatments using t tests. The initial frequency varied widely between patches, but significant decreases were found with Eco (100 μM) and VX (50 μM). None of the other organophosphates affected the initial opening frequency (Table 2). In order to compare the rates of decrease of channel opening, the frequency was expressed as a percentage of the maximum frequency for each patch, and plotted on a logarithmic scale against time. The slope of the plot was found by linear regression. In control patches, the frequency of channel openings decreased exponentially with time after the formation of the gigaseal (Figure 7). The points before 1 min appeared to deviate from the straight line fit, suggesting there may have been a second, faster phase of decrease prior to this time. Of the five organophosphates studied, only sarin and soman caused any significant increase in the rate of decay of opening frequency (Figure 7 and Table 2). The results for soman showed a deviation from the straight line fit at times before about 3 min, providing further evidence of a second, faster phase of decay.

Table 2 Effects of organophosphates on channel opening frequencies

	Initial frequency (openings per 30 s)	*Slope of log (frequency) versus time*	*Time constant of decrease* (min)
Control (n = 15)	526 ± 351	−0.072 ± 0.040	10.7 ± 8.6
DFP 100 μM (n = 5)	304 ± 158	−0.078 ± 0.036	6.2 ± 1.9
Eco 100 μM (n = 5)	117 ± 62**	−0.032 ± 0.022	17.8 ± 8.5
Sarin 100 μM (n = 6)	269 ± 178	−0.174 ± 0.043***	2.6 ± 0.7*
Soman 100 μM (n = 6)	817 ± 471	−0.145 ± 0.043***	3.2 ± 0.7*
VX 50 μM (n = 7)	144 ± 101**	−0.055 ± 0.019	7.8 ± 4.4

DFP = diisopropylfluorophosphate; Eco = ecothiopate; VX = O-ethyl S-[2-(diisopropylamino) ethyl]methyl phosphonothiolate. Figures are mean ± s.d. Significant difference from control values (t test) are indicated by asterisks: * $P < 0.05$; ** $P < 0.02$; *** $P < 0.002$.

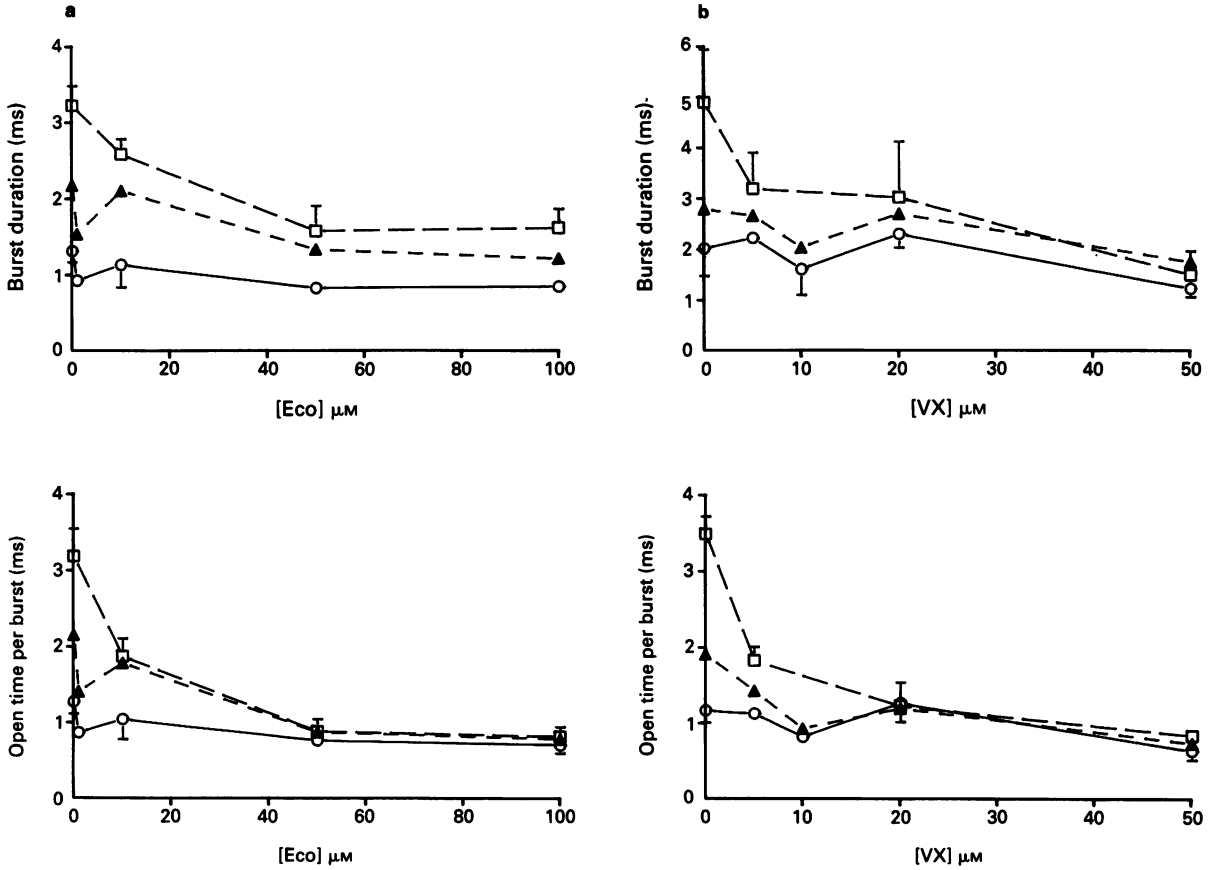

Figure 6 Concentration-dependent effects of ecothiopate (Eco, a) and O-ethyl S-[2-(diisopropylamino) ethyl]methyl phosphonothiolate (VX, b) on mean burst duration (top) and mean total open time per burst (bottom) at membrane potentials of −40 (○), −80 (▲) and −120 (□) mV. Each point represents the mean of at least 4 patches. Error bars show standard deviations.

Effect of organophosphates on channel conductance

None of the compounds tested was found to cause any statistically significant change in the conductance of single ACh-activated channels.

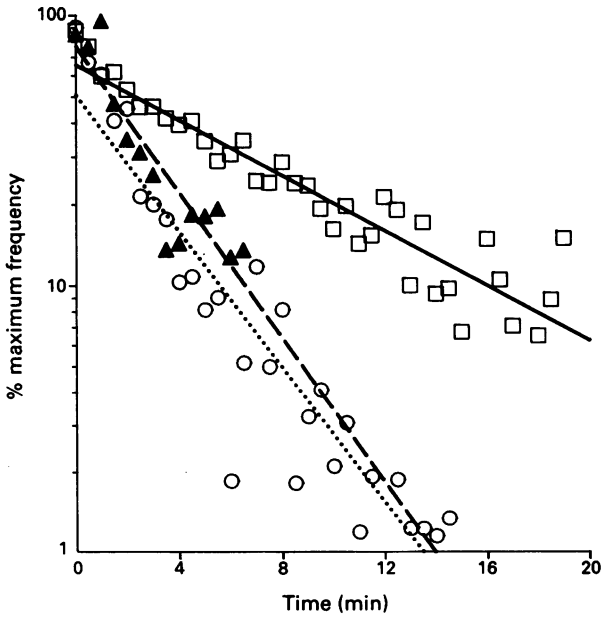

Figure 7 Time-dependent decrease in the frequency of channel opening (logarithmic scale) following gigaseal formation in control patches (□) and in the presence of 100 μM sarin (▲), or 100 μM soman (○). Each point represents the mean of at least five patches. Lines were fitted by linear regression.

Organophosphate-induced channel openings

Of the compounds tested, only Eco showed any agonist activity as determined by its ability to induce openings of single channels in the absence of ACh. Channel openings induced by 50 μM Eco were much less frequent than those activated by 300 nM ACh (mean closed time 3–20 s). They showed similar open times, bursting behaviour and conductance to those observed in the presence of ACh and Eco together.

Discussion

In the dissociated muscle fibre preparation, the presynaptic terminals and the synaptic acetylcholinesterase have been removed during the enzymatic dissociation (Hall & Kelly, 1971; Betz & Sakmann, 1973), allowing postsynaptic actions of drugs to be observed in the absence of any other effects. None of the organophosphates studied (up to a concentration of 100 μM) was found to have any effect on the resting potential of the fibres, suggesting that they were unable to produce any appreciable activation of ion channels. Of the five agents tested, only Eco and VX showed any marked open channel-blocking action in the range of concentrations used. Both of these compounds have a nitrogen-containing side chain, in contrast to sarin, soman and DFP, which have a fluorine atom in this position. This is consistent with the observation that open channel blockers and other noncompetitive antagonists of the nicotinic cholinoceptor are generally amines or quarternary ammonium compounds (Aracava *et al.*, 1988). Kuba *et al.* (1973; 1974) reported that high concentrations (0.25–1.1 mM) of DFP both decreased the amplitude and shortened the falling phase of the endplate current in frog sartorius muscle, and they suggested that this was due to direct effects on the ion channel of the nicotinic cholinoceptor.

However, no such effects of DFP were found at the lower concentrations (up to 100 μM) used in the present study.

The results obtained with Eco and VX showed a blockade of the open state of the nicotinic cholinoceptor ion channel. Analysis of burst durations, however, revealed inconsistencies with the simple sequential model proposed by Adams (1976), Adler *et al.* (1978) and Neher & Steinbach (1978): the mean burst duration and the mean total open duration per burst both decreased as the concentration of Eco or VX was increased. Similar behaviour has been reported for the local anaesthetic QX-222 (Neher, 1983) and for the bispyridinium compound SAD-128 (Alkondon & Albuquerque, 1989). These observations suggest that the blocked open channel is not an end-state as proposed by the simple sequential model. The discrepancy does not invalidate this model, but merely requires the addition of a further reaction step leading out of the blocked state. The additional state must be relatively long-lived compared with the blocked state in order to cause the observed reduction in mean burst duration, and it is still possible to distinguish the blocked state as the short closing within bursts. Furthermore, as there are still only two pathways leading away from the open state, it therefore remains valid to use the model to derive the rate constants for the forward and backward steps of the blocking reaction as described in the Methods. The forward rate constants derived from this model for Eco and VX were very similar, 2.73×10^7 and $3.36 \times 10^7\,\text{M}^{-1}\text{s}^{-1}$, respectively, at -120 mV membrane potential. These values are within the range reported for a wide variety of blocking drugs (Dreyer, 1982; Lambert *et al.*, 1983).

Both Eco and VX reduced the voltage-dependence of the mean open time of ACh-activated channels, but this effect was more marked with Eco. The greater voltage-dependence of the blockade by Eco was wholly explained by the greater voltage sensitivity of G (the forward rate constant) for Eco compared to that for VX, since the voltage sensitivity of F (the backward rate constant) was the same for both compounds. The difference in the voltage sensitivity of G between the two compounds is to be expected, since the quaternary Eco molecule carries a positive charge, whereas the tertiary VX molecule is predominantly uncharged at physiological pH (Epstein *et al.*, 1974). What is surprising is the similarity in the voltage-dependence of F for the two compounds, since the unblocking rates for other uncharged drugs are independent of membrane potential (Lambert *et al.*, 1983). At least two alternative explanations seem possible for this observation. Firstly, the voltage-dependence of the unblocking step could result from the influence of the transmembrane electrical field on the nicotinic cholinoceptor ion channel, rather than on the blocking molecule. This would suggest that there is an allosteric binding site for the blocking drug outside of the ionic channel, which is unlikely, since the conformational changes involved would need to occur at improbably large rates, and their voltage-dependence would be the opposite of that associated with normal channel closing (Neher & Steinbach, 1978).

An alternative explanation is that VX may gain a positive charge by interaction with the ion channel. This idea seems more likely, as the lone pair of electrons on the tertiary nitrogen atom of VX makes it susceptible to protonation (Epstein *et al.*, 1974). It is therefore possible that, as the VX molecule approaches its noncompetitive binding site, it gains a positive charge by protonation of the nitrogen atom. The species which leaves the channel during the unblocking step would then behave in a similar way to the positively-charged Eco molecule under the influence of the membrane electrical field.

Whilst there have been no previous single-channel studies on the effects of quaternary organophosphorus compounds such as Eco, channel-blocking by VX has been demonstrated at frog endplate nicotinic cholinoceptors (Albuquerque *et al.*, 1985; Rao *et al.*, 1987; Aracava *et al.*, 1988). VX has also been reported to be a potent inhibitor of [^{3}H]-perhydrohistrionicotoxin binding to the nicotinic cholinoceptor ion channel in both closed and open states, but predominantly in the latter (Eldefrawi *et al.*, 1985). This is consistent with the open channel-blocking action described here and in the frog. Interestingly, Eco does not displace the binding of phencyclidine (PCP) to the nicotinic cholinoceptor (Henderson *et al.*, 1983; 1988; Bakry *et al.*, 1988), even though it was found to be a potent blocker of open channels in the present study.

Bakry *et al.* (1988) and Eldefrawi *et al.* (1988), in experiments on α-bungarotoxin binding and ^{22}Na uptake, suggested that DFP, Eco, soman and VX may enhance desensitization of the nicotinic cholinoceptor in *Torpedo* membranes. In the single-channel studies reported here, sarin and soman increased the rate of rundown of channel opening, but had no effect on the initial frequency. In contrast, a significant reduction of the initial frequency of opening was found with Eco and VX, but neither of these compounds increased the rate of decay of the opening frequency. These results indicate that sarin and soman accelerated desensitization of the nicotinic cholinoceptor, and suggest that Eco and VX may have enhanced a faster phase of desensitization (Ochoa *et al.*, 1989) which was largely complete by the time the gigaseal had formed; alternatively, Eco and VX may have acted on closed channels to reduce their probability of opening. These effects were rather small in magnitude, however, and were seen only at high concentrations of the organophosphates (100 μM). It could be argued that the low resting potential of the dissociated fibres (-50 mV compared with -75 mV in undissociated fibres) may have resulted in an already high degree of receptor desensitization, which could have masked the effects of the organophosphates. However, a considerable decline in the opening frequency was observed in many patches following gigaseal formation, which makes it unlikely that the receptors were initially in a highly desensitized state.

Bakry *et al.* (1988) reported that soman and Eco were partial agonists of the *Torpedo* nicotinic cholinoceptor. The present experiments confirmed the weak agonist action of Eco, but no such activity could be demonstrated for soman or any of the other compounds used. In aqueous solution, Eco undergoes hydrolysis to thiocholine, which is an agonist at the nicotinic cholinoceptor (Amitai & Chapman, 1988). It is quite possible, therefore, that the agonist action observed here may have been due to the presence of this hydrolysis product, rather than an effect of the Eco itself.

It has been proposed that direct effects of organophosphorus esters on ion channels may contribute to their toxicity (Albuquerque *et al.*, 1985; Aracava *et al.*, 1988; Bakry *et al.*, 1988). All of the direct actions that have been demonstrated, however, are readily reversible, in contrast to the irreversible nature of the anticholinesterase action of these agents, and occur at much higher concentrations than those which inhibit acetylcholinesterase. For example, the IC_{50} values for inhibition of *Torpedo* acetylcholinesterase by these agents, measured after a 30 min exposure, range between 10 and 40 nM (Bakry *et al.*, 1988). This is three orders of magnitude lower than the dissociation constants for reversible channel block by Eco and VX (Table 3). Other direct actions which have been reported, such as enhancement of desensitization, also occur only at concentrations greater than the micromolar range (Aracava *et al.*, 1988; Bakry *et al.*, 1988; Eldefrawi *et al.*, 1988). The LD_{50} values which have been measured for the organophosphates show a good correlation with the second order rate constant for inhibition of acetylcholinesterase, K_i (Table 3), consistent with this being the most important toxic effect. It is possible, however, that direct effects on ion channels could modify the toxicity of certain compounds, and these effects could be significant in cases where the lethal dose of organophosphate has been raised by therapy with anticholinoceptor drugs and acetylcholinesterase reactivators (Gall, 1981; Leadbeater *et al.*, 1985). This is a problem which may need to be addressed as treatments against the anticholinesterase effects of organophosphate poisoning become more effective.

The direct actions on the nicotinic cholinoceptor described here may complicate some of the pharmacology of

Table 3 Toxicity values and binding constants of organophosphates

	DFP	*Eco*	*Sarin*	*Soman*	*VX*
LD_{50} s.c. ($\mu g\,kg^{-1}$)	4670[a]	141[c]	160[e]	156[c]	23[d]
	4700[b]		207[f]	120[e]	22[e]
			184[g]		
K_D of blocked channel (μM)		133			19
K_i ($mol^{-1}\,min^{-1}$)	2.9×10^{4}[i]	1.2×10^{5}[h]	1.2×10^{7}[i]	3.6×10^{7}[i]	
			1.5×10^{7}[j]	9.0×10^{7}[j]	3.42×10^{7}[j]
IC_{50} (30 min) (nM)		14[k]	40[k]	20[k]	10[k]

DFP = diisopropylfluorophosphate; Eco = ecothiopate; VX = O-ethyl S-[2-(diisopropylamino) ethyl]methyl phosphonothiolate.
LD_{50} value is by the subcutaneous route; K_D is the dissociation constant of the blocked nicotinic cholinoceptor ion channel at a membrane potential of -80 mV; K_i, is the second order rate constant for binding to acetylcholinesterase; IC_{50} (30 min) is the concentration required to produce 50% inhibition of acetylcholinesterase after a 30 min exposure. All values were measured in the mouse unless otherwise stated.
References: [a] Streicher, 1951; [b] Ellin, 1982; [c] Aronstam *et al.*, 1986; [d] Simeon *et al.*, 1979 (rat); [e] Maksimovic *et al.*, 1980; [f] Brimblecombe *et al.*, 1970; [g] Fonnum & Sterri, 1981; [h] Cohen & Osterbaan, 1963 (*Torpedo*); [i] Andersen *et al.*, 1977 (rat); [j] Gray & Dawson, 1987 (rabbit); [k] Bakry *et al.*, 1988 (*Torpedo*).

organophosphates. For example, the channel-blocking action would tend to shorten endplate currents and miniature endplate currents, thus counteracting the prolongation of these currents caused by the inhibition of acetylcholinesterase. The channel-blocking activity and acceleration of desensitization could also have presynaptic influences on autoreceptors for acetylcholine (Jones, 1987). Clearly, such effects should be taken into account when studying the actions of organophosphates on cholinergic transmission.

References

ADAMS, P.R. (1976). Drug blockade of open end-plate channels. *J. Physiol.*, **260**, 531–552.

ADAMS, P.R. (1987). Transmitter action at endplate membrane. In *The Vertebrate Neuromuscular Junction*. ed. Salpeter, M.M. pp. 317–359. New York: Alan R. Liss Inc.

ADLER, M., ALBUQUERQUE, E.X. & LEBEDA, F.J. (1978). Kinetic analysis of endplate currents altered by atropine and scopolamine. *Mol. Pharmacol.*, **14**, 514–529.

ALBUQUERQUE, E.X., AKAIKE, A., SHAW, K.-P. & RICKETT, D.L. (1984). The interaction of anticholinesterase agents with the acetylcholine receptor-ionic channel complex. *Fundam. Appl. Toxicol.*, **4**, S27–S33.

ALBUQUERQUE, E.X., DESHPANDE, S.S., KAWABUCHI, M., ARACAVA, Y., IDRISS, M., RICKETT, D.L. & BOYNE, A.F. (1985). Multiple actions of anticholinesterase agents on chemosensitive synapses: molecular basis for prophylaxis and treatment of organophosphate poisoning. *Fundam. Appl. Toxicol.*, **5**, S182–S203.

ALKONDON, M. & ALBUQUERQUE, E.X. (1989). The nonoxime dispyridinium compound SAD-128 alters the kinetic properties of the nicotinic acetylcholine receptor ion channel: a possible mechanism for antidotal effects. *J. Pharmacol. Exp. Ther.*, **250**, 842–852.

AMITAI, G. & CHAPMAN, S. (1988). Thiocholine is an agonist of the nicotinic acetylcholine receptor. *Abstr. Soc. Neurosci.*, **14**, 640.

ANDERSEN, R.A., MALTHE-SØRENSSEN, D., ODDEN, E. & FONNUM, F. (1977). Effects of organophosphates on presynaptic events in the vascularly perfused phrenic nerve-hemidiaphragm preparation from the rat. *Biochem. Pharmacol.*, **36**, 1107–1117.

ARACAVA, Y., DESHPANDE, S.S., RICKETT, D.L., BROSSI, A., SCHÖNENBERGER, B. & ALBUQUERQUE, E.X. (1988). The molecular basis of anticholinesterase actions on nicotinic and glutamatergic synapses. *Ann. N.Y. Acad. Sci.*, **505**, 226–255.

ARONSTAM, R.S., SMITH, M.D. & BUCCAFUSCO, J.J. (1986). Clonidine protection from soman and ecothiopate toxicity in mice. *Life Sci.*, **39**, 2097–2102.

BAKRY, N.M.S., EL-RASHIDY, A.H., ELDEFRAWI, A.T. & ELDEFRAWI, M.E. (1988). Direct actions of organophosphate anticholinesterases on nicotinic and muscarinic acetylcholine receptors. *J. Biochem. Toxicol.*, **3**, 235–259.

BARRANTES, F.J. (1988). Muscle endplate cholinoreceptors. *Pharmacol. Ther.*, **38**, 331–385.

BEKOFF, A. & BETZ, W.J. (1977). Physiological properties of dissociated muscle fibres obtained from innervated and denervated adult rat muscle. *J. Physiol.*, **271**, 25–40.

BETZ, W. & SAKMANN, B. (1973). Effects of proteolytic enzymes on function and structure of frog neuromuscular junctions. *J. Physiol.*, **230**, 673–688.

BREHM, P. & KULLBERG, R. (1987). Acetylcholine receptor channels on adult mouse skeletal muscle are functionally identical in synaptic and nonsynaptic membrane. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 2550–2554.

BRIMBLECOMBE, R.W., GREEN, D.M., STRATTON, J.A. & THOMPSON, P.A. (1970). The protective actions of some anticholinergic drugs in sarin poisoning. *Br. J. Pharmacol.*, **39**, 822–850.

COHEN, J.A. & OOSTERBAAN, R.A. (1963). The active site of acetylcholinesterase and related esterases and its reactivity towards substrates and inhibitors. In *Handbuch Exp. Pharmacol.*, Vol. 15, *Cholinesterases and Anticholinesterase Agents*. ed. Koelle, G.B. pp. 299–373. Berlin: Springer-Verlag.

COLQUHOUN, D. & HAWKES, A.G. (1983). The principles of the stochastic interpretation of ion-channel mechanism. In *Single Channel Recording*. ed. Sakmann, B. & Neher, E. pp. 135–175. New York: Plenum Press.

DREYER, F. (1982). Acetylcholine receptor. *Br. J. Anaesth.*, **54**, 115–130.

ELDEFRAWI, M.E., VALDES, J.J. & SCHWEIZER, G. (1985). Interactions of organophosphate nerve agents with the nicotinic acetylcholine receptor. *Proceedings of the Fifth Annual Chemical Defense Bioscience Review*, p. 80. United States Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD.

ELDEFRAWI, M.E., SCHWEIZER, G., BAKRY, N.B.S. & VALDES, J.J. (1988). Desensitization of the nicotinic acetylcholine receptor by diisopropylfluorophosphate. *J. Biochem. Toxicol.*, **3**, 21–32.

ELLIN, R.I. (1982). Anomalies in theories and therapy of intoxication by potent organophosphorus anticholinesterase compounds. *Gen. Pharmacol.*, **13**, 457–466.

EPSTEIN, J., CALLAHAN, J.J. & BAUER, V.E. (1974). The kinetics and mechanisms of hydrolysis of phosphonothiolates in dilute aqueous solutions. *Phosphorus*, **4**, 157–163.

FONNUM, F. & STERRI, S.H. (1981). Factors modifying the toxicity of organophosphorus compounds including soman and sarin. *Fundam. Appl. Toxicol.*, **1**, 143–147.

GALL, D. (1981). The use of therapeutic mixtures in the treatment of cholinesterase inhibition. *Fundam. Appl. Toxicol.*, **1**, 214–216.

GRAY, P.J. & DAWSON, R.M. (1987). Kinetic constants for the inhibition of eel and rabbit brain acetylcholinesterase by some organophosphates and carbamates of military significance. *Toxicol. Appl. Pharmacol.*, **91**, 140–144.

HALL, Z.W. & KELLY, R.B. (1971). Acetylcholinesterase: enzymatic detachment of end plate acetylcholinesterase from muscle. *Nature*, **232**, 62–63.

HENDERSON, E.G., VOLLE, R.L., REYNOLDS, L.S., LAMBERT, J.J. & EPSTEIN, P.M. (1983). Ecothiopate (217MI) and its tertiary amine analogue (217AO) decrease voltage dependence of endplate current (e.p.c.) decay in frog skeletal muscle. *Fed. Proc.*, **42**, 991.

HENDERSON, E.G., POST, D.J., BURKARD, M.R. & PAPPAS, C.A. (1988). Alteration of endplate currents by Tetram (an irreversible anticholinesterase agent). *Abstr. Soc. Neurosci.*, **14**, 506.

HENDERSON, L.P., LECHLEITER, J.D. & BREHM, P. (1987). Single channel properties of newly synthesized acetylcholine receptors following denervation of mammalian skeletal muscle. *J. Gen. Physiol.*, **89**, 999–1014.

JONES, S.W. (1987). Presynaptic mechanisms at vertebrate neuromuscular junctions. In *The Vertebrate Neuromuscular Junction.* ed. Salpeter, M.M. pp. 187–245. New York: Alan R. Liss.

KUBA, K., ALBUQUERQUE, E.X. & BARNARD, E.A. (1973). Diisopropylfluorophosphate: suppression of ionic conductance of the cholinergic receptor. *Science*, **181**, 853–856.

KUBA, K., ALBUQUERQUE, E.X., DALY, J.W. & BARNARD, E.A. (1974). A study of the irreversible cholinesterase inhibitory, diisopropylfluorophosphate, on time course of end-plate currents in frog sartorius muscle. *J. Pharmacol. Exp. Ther.*, **189**, 499–512.

LAMBERT, J.J., DURANT, N.N. & HENDERSON, E.G. (1983). Drug induced modification of ionic conductance at the neuromuscular junction. *Ann. Rev. Pharmacol. Toxicol.*, **23**, 505–539.

LEADBEATER, L.L., INNS, R.H. & RYLANDS, J.M. (1985). Treatment of poisoning by soman. *Fundam. Appl. Toxicol.*, **5**, S225–S231.

MAKSIMOVIC, M., BOSKOVIC, B., RADOVIC, L., TADIC, V., DELJAC, V. & BINENFELD, Z. (1980). Antidotal effects of bis-pyridinium-2-monooxime carbonyl derivatives in intoxications with highly toxic organophosphorus compounds. *Acta Pharmacol. Jugosl.*, **30**, 151–160.

NEHER, E. (1983). The charge carried by single-channel currents of rat cultured muscle cells in the presence of local anaesthetics. *J. Physiol.*, **339**, 663–678.

NEHER, E. & STEINBACH, J.H. (1978). Local anaesthetics transiently block currents through single acetylcholine-receptor channels. *J. Physiol.*, **277**, 153–176.

OCHOA, E.L.M., CHATTOPADHYAY, A. & MCNAMEE, M.G. (1989). Desensitization of the nicotinic acetylcholine receptor: molecular mechanisms and effect of modulators. *Cell. Mol. Neurobiol.*, **9**, 141–178.

RAO, K.S., ARACAVA, Y., RICKETT, D.L. & ALBUQUERQUE, E.X. (1987). Noncompetitive blockade of the nicotinic acetylcholine receptor-ion channel complex by an irreversible cholinesterase inhibitor. *J. Pharmacol. Exp. Ther.*, **240**, 337–344.

SIMEON, V., WILHELM, K., GRANOV, A., BESAROVIC-LAZAREV, S., BUNTIC, A., FAJDETIC, A. & BINENFELD, Z. (1979). 1,3-bispyridinium-dimethylether mono- and dioximes: synthesis, reactivating potency and therapeutic effect in experimental poisoning by organophosphorus compounds. *Arch. Toxicol.*, **41**, 301–306.

STREICHER, E. (1951). Toxicity of colchicine, di-isopropyl fluorophosphate, intracostin and potassium cyanide in mice. *Proc. Soc. Exp. Biol. Med.*, **76**, 536–538.

TATTERSALL, J.E.H. (1989). Ecothiopate, an organophosphorus anticholinesterase, blocks open ion channels in dissociated adult mouse muscle fibres. *J. Physiol.*, **412**, 73P.

WOODHULL, A.M. (1973). Ionic blockage of sodium channels in nerve. *J. Gen. Physiol.*, **61**, 687–708.

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Adrenalectomy abolishes antagonism of α-adrenoceptor-mediated hypotension by a β-blocker in conscious rats

[1]R. Tabrizchi & [2]C.C.Y. Pang

Department of Pharmacology & Therapeutics, Faculty of Medicine, The University of British Columbia, 2176 Health Sciences Mall, Vancouver, B.C., V6T 1W5, Canada

1 The effects of a single bolus injection of propranolol, atenolol or ICI 118,551, non-selective β-, selective β_1- and selective β_2-adrenoceptor antagonists, respectively, on mean arterial pressure (MAP) and plasma catecholamine concentrations were examined in seven groups of conscious and unrestrained adrenalectomized rats receiving a continuous infusion of the α-adrenoceptor antagonist phentolamine. In all rats adrenaline was undetectable in the plasma four days after adrenalectomy.

2 In the first three groups, phentolamine significantly decreased MAP and increased the plasma concentrations of noradrenaline. The injection of propranolol, atenolol or ICI 118,551 in Groups I, II and III, respectively, caused a small increase in MAP and a small, but not significant, decrease in plasma noradrenaline concentrations.

3 Groups IV, V and VI were given a continuous infusion of adrenaline for 1 h prior to the infusion of phentolamine, followed by a bolus injection of propranolol, atenolol or ICI 118,551, respectively. Group VII was treated similarly to IV, but was also given daily cortisone replacement after adrenalectomy. Adrenaline slightly, but not significantly, decreased MAP while phentolamine significantly decreased MAP and increased plasma noradrenaline concentrations in all groups. A subsequent injection of a β-blocker caused a significant increase in MAP in each group and a slight decrease in the plasma level of noradrenaline which reached statistical significance in group VII. The pressor effect of propranolol was significantly greater in the cortisone-treated rats (Group VII) than in Group IV.

5 The results suggest that adrenaline and adrenocortical steroids are both involved in the antagonism of α-adrenoceptor-induced hypotension by a β-blocker.

Introduction

A paradoxical pressor response to the administration of a β-adrenoceptor antagonist has been shown to occur in rats anaesthetized with urethane (Kayaalp & Turker, 1967; Dasgupta, 1968; Yamamoto & Sekiya, 1969; 1972; Regoli, 1970; Sugawara *et al.*, 1980; Himori *et al.*, 1984). The pressor response in the anaesthetized rat was enhanced in the presence of an α-adrenoceptor antagonist (Yamamoto & Sekiya, 1969; Regoli, 1970; Sugawara *et al.*, 1980). In contrast to these observations, we have recently found that the injection of propranolol into conscious rats (that were not previously treated with an α-adrenoceptor antagonist) did not produce a pressor response (Tabrizchi & Pang, 1989). However, we have observed that in conscious rats given a continuous infusion of the α-adrenoceptor antagonist phentolamine, a bolus injection of a β_1-, β_2- or non-selective β-adrenoceptor antagonist did cause a dose-dependent increase in mean arterial pressure (MAP) (Tabrizchi *et al.*, 1988). This discrepancy between the response to a β-adrenoceptor antagonist in the intact conscious and anaesthetized rat is not readily explained. However, urethane has been reported to antagonize the pressor response induced by an α-adrenoceptor agonist and increase the release of adrenaline from the adrenal medulla (Armstrong *et al.*, 1982). It is interesting that, in most reports where the administration of a β-adrenoceptor antagonist produced a pressor response, high plasma concentrations of catecholamines were found (Pritchard & Ross, 1966; Berlyne *et al.*, 1974; McMurtry, 1974; Tabrizchi *et al.*, 1988). The role of adrenaline in the pressor response to propranolol was suggested in a study by Himori *et al.* (1984) which showed that acute adrenalectomy prevented the pressor effect of propranolol in urethane-anaesthetized rats. Moreover, Grewal & Kaul (1970) reported that adrenal gland enucleation abolished the pressor response to propranolol in guanethidine-treated renal hypertensive rats.

In the present study we investigated whether adrenaline was involved in the pressor response to a β_1-, β_2- or non-selective β-blocker in conscious α-adrenoceptor-blocked rats. Preliminary experiments had shown that adrenal enucleation did not completely eliminate adrenaline from the circulation and thus all rats were totally adrenalectomized four days before the study. Then the response to β-blockade was assessed in the absence or presence of an i.v. adrenaline infusion.

Methods

Surgical preparation

Four days prior to the study, male Sprague-Dawley rats (360–390 g, 12–13 weeks old) were prepared under halothane anaesthesia (1.5% halothane in air). A one-inch midline incision was made on the skin along the back. After moving the incision towards either side of the kidney, a small cut was made on the muscle posterior to the last rib. Both kidneys were located and the adrenals excised. Following surgery, the rats were kept warm (25°C) in the laboratory for observation for 6 h after which they were transferred to the animal housing unit, individually housed at a controlled temperature of 22°C with 12 h light/dark cycle and given free access to food (Purina rat chow) and water. None of the rats died from the surgical procedure. After recovery from surgery for four days, the rats were again anaesthetized with halothane. Cannulae were inserted into the iliac arteries for the measurement of MAP by a pressure transducer (P 23 ID, Statham, CA, U.S.A.) and heart rate (HR) by a tachograph (Model 7P4G, Grass Instrument, Mass, U.S.A.) and into both iliac veins for the administrations of drugs. In some rats, additional cannulae were

[1] Present address: Dept. of Pharmacology & Therapeutics, Medical School, The University of Calgary, Calgary, Alberta, Canada T2N 4N1.

[2] Author for correspondence.

placed in the right external jugular vein for the infusion of drugs. All cannulae were filled with heparinized saline (25 iu ml^{-1}) and tunnelled subcutaneously to the back of the neck, exteriorized and secured. The rats were allowed a recovery period of 6 h in the laboratory before experiments were started.

Experimental protocol

MAP and HR were continuously monitored in these conscious, unrestrained adrenalectomized rats prior to and during the administration of drugs. The rats were given >30 min to familiarize with the environment. There was no sign of any distress during the experiments. The rats were randomly divided into seven groups ($n = 8$ in each group). The first three groups received a continuous i.v. infusion of phentolamine (0.3 mg kg^{-1} min^{-1}) prior to the bolus injection of a β-blocker. At 10 min after the start of the infusion of phentolamine, propranolol (100 $\mu g kg^{-1}$), atenolol (100 $\mu g kg^{-1}$) or ICI 188,551 (30 $\mu g kg^{-1}$) was injected intravenously into rats in groups I, II and III, respectively. The doses of β-blocker chosen were those that completely restored MAP to control levels in intact animals treated with phentolamine (Tabrizchi *et al.*, 1988). These doses of atenolol and ICI 118,551 were selective for the antagonism of β_1- and β_2-adrenoceptors, respectively. Another three groups were given a continuous i.v. infusion of adrenaline (0.3 mg kg^{-1} min^{-1}). After a 1 h period of adrenaline infusion, a continuous i.v. infusion of phentolamine was also started followed 10 min later by an i.v. bolus injection of propranolol, atenolol or ICI 118,551 in groups IV, V and VI, respectively (doses as before). Rats in Group VII were given cortisone (50 mg kg^{-1} i.p., twice daily) after adrenalectomy. They were later given adrenaline and phentolamine infusions followed by propranolol as described in group IV. In all rats, three 1 ml arterial blood samples (equivalent to 4% of blood volume each) were taken for the measurement of plasma adrenaline and noradrenaline concentrations. In Groups I to III, a blood sample was taken 5 min before and 6 min following the administration of phentolamine and 1 min after the injection of a β-blocker. In Groups IV to VII, blood samples were taken prior to the infusion of adrenaline, 6 min after the infusion of phentolamine and 1 min after the injection of a β-blocker.

Catecholamine assays

Blood samples were slowly withdrawn into a 1 ml syringe over 20–30 s from the arterial cannula in order to avoid disturbance to the rat. All blood samples removed were replaced by an equal volume of normal saline. As observed previously (King *et al.*, 1985), there was no change in MAP following each blood sampling. The samples were immediately placed on ice, centrifuged and the plasma was removed and stored at −80°C. Plasma samples were assayed within 2 weeks for noradrenaline and adrenaline content by a modified method of reverse-phase ion pair high performance liquid chromatography (h.p.l.c.) with electrochemical detection (Davis *et al.*, 1981). The sensitivity of the catecholamine assay is 0.02 ng ml^{-1}. The coefficient of variation within the same assay (s.d./mean value × 100%) for 9 repeated measurements is 16 for noradrenaline and 6 for adrenaline. The h.p.l.c. method has been described previously (Tabrizchi *et al.*, 1988).

Drugs

All drugs were made up fresh daily and dissolved in normal saline, except for cortisone (11-dehydrocortisol) which was made into a suspension in 1% Ficoll 70 in normal saline. The following drugs were used: phentolamine HCl (CIBA Pharmaceutical Co., N.J., U.S.A.), propranolol, adrenaline, cortisone and atenolol (Sigma Chemical Co., MO, U.S.A.), ICI 118,551 (ICI, Macclesfield, England).

Statistical analysis

The data were assessed for statistical significance by analysis of variance. Repeated measures design was used for comparison of results obtained within the same group of rats and random design, for comparison between different groups of rats. Duncan's multiple range test was used to compare group means. In all cases a probability of error <0.05 was preselected as the criterion for statistical significance. All results are presented as mean ± s.e.

Results

Effects of β-antagonists in adrenalectomized rats

The infusion of phentolamine in adrenalectomized rats in Groups I, II and III significantly decreased MAP (Figure 1a). HR was increased in each of the groups, but the changes were

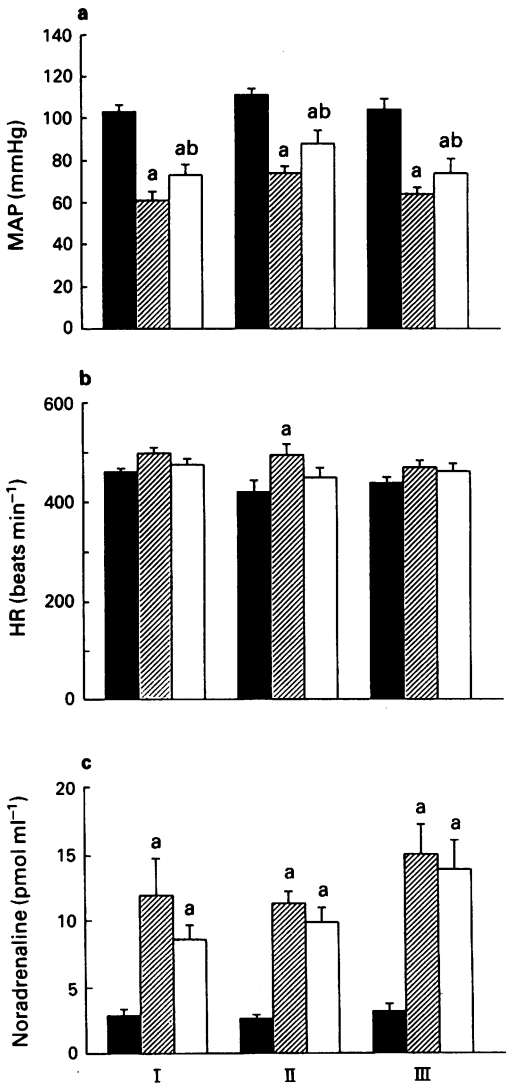

Figure 1 Mean arterial pressure (MAP, a), heart rate (HR, b) and plasma noradrenaline concentrations (c) of conscious adrenalectomized rats ($n = 8$ in each group) during control conditions (solid columns), after a continuous infusion of phentolamine (hatched columns) and after a bolus injection of propranolol, atenolol or ICI 118,551 (open columns) in Groups I, II and III, respectively. Values represent mean with s.e. shown by vertical bars. [a] Significantly different from control; [b] significantly different from phentolamine-treatment.

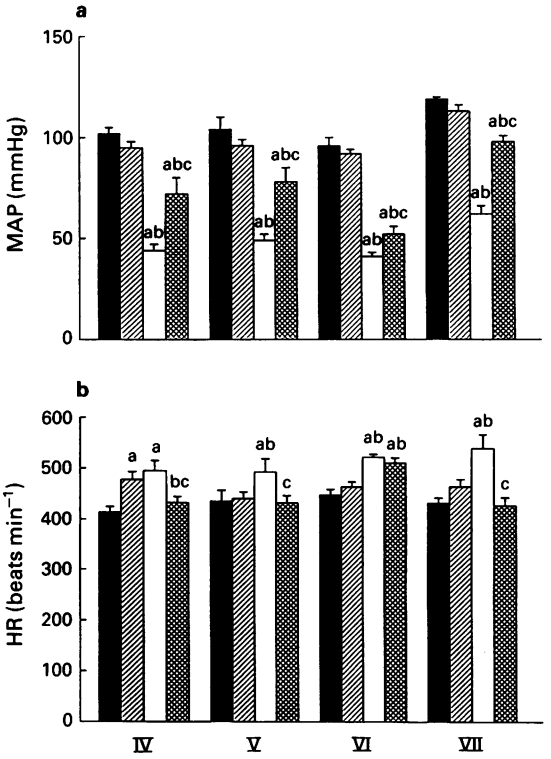

Figure 2 Mean arterial pressure (MAP, a) and heart rate (HR, b) of conscious adrenalectomized rats ($n = 8$ in each group) during control conditions (solid columns), during a continuous infusion of adrenaline (hatched columns), during a continuous infusion of phentolamine (open columns), and after a bolus injection of propranolol, atenolol or ICI 118,551 (cross-hatched columns) in non-cortisone-treated rats (Groups IV, V and VI, respectively), or after the injection of propranolol in cortisone-treated rats (Group VII). Values represent mean with s.e.mean shown by vertical bars. [a] Significantly different from control; [b] significantly different from adrenaline infusion; [c] significantly different from phentolamine infusion.

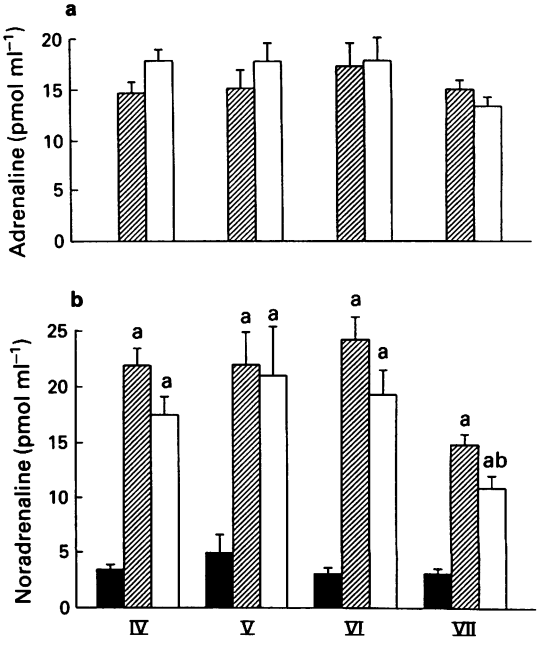

Figure 3 Plasma adrenaline (a) and noradrenaline (b) concentrations of conscious adrenalectomized rats ($n = 8$ in each group) in control conditions (solid columns), during continuous infusions of adrenaline and phentolamine (hatched columns), and after a bolus injection of propranolol, atenolol or ICI 118,551, respectively (open columns), in non-cortisone-treated rats (Groups IV, V and VI, respectively), and after the injection of propranolol in cortisone-treated rats (Group VII). Values represent mean with s.e. shown by vertical bars. [a] Significantly different from control; [b] Significantly different from phentolamine.

only significant in Group II (Figure 1b). The adrenaline level was below the detection limit (<0.1 pmol ml^{-1}) in all rats. The infusion of phentolamine significantly increased the plasma levels of noradrenaline in all groups (Figure 1c). A single bolus injection of propranolol, atenolol or ICI 118,551 in the adrenalectomized rats in Groups I, II and III, respectively, caused a small, but significant, increase in MAP (Figure 1a) and a small, and not significant, decrease in plasma noradrenaline level (Figure 1c). However, MAP after β-blocker injection was significantly less than that prior to phentolamine infusion. HR was not affected by any one of the β-blockers (Figure 1b).

Effects of β-antagonists in adrenalectomized rats given adrenaline infusion

The infusion of adrenaline over a 1 h-period in adrenalectomized rats in Groups IV, V, VI and VII caused a slight, but not significant, decrease in MAP (Figure 2a). The subsequent infusion of phentolamine significantly further decreased MAP in all groups. Against this background of hypotension, the bolus injection of propranolol, atenolol or ICI 118,551 in the non-cortisone-treated rats (Groups IV, V and VI, respectively) and propranolol in the cortisone-treated rats (Group VII) caused an increase in MAP. This pressor response to propranolol in Group VII was significantly greater than that in Group IV. In all cases, MAP after the injection of a β-blocker was significantly less than that measured prior to phentolamine infusion.

HR was slightly, but not significantly, increased by the infusion of adrenaline in Groups V, VI and VII, but significantly increased in Group IV (Figure 2b). The subsequent infusion of phentolamine caused a small increase in HR which reached statistical significance in all groups, except IV. HR after the infusion of both adrenaline and phentolamine in each of the groups was, therefore, significantly greater than the corresponding values in the control condition. The injection of a β-blocker decreased HR which reached statistical significance in all groups, except Group VI.

Plasma adrenaline concentrations were not detectable in the control condition in all groups of adrenalectomized rats (Figure 3a). The infusion of adrenaline in all groups raised plasma concentrations of adrenaline (concentrations were determined during the infusion of phentolamine). Plasma adrenaline concentration was not altered by the injection of any one of the β-antagonists.

The infusion of phentolamine significantly increased plasma concentrations of noradrenaline in all groups (Figure 3b). The injection of a β-antagonist slightly decreased the plasma concentration of noradrenaline, but the decrease was only significant in group VII.

Discussion

We have previously reported that in the conscious rat treated with phentolamine, the injection of atenolol, ICI 118,551 or propranolol caused a pressor response which restored MAP to the level prior to the infusion of phentolamine (Tabrizchi *et al.*, 1988). Furthermore, we have observed that propranolol completely antagonized hypotension induced by an α-blocker, but not that by methacholine or sodium nitroprusside (Tabrizchi & Pang, 1989). Our results suggested the possible existence of an interaction between α- and β-adrenoceptor antagonists. Plasma catecholamine concentrations were markedly elevated following the infusion of phentolamine (Tabrizchi *et al.*, 1988). In order to examine the possibility that adrenaline is essential for the reversal of phentolamine-induced hypotension by a selective or non-selective β-blocker, a series of experiments was conducted in adrenalectomized

rats, both in the absence and presence of infused adrenaline. Arterial plasma concentrations of adrenaline were below the detection limit of 0.1 pmol ml^{-1} in all rats, suggesting the completeness of adrenalectomy.

A study of Gardiner & Bennett (1988) showed that adrenal-demedullation of Long Evans rats significantly impaired the hypotensive effects of prazosin and idazoxan. This suggests that in the intact rat, β_2-adrenoceptor-mediated vasodilatation by adrenaline, which was released as a result of the infusion of α-blockers, contributed to the hypotensive response of α-adrenoceptor antagonists. In the present study we have shown that complete adrenalectomy did not significantly affect the hypotensive action of phentolamine. It should be noted that the conditions in the two studies were quite different. Adrenal-demedullation leaves the adrenal cortex intact. However, we have observed that adrenal-demedullation did not completely eliminate plasma adrenaline in rats (Tabrizchi & Pang, unpublished observation). It is not clear if circulating adrenaline was completely eliminated in the study carried out by Gardiner & Bennett (1988). The administration of propranolol, atenolol or ICI 118,551 in Group I, II and III, respectively, in the phentolamine-treated adrenalectomized animals caused a small increase in MAP. The ability of atenolol, at a dose which is selective for β_1-adrenoceptors in the intact rat (Tabrizchi *et al.*, 1988), to cause a pressor response in α-blocked rats confirms previous findings that pressor response to a β-blocker was not due to the blockade of vasodilator β_2-adrenoceptors (Tabrizchi *et al.*, 1988). The pressor response obtained with each one of the β-antagonists in the adrenalectomized rat was significantly less than that obtained in the intact rats from a previous study (Tabrizchi *et al.*, 1988). Plasma noradrenaline concentrations in these rats prior to and during the infusion of phentolamine, and after the injection of a β-blocker, were similar to the corresponding concentrations reported in a previous study (Tabrizchi *et al.*, 1988). The attenuated pressor response with a β-blocker might suggest that the presence of adrenaline is essential for the interaction between an α- and a β-blocker. Further experiments were carried out to examine the role of adrenaline in the pressor response to a β-blocker.

Adrenalectomized animals were given a continuous infusion of adrenaline. Adrenaline concentrations at 1 h following the start of adrenaline infusion were slightly higher than the concentrations attained in intact rats infused with phentolamine in a previous study (Tabrizchi *et al.*, 1988). These rats were also given phentolamine, which markedly raised noradrenaline concentrations, followed by a bolus injection of propranolol, atenolol or ICI 118,551 in Groups IV, V and VI, respectively. All three β-blockers significantly raised MAP. However, unlike results from a previous study in which the injection of propranolol, atenolol or ICI 118,551 in the intact rat completely antagonized the hypotensive response to phentolamine (Tabrizchi *et al.*, 1988), the β-blockers only partially antagonized the hypotension induced by phentolamine, i.e., each one of the β-blockers raised MAP to levels significantly less than MAP measured 1 h after adrenaline infusion prior to the infusion of phentolamine. Thus, an acute replacement of adrenaline did not completely restore pressor response to a β-blocker. It is possible that the lack of a full restoration of MAP to the control level by a β-antagonist may have been due to morphological changes which may have occurred in the vasculature as a result of the absence of adrenaline. Structural changes occurred in the vascular smooth muscle of animals treated with reserpine, an adrenergic neurone blocking agent, (Hudgins & Harris, 1970; Aprigliano & Hermsmeyer, 1977; Bevan & Tsuru, 1979) resulting in an increase in the sensitivity to noradrenaline as well as other neurotransmitters such as acetylcholine (Hudgins & Fleming, 1966).

Adrenalectomized rats in Group VII were given daily injections of cortisone which has glucocorticoid and mineralocorticoid activities. The injection of propranolol in this latter group raised MAP to 87 $\pm$ 3% of MAP that was measured immediately prior to the infusion of phentolamine in the adrenaline-treated rats. Although MAP after the injection of propranolol in the cortisone-treated rats (Group VII) was still significantly less than MAP measured immediately prior to the infusion of phentolamine, it was significantly higher than the corresponding MAP (74 $\pm$ 5% of control MAP) in rats (Group IV) not treated with cortisone. This suggests that the presence of both adrenal corticosteroids and adrenaline contribute to the pressor response of a β-blocker.

Glucocorticoids are known to be important in the maintenance of cardiac contractility and vascular tone (Baxter & Rousseau, 1979). Reduced pressor response and cardiac contractile force to low doses of catecholamines have been reported in anaesthetized adrenalectomized dogs (Lefer & Sutfin, 1964). The 'permissive' action of glucocorticoids in the responses to catecholamines and other hormones (glucagon, insulin, sex hormones, etc.) is well described (Granner, 1979). Decreased hyperglycaemic (Schaeffer *et al.*, 1969) or hepatic gluconeogenic (Exton *et al.*, 1972) responses to adrenaline in adrenalectomized rats have been reported; these deficits were restored by the administration of a glucocorticoid. However, it should be noted that in the present study, chronic replacement of cortisone in adrenalectomized rats enhanced but did not completely restore the pressor response to propranolol.

In conclusion, the injection of propranolol, atenolol or ICI 118,551 in the α-blocked, adrenalectomized rats caused a small increase in MAP. Following the infusion of adrenaline, the injection of propranolol, atenolol or ICI 118,551 in the phentolamine-treated adrenalectomized rat also caused a small increase in MAP. This is in contrast to responses in the intact rat in which the injection of each one of the three β-blockers completely antagonized the hypotension induced by phentolamine. The pressor response to propranolol was significantly enhanced after replacements of both adrenal corticosteroid and adrenaline. These findings suggest that adrenaline and adrenocortical steroids are involved in the antagonism of α-adrenoceptor-induced hypotension by a β-blocker.

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References

APPRIGLIANO, O. & HERMSMEYER, K. (1977). Trophic influences of the sympathetic nervous system on the rat portal vein. *Circ. Res.*, **41**, 198–206.

ARMSTRONG, J.M., LEFEVRE-BORG, F., SCATTON, B. & CAVERO, O. (1982). Urethane inhibits cardiovascular response mediated by the stimulation of alpha-2 adrenoceptors in the rat. *J. Pharmacol. Exp. Ther.*, **233**, 524–535.

BAXTER, J.D. & ROUSSEAU, G.G. (1979). Glucocorticoid hormone action: an overview. In *Glucocorticoid Hormone Action*. ed. Baxter, J.D. & Rousseau, G.G. pp. 1–24. New York: Springer-Verlag.

BERLYNE, G.M., FINBERG, J.P.M. & YORAN, C. (1974). The effect of β-adrenoceptor blockade on body temperature and plasma renin activity in heat-exposed man. *Br. J. Clin. Pharmacol.*, **1**, 307–312.

BEVAN, R.D. & TSURU, H. (1979). Long-term denervation of vascular smooth muscle causes not only functional but structural changes. *Blood Vessels*, **16**, 109–112.

DASGUPTA, N.K. (1968). On the mechanism of the pressor response due to propranolol. *Br. J. Pharmacol.*, **34**, 200–201P.

DAVIS, G.C., KISSINGER, P.T. & SHOUP, R.E. (1981). Strategies for the determination of serum or plasma norepinephrine by reverse-phase liquid chromatography. *Anal. Chem.*, **53**, 156–159.

EXTON, J.H., FRIEDMANN, N., WONG, E.H., BRINEAUX, J.P., CORBIN, J.D. & PARK, C.R. (1972). Interaction of glycocorticoids with glucagon and epinephrine in the control of gluconeogenesis and glycogenolysis in liver and of lipolysis in adipose tissue. *J. Biol. Chem.*, **247**, 3579–3588.

GARDINER, S.M. & BENNETT, T. (1988). Involvement of β_2-adrenoceptor-mediated mechanisms in the cardiovascular responses to α_1- and α_2-adrenoceptor antagonism in conscious, unrestrained, Long Evans and Brattleboro rats. *Br. J. Pharmacol.*, **93**, 9–22.

GRANNER, D.K. (1979). The role of glucocorticoid hormones as biological amplifiers. In *Glucocorticoid Hormone Action*. ed. Baxter, J.D. & Rousseau, G.G. pp. 593–611. New York: Springer-Verlag.

GREWAL, R.S. & KAUL, C.L. (1970). Mechanism of the antagonism of hypotensive action of guanethidine by propranolol. *Br. J. Pharmacol.*, **38**, 771–775.

HIMORI, N., ISHIMORI, T., SHIRATSUHI, K. & IZUMI, A. (1984). Role of β_2-adrenoceptor blockade and circulating adrenaline level for the pressor responses to β-adrenoceptor blocking drugs in rats. *Naunyn Schmiedebergs Pharmacol. Ther.*, **325**, 314–319.

HUDGINS, P.M. & FLEMING, W.W. (1966). A relatively nonspecific supersensitivity in aortic strips resulting from pretreatment with reserpine. *J. Pharmacol. Exp. Ther.*, **153**, 70–80.

HUDGINS, P.M. & HARRIS, T.M. (1970). Further studies on the effects of reserpine pretreatment on rabbit aorta: Calcium and histologic changes. *J. Pharmacol. Exp. Ther.*, **173**, 609–618.

KAYAALP, S.O. & TURKER, R.K. (1967). Further observation on the pressor action of propranolol. *Br. J. Pharmacol. Chemother.*, **30**, 668–675.

KING, K.A., MACKIE, G., PANG, C.C.Y. & WALL, R.A. (1985). Central vasopressin in the modulation of catecholamine release in conscious rats. *Can. J. Physiol. Pharmacol.*, **63**, 1501–1505.

LEFER, A.M. & SUTFIN, D.C. (1964). Cardiovascular effects of catecholamines in experimental adrenal insufficiency. *Am. J. Physiol.*, **206**, 1151–1155.

MCMURTRY, R.J. (1974). Propranolol, hypoglycemia and hypertension crisis. *Ann. Intern. Med.*, **80**, 669–670.

PRITCHARD, B.N.C. & ROSS, E.J. (1966). Use of propranolol in conjunction with alpha receptor blocking drugs in pheochromocytoma. *Am. J. Cardiol.*, **18**, 394–398.

REGOLI, D. (1970). Pressor action of beta blocking agents in rats. *Can. J. Physiol. Pharmacol.*, **48**, 481–489.

SCHAEFFER, L.D., CHENOWETH, M. & DUNN, A. (1969). Adrenal corticosteroid involvement in the control of liver glycogen phosphorylation activity. *Biochim. Biophys. Acta*, **192**, 292–303.

SUGAWARA, K., TAKAMI, N., MAEMURA, S., NIWA, M. & MASAYORI, O. (1980). β-adrenoceptor blocking agents release catecholamine from rat adrenal medulla. *Eur. J. Pharmacol.*, **62**, 287–295.

TABRIZCHI, R., KING, K.A. & PANG, C.C.Y. (1988). Pressor response to β_1- and β_2-blockers in conscious rats treated with phentolamine. *Pharmacology*, **37**, 385–393.

TABRIZCHI, R. & PANG, C.C.Y. (1989). Propranolol antagonizes hypotension induced by α-blockers but not by sodium nitroprusside or methacholine. *Can. J. Physiol. Pharmacol.*, **67**, 83–87.

YAMAMOTO, J. & SEKIYA, A. (1969). On the pressor actions of propranolol in the rat. *Arch. Int. Pharmacodyn.*, **179**, 372–380.

YAMAMOTO, J. & SEKIYA, A. (1972). Further studies on the pressor actions of propranolol in the rat. *Arch. Int. Pharmacodyn.*, **198**, 347–354.

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Chronic β_1-adrenoceptor antagonist treatment sensitizes β_2-adrenoceptors, but desensitizes M_2-muscarinic receptors in the human right atrium

S. Motomura, N.M. Deighton, *H.-R. Zerkowski, *N. Doetsch, M.C. Michel & [1]O.-E. Brodde

Biochemical Research Laboratory, Medizinische Klinik & Poliklinik, Division of Renal & Hypertensive Diseases, and *Division of Thoracic & Cardiovascular Surgery, University of Essen, D-4300 Essen, F.R.G.

1 In 64 patients undergoing coronary artery bypass grafting the effects of chronic β_1-adrenoceptor antagonist (metoprolol, atenolol, bisoprolol) treatment on right atrial β-adrenoceptor and muscarinic M_2-receptor number and functional responsiveness were investigated.

2 The β_1-adrenoceptor antagonists increased right atrial β_1-adrenoceptor number, did not affect β_2-adrenoceptor number, and decreased muscarinic M_2-receptor number.

3 Concomitantly, activation of right atrial adenylate cyclase by 10 μM GTP, 10 μM isoprenaline and 1 μM forskolin was enhanced and inhibition by 100 μM carbachol was diminished.

4 On isolated, electrically driven right atria the β_1-adrenoceptor-mediated positive inotropic effect of noradrenaline was – even with β_1-adrenoceptor number increased – not altered, while the β_2-adrenoceptor-mediated effect of procaterol was markedly enhanced. However, the carbachol-induced negative inotropic effect was decreased.

5 It is concluded that chronic β_1-adrenoceptor antagonist treatment increases β_1-adrenoceptor number and concomitantly sensitizes β_2-adrenoceptor function, but desensitizes muscarinic M_2-receptor function in the human heart.

Introduction

In the human heart, as in numerous other species, adenosine 3':5'-cyclic monophosphate (cyclic AMP) is a second messenger which mediates positive inotropic effects (Ikezono *et al.*, 1987). Cyclic AMP is formed by the enzyme adenylate cyclase, which is under dual control of stimulating and inhibitory receptors: for example, stimulation of cardiac β-adrenoceptors activates (Brodde *et al.*, 1984; Gille *et al.*, 1985; Kaumann & Lemoine, 1987; Bristow *et al.*, 1989) and stimulation of muscarinic receptors inhibits adenylate cyclase (Waelbroeck *et al.*, 1984). In the human heart, β_1- and β_2-adrenoceptors coexist (for recent reviews see Brodde, 1987; Jones *et al.*, 1989), whereas muscarinic receptors belong predominantly (if not exclusively) to the M_2-subtype (Giraldo *et al.*, 1988; Deighton *et al.*, 1990).

β-Adrenoceptor antagonists are commonly used in the therapy of hypertension and angina pectoris (Scriabine, 1979; McDevitt, 1979). We have recently shown in patients undergoing coronary artery bypass grafting that chronic treatment with different β-adrenoceptor antagonists without intrinsic sympathomimetic activity (ISA) increases the density of β-adrenoceptors in the right atrium in a subtype-selective manner: β_1-selective antagonists only increase β_1-adrenoceptor density whereas non-selective antagonists increase both β_1- and β_2-adrenoceptor number (Michel *et al.*, 1988). While in that study the functional significance of such subtype-selective increases in β-adrenoceptor number was not assessed, Hall *et al.* (1988) simultaneously provided evidence that in right atria from patients chronically treated with the β_1-adrenoceptor antagonist atenolol not β_1-, but β_2-adrenoceptors appear to be hyperresponsive to β-adrenoceptor agonist stimulation. The present studies were, therefore, undertaken in order to obtain further insight into changes of number and function of human cardiac β-adrenoceptor subtypes following chronic β-adrenoceptor antagonist treatment. For this purpose we have investigated the effects of chronic β_1-adrenoceptor antagonist treatment on the density and functional responsiveness of human right atrial β_1- and β_2-adrenoceptors. Since nothing is known of whether and how an increase in β-adrenoceptors might affect receptors which inhibit adenylate cyclase, we also determined density and function of right atrial muscarinic receptors.

[1] Author for correspondence at: Biochem. Forschungslabor, Med. Klinik & Poliklinik, Abtlg. für Nieren- & Hochdruckkrankheiten, Universitätsklinikum, Hufelandstr. 55, D-4300 Essen 1, F.R.G.

Methods

Human right atrial appendages were obtained from 64 patients (42–73 years) undergoing coronary artery bypass grafting (NYHA functional class I–II). No patient suffered from acute heart failure or had been treated with sympathomimetic or parasympathomimetic drugs for at least 3 weeks before surgery. Patients were divided into two age-matched groups according to their chronic pretreatment with β-adrenoceptor antagonists: group I (38 patients, 27 males, 11 females, mean age: 58.1 ± 1.2 years) had received no β-adrenoceptor antagonists for at least six weeks, group II (26 patients, 23 males, 3 females, mean age: 59.9 ± 1.5 years) was chronically treated with the β_1-selective antagonists metoprolol (1–4 × 50 mg day^{-1}; mean dose: 108 ± 14 mg day^{-1}, $n = 12$), bisoprolol (1 × 10 mg day^{-1}, $n = 4$) or atenolol (1–2 × 25 mg day^{-1}, mean dose: 48 ± 7 mg day^{-1}, $n = 10$). Additionally, some patients in both groups were treated with nitrates ($n = 50$), calcium antagonists ($n = 44$), or diuretics ($n = 13$), and occasionally with digitalis glycosides ($n = 6$) and angiotensin-converting enzyme (ACE)-inhibitors ($n = 7$), alone or in combination. In both groups left ventricular function of the patients was not significantly different and treatment with these additional drugs was equally distributed.

Premedication for anaesthesia usually consisted of 2 mg flunitrazepam given orally the evening before and on the morning of surgery. The operation was carried out under neurolept anaesthesia with flunitrazepam and fentanyl; controlled ventilation was performed with a 1:1 mixture of oxygen and N_2O; isoflurane was added up to 1.0% (v/v). Pancuronium was used as a muscle relaxant. Right atrial appendages were removed during installation of the cardiopulmonary bypass. Immediately after excision, the specimens were either

quickly frozen in liquid nitrogen or placed in sealed vials containing Krebs-Henseleit solution (composition below) aerated by carbogen (95% O_2, 5% CO_2).

Preparation of tissues

Preparation of fresh tissues for the organ bath experiments usually began within 5–20 min of surgical removal in oxygenated Krebs-Henseleit solution at room temperature in order to minimize inadequate oxygenation. The right atrial appendages were dissected to yield trabecular strips (4–5 mm in length and 1 mm or less in diameter) without endocardial damage. Whenever possible, part of the same specimen (macroscopically freed from adipose or fibrous tissue) was used for radioligand binding studies.

Organ-bath experiments

The preparations were mounted in 25 ml organ baths containing Krebs-Henseleit solution of the following composition (mM): NaCl 119, $CaCl_2$ 2.5, KCl 4.8, MgSO 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.9, glucose 10.0, EDTA 0.1, ascorbic acid 0.057; aerated with carbogen at 37°C. The myocardial strips were electrically stimulated by square wave impulses of 5 ms duration and a voltage about 50% above threshold (3.0–8.0 V, mean 5.0 V) at a frequency of 1.0 Hz. The developed tension of the preparations (maintained under a resting tension of 4.9 mN) was recorded via a strain gauge on a Hellige recorder. All preparations were allowed to equilibrate for at least 1 h in Krebs-Henseleit solution. After equilibration, a submaximally effective concentration of isoprenaline (10^{-7} M) was administered two or three times, until the successive response remained unchanged. Cumulative concentration-effect curves for the β-adrenoceptor agonists were performed by adding 0.1 ml drug solution, thus increasing the final bath concentration in steps of 0.5 log units. When a steady state of developed tension was reached – usually after 2–5 min – the next higher concentration was applied. When maximum contractile responses were obtained (usually at 3×10^{-7} M procaterol, 3×10^{-6} M isoprenaline, and 3×10^{-5} M noradrenaline), the β-adrenoceptor agonists were added to reach final concentrations of 10^{-6} M (procaterol), 10^{-5} M (isoprenaline) or 10^{-4} M (noradrenaline); under these conditions contractile force was stable for at least 40–60 min (Deighton *et al.*, 1990). Thereafter, cumulative concentration-effect curves for carbachol were carried out as described above. In order to exclude desensitization phenomena, only one concentration-effect curve for the β-adrenoceptor agonists and/or carbachol was assessed in each preparation.

In some experiments the negative inotropic effect of carbachol on basal developed tension was investigated. In these experiments the cumulative concentration-effect curves of carbachol were assessed directly after 1 h of equilibration of the strips in Krebs-Henseleit solution.

Radioligand binding studies

Tissues were minced with scissors and homogenized in 10 volumes of ice-cold 1 mM $KHCO_3$ with an Ultra Turrax (Jahnke & Kunkel, Stauffen, F.R.G.) for 10 s at full speed and twice for 20 s at half-maximal speed at 1 min intervals. The homogenate was diluted to 50 ml with 1 mM $KHCO_3$ and centrifuged at 700 *g* for 15 min; the supernatant was passed through 4 layers of cheesecloth and centrifuged at 21,000 *g* for 45 min. For β-adrenoceptor determination the final pellets were resuspended in 10 mM Tris-HCl, 154 mM NaCl buffer (pH 7.4) containing 0.55 mM ascorbic acid at a protein concentration of 0.1 to 0.2 mg ml^{-1}. Protein content was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

The density of β-adrenoceptors in atrial membranes was determined by (−)-[^{125}I]-iodocyanopindolol (ICYP) binding at 6–8 concentrations of ICYP ranging from 5–200 pM as recently described (Michel *et al.*, 1988). Non-specific binding of ICYP was defined as binding to membranes which was not displaced by a high concentration of the non-selective β-adrenoceptor antagonist (±)-CGP 12177 (1 μM). Specific binding of ICYP was defined as total binding minus non-specific binding and was usually 70–80% at 50 pM of ICYP.

To determine the relative amount of β_1- and β_2-adrenoceptors, membranes were incubated with ICYP (50 pM) in the presence or absence of 21 concentrations of the selective β_2-adrenoceptor antagonist ICI 118,551 (Bilski *et al.*, 1983) and specific binding was determined as described above. ICI 118,551 competition curves were analysed by the iterative curve fitting programme LIGAND (McPherson, 1985). Statistical analysis was performed by use of the *F*-ratio test to measure the goodness of fit of the competition curves for either one or two sites.

For determination of muscarinic receptors the final pellets were resuspended in 10 mM Na_2HPO_4, 10 mM NaH_2PO_4 buffer (pH 7.4) to yield a protein concentration of 0.6 to 0.7 mg ml^{-1}. The density of muscarinic receptors was determined by [N-Methyl-^{3}H]-scopolamine ([^{3}H]-NMS) binding at 7–9 concentrations of [^{3}H]-NMS ranging from 0.1 to 10 nM. Incubations were carried out for 60 min at 25°C and terminated by adding 10 ml of washing buffer to the entire incubation mixture, followed by rapid filtration over Whatman GF/C glass fibre filters that had previously been soaked in 1 mM unlabelled NMS (Deighton *et al.*, 1990). Each filter was washed with an additional 10 ml of washing buffer. The filters were then dried, transferred to vials containing 5 ml of a Triton X-100/toluene scintillation mixture, and the radioactivity determined in a Beckman LS 9000 scintillation counter at an efficiency of 44%. 'Non-specific' binding of [^{3}H]-NMS was defined as radioactivity bound to membranes which was not displaced by a high concentration (1 μM) of atropine. 'Specific' binding of [^{3}H]-NMS was defined as total binding minus non-specific binding and was 80–90% at 0.5–3 nM and 70% at 10 nM [^{3}H]-NMS.

For determination of adenylate cyclase activity cardiac membranes were suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 2 mM $MgCl_2$ and 0.1 mM EGTA. Membranes (5–10 μg of protein) were incubated at 37°C for 10 min in total volume of 100 μl containing 25 mM Tris-HCl buffer, 5 mM $MgCl_2$, 0.5 mM ATP, 100 μM isobutylmethylxanthine (IBMX) 5 mM creatine phosphate, 50 iu ml^{-1} creatine phosphokinase, and (unless otherwise stated) 10 μM GTP at pH 7.4. Isoprenaline (10 μM), forskolin (1 μM), and/or carbachol (100 μM) were added to some tubes. The reaction was stopped by immersing the incubation tubes in boiling water for 5 min. After being cooled the samples were centrifuged at 12000 *g* for 10 min and the cyclic AMP concentration determined in aliquots of the supernatant with a radioimmunoassay kit by the non-acetylated procedure.

Statistical evaluations

The experimental data given in the text, figures and tables are expressed as the means ± s.e.mean of *n* experiments. The equilibrium dissociation constants (K_D) and the maximal number of binding sites (B_{max}) for ICYP and [^{3}H]-NMS were calculated either from plots according to Scatchard (1949) or by the computer programme LIGAND (McPherson, 1985). Both methods yielded similar results. The significance of differences was estimated by non-paired Student's *t* test. A *P* value smaller than 0.05 was considered to be significant. The pD_2 values for the β-adrenoceptor agonists and for carbachol (i.e. the negative logarithm of the molar concentration of agonists causing half-maximal effects) were determined as described by Van Rossum (1963).

Drugs used

ICI 118,551 hydrochloride (erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol) was kindly donated by

ICI Pharma (Plankstadt, F.R.G.), (±)-CGP 12177 hydrochloride (4-(3-tertiarybutylamino-2-hydroxypropoxy)-benzimidazole-2-on) by Ciba Geigy (Basel, Switzerland), and procaterol hydrochloride by Otsuka Pharmaceutical Co. Ltd (Tokushima, Japan). [^{3}H]-NMS (specific radioactivity 85 Ci mmol^{-1}), ICYP (specific radioactivity 2,200 Ci mmol^{-1}), and a radioimmunoassay for cyclic AMP were obtained from New England Nuclear (Dreieich, F.R.G.). Isoprenaline bitartrate, noradrenaline bitartrate, carbachol (carbamylcholine chloride), atropine sulphate and forskolin were purchased from Sigma (Deisenhofen, F.R.G.), N-methyl-scopolamine nitrate from Merck (Darmstadt, F.R.G.). All other chemicals used were of reagent grade or of the purest commercially available grade.

Results

The mean number of right atrial β-adrenoceptors in patients not treated with the β_1-selective adrenoceptor antagonists was 73.6 ± 5.9 fmol ICYP specifically bound mg^{-1} protein (n = 21), the mean β_1-:β_2-adrenoceptor ratio was 69:31%. In the group of patients chronically treated with the β_1- adrenoceptor selective antagonists the mean β-adrenoceptor number was significantly higher (106.2 ± 9.8 fmol mg^{-1} protein, n = 25, $P < 0.01$) (Figure 1), and mean right atrial β_1:β_2-adrenoceptor ratio was shifted towards β_1-adrenoceptors (75:25%); accordingly, the increase in β-adrenoceptors was predominantly due to an increase in β_1-adrenoceptor number, while β_2-adrenoceptor number was not significantly changed (Figure 1).

Concomitant with the increase in β-adrenoceptor number activation of adenylate cyclase by 10 μM isoprenaline and 10 μM guanosine 5'-triphosphate (GTP) was significantly higher in right atrial membranes from treated patients, while basal adenylate cyclase activity was not significantly different between the two groups (Figure 2). Stimulation of the enzyme by 1 μM forskolin was also enhanced, although this did not reach statistical significance (Figure 2) perhaps because the concentration of forskolin used in these experiments was quite low.

The number of right atrial muscarinic receptors was approximately 25 to 30% lower in the patients chronically treated with the β_1-selective adrenoceptor antagonists than in the control subjects (Figure 1). This loss in receptor number was associated with a significantly weaker inhibition of adenylate cyclase activity by the muscarinic receptor agonist, carbachol (Figure 3).

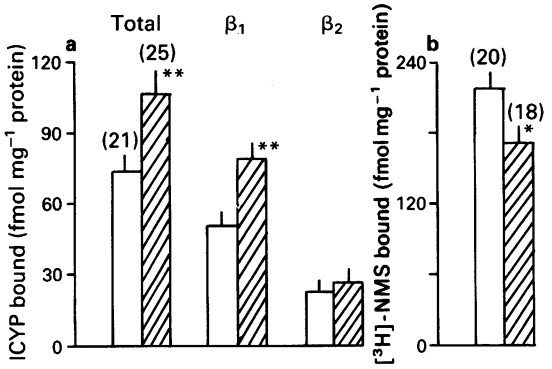

Figure 1 β-Adrenoceptor (a) and muscarinic (b) receptor densities in right atrial membranes from control subjects (open columns) and patients chronically treated with different β_1-selective adrenoceptor antagonists (hatched columns). For details see Methods section. Ordinates (a): right atrial total β-, β_1- and β_2-adrenoceptor density, respectively, in fmol (−)-[^{125}I]-iodocyanopindolol (ICYP) specifically bound mg^{-1} protein; (b): right atrial muscarinic receptor density in fmol [N-methyl-^{3}H]-scopolamine ([^{3}H]-NMS) specifically bound mg^{-1} protein. Means are given with vertical lines showing s.e.mean; number of experiments in parentheses. ** $P < 0.01$, * $P < 0.05$ vs the corresponding control.

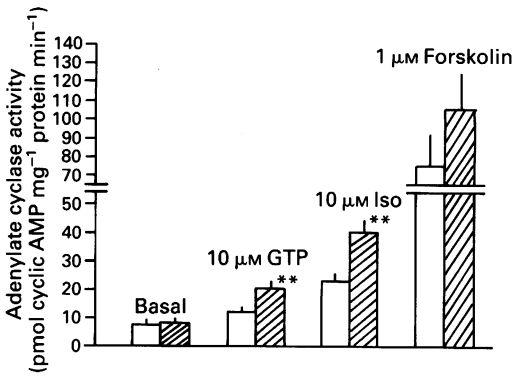

Figure 2 Stimulation of adenylate cyclase activity in right atrial membranes from control subjects (open columns) and patients chronically treated with different β_1-selective adrenoceptor antagonists (hatched columns) by 10 μM GTP, 10 μM isoprenaline (Iso) or 1 μM forskolin. For details see Methods. Means are given with vertical lines showing s.e.mean; n = 6 experiments. ** $P < 0.01$ vs the corresponding control.

We also asked whether the biochemical changes in β-adrenoceptors and muscarinic receptors may be accompanied by similar changes in the contractile responses of the atria to β-adrenoceptor and muscarinic agonists, respectively. For this purpose we determined the positive inotropic effects of isoprenaline (acts via β_1- and β_2-adrenoceptor stimulation; Zerkowski *et al.*, 1986; Motomura *et al.*, 1990), noradrenaline (acts solely through β_1-adrenoceptor stimulation; Gille *et al.*, 1985; Brodde, 1986; Motomura *et al.*, 1990) and procaterol (acts solely through β_2-adrenoceptor stimulation; Zerkowski *et al.*, 1986) on isolated, electrically driven right atria obtained from untreated and β_1-selective adrenoceptor antagonist-treated patients. The concentration-effect curve for the positive inotropic effect of noradrenaline in atria of treated patients was nearly superimposable on that of untreated subjects, while that of isoprenaline was slightly, but not significantly, shifted to the left by about 0.2 log units (Figure 4, Table 1). On the other hand, the concentration-effect curve for the positive inotropic effect of procaterol was significantly shifted to the left by about 0.6 log units (Figure 4, Table 1) in the group of patients chronically treated with β_1-selective adrenoceptor antagonists. In addition, in these patients procaterol always produced the same maximum positive inotropic effect as isoprenaline, while in atria from untreated patients the maximum positive inotropic effect of procaterol was only 77.4 ± 5.4% (n = 15) of that of isoprenaline.

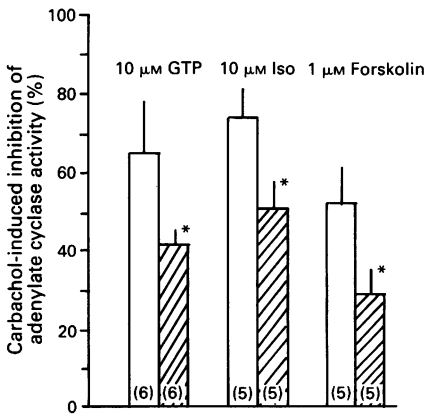

Figure 3 Inhibition of 10 μM GTP-, 10 μM isoprenaline (Iso)- and 1 μM forskolin-stimulated adenylate cyclase activity by 100 μM carbachol in right atrial membranes from control subjects (open columns) and patients chronically treated with different β_1-selective adrenoceptor antagonists (hatched columns). For details see Methods. Means are given with vertical lines showing s.e.mean; number of experiments in parentheses. * $P < 0.05$ vs the corresponding control.

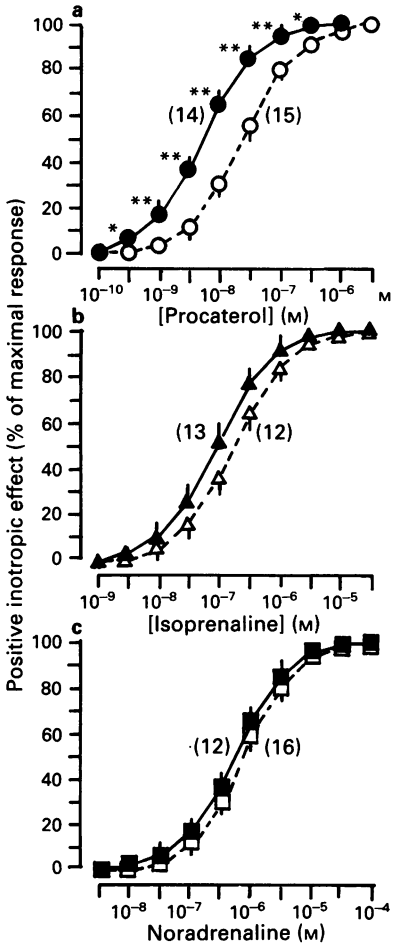

Figure 4 Positive inotropic effects of procaterol (a), isoprenaline (b) and noradrenaline (c) on isolated electrically driven right atria from control subjects (open symbols) and patients chronically treated with different β_1-selective adrenoceptor antagonists (closed symbols). For details see Methods. Abscissae: molar concentrations of the β-adrenoceptor agonists. Means are given with vertical lines showing s.e.mean, number of experiments in parentheses. ** $P < 0.01$, * $P < 0.05$ vs the corresponding control.

Table 1 pD_2 values for the positive inotropic effects of isoprenaline, procaterol and noradrenaline on isolated, electrically driven right atria from untreated and β_1-adrenoceptor antagonist-treated patients

	pD_2 values	
	Untreated	*Treated*
Isoprenaline	7.29 ± 0.09 (12)	7.53 ± 0.11 (13)
Procaterol	7.63 ± 0.07 (15)	8.24 ± 0.13 (14)**
Noradrenaline	6.16 ± 0.06 (16)	6.30 ± 0.13 (12)

Means ± s.e.mean; number of experiments in parentheses.
** $P < 0.01$ vs untreated.

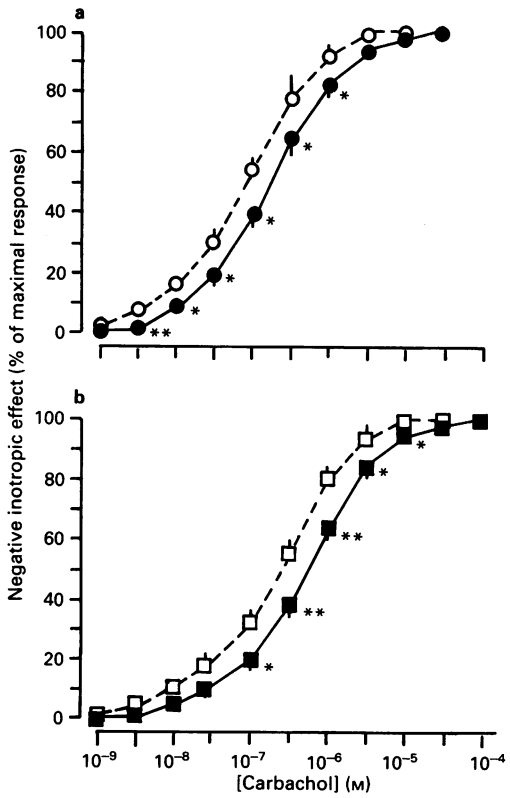

Figure 5 Negative inotropic effect of carbachol on 10^{-6} M procaterol (a)- or 10^{-4} M noradrenaline (b)-prestimulated isolated, electrically driven, right atria from control subjects (open symbols) and patients chronically treated with different β_1-selective adrenoceptor antagonists (closed symbols). For details see Methods. Abscissae: molar concentrations of carbachol. Means are given with vertical lines showing s.e.mean; $n = 11$ experiments. ** $P < 0.01$, * $P < 0.05$ vs the corresponding control.

On the other hand, the concentration-effect curve for the negative inotropic effect of carbachol (mediated by M_2-receptor stimulation; Deighton *et al.*, 1990) was significantly shifted to the right by about 0.3–0.4 log units in atria from patients chronically treated with β_1-selective adrenoceptor antagonists. This was found for the negative inotropic effect of carbachol on basal developed tension of the atria as well as on atria, prestimulated with either 1 μM procaterol, 10 μM isoprenaline, or 100 μM noradrenaline (Figure 5, Table 2). The pD_2 value for the negative inotropic effect of carbachol was significantly higher in atria prestimulated with procaterol than in noradrenaline-prestimulated atria (Table 2), regardless of whether the patients were treated with β-blockers or not.

Discussion

We have investigated the effects of β_1-selective adrenoceptor antagonists on the number and function of β-adrenoceptors

Table 2 pD_2 values for the negative inotropic effect of carbachol on isolated, electrically driven right atria from untreated and β_1-adrenoceptor antagonist-treated patients

	pD_2 values	
Experimental conditions	*Untreated*	*Treated*
Basal developed tension	7.23 ± 0.08 (20)	6.93 ± 0.11 (11)*
Pre-contracted by 10^{-5} M isoprenaline	6.89 ± 0.08 (22)	6.58 ± 0.07 (12)**
Pre-contracted by 10^{-6} M procaterol	7.07 ± 0.10 (11)	6.76 ± 0.11 (11)*
Pre-contracted by 10^{-4} M noradrenaline	6.63 ± 0.11 (11)†	6.29 ± 0.12 (11)*†

Means ± s.e.mean; number of experiments in parentheses.
** $P < 0.025$, * $P < 0.05$ vs untreated.
† $P < 0.01$ vs procaterol.

and muscarinic receptors in the human heart, and found some expected as well as some unexpected results. As expected from previous studies (Michel *et al.*, 1988), patients treated with β_1-selective antagonists without ISA had more β-adrenoceptors than control subjects; moreover, the β_1-selective antagonists only increased the density of β_1-adrenoceptors but did not significantly alter that of β_2-adrenoceptors. We have now extended these observations by studying the functional relevance of elevated receptor number, and have demonstrated that adenylate cyclase activation by the β-adrenoceptor agonist isoprenaline is also enhanced. Thus, not only the number but also the functional response of β-adrenoceptors was higher in the patients chronically treated with β_1-selective adrenoceptor antagonists.

As the increase in receptor number was limited to β_1-adrenoceptors, we also studied the involvement of β_1- and β_2-adrenoceptors in the enhanced β-adrenoceptor responsiveness by using noradrenaline and procaterol. These two agonists elicit their positive inotropic effects in the human heart solely via β_1- and β_2-adrenoceptors, respectively (Gille *et al.*, 1985; Zerkowski *et al.*, 1986; Brodde, 1986; Motomura *et al.*, 1990). The concentration-effect curve for the β_1-selective agonist noradrenaline was similar in patients treated with β-blockers and control subjects, suggesting that this pathway had not been sensitized even though β_1-adrenoceptor density was increased. However, the concentration-effect curve for the β_2-selective agonist procaterol was shifted to the left towards lower concentrations by approx. 0.6 log units. In addition, in the β-blocker-treated patients procaterol was a full β-adrenoceptor agonist, whereas in untreated patients it was only a partial agonist (intrinsic activity = 0.77). These results confirm recently obtained data by Hall *et al.* (1988, 1989) and Kaumann *et al.* (1989) who demonstrated that, in patients chronically treated with atenolol, the potency of adrenaline (acting via β_1- and β_2-adrenoceptors in the human heart; Gille *et al.*, 1985; Kaumann & Lemoine, 1987; Motomura *et al.*, 1990) and the β_2-adrenoceptor-selective partial agonist salbutamol for induction of the positive inotropic effects was markedly enhanced, while that of noradrenaline (acting via β_1-adrenoceptors only in the human heart) was only slightly changed, when compared with untreated patients. These authors also demonstrated that in atenolol-treated patients the intrinsic activity of salbutamol was markedly enhanced (Hall *et al.*, 1989).

The increased β_2-adrenoceptor responsiveness in the presence of an unaltered β_2-adrenoceptor density indicates enhanced receptor-effector coupling. In fact, receptor-independent stimulation of adenylate cyclase by GTP (acting directly on G proteins) was greater in the β-blocker-treated patients than in the control subjects. Receptor-independent stimulation of the enzyme by forskolin (acting directly on the catalytic subunit of the enzyme, but also capable of activating G_s) was also enhanced although this did not reach statistical significance. Thus, β-blocker treatment was accompanied by a heterologous sensitization of adenylate cyclase stimulation. These data suggest that the patients treated with the β_1-selective antagonists either had an increased activity of the stimulating G protein G_s, which enhanced responsiveness of β_2-adrenoceptors, or had an increased efficiency of coupling of the β_2-adrenoceptor to the G_s protein or adenylate cyclase leading to enhanced responsiveness.

Although we were not able to assess directly G protein function in the present study, several pieces of evidence support the idea of altered G protein function in chronically β-blocker treated patients. Firstly, patients with congestive heart failure have a heightened sympathetic activity and a decreased ratio of functional G_s/G_i in their heart (Feldman *et al.*, 1988; Neumann *et al.*, 1988). Secondly, long-term *in vitro* treatment of rat cardiomyocytes with noradrenaline causes a shift in the G_s/G_i-ratio towards elevated G_i-protein (Reithmann *et al.*, 1989). Thirdly, long-term *in vitro* treatment of a variety of cell types with agonists inhibiting adenylate cyclase (such as somatostatin, opiates, muscarinic cholinoceptor, α_2-adrenoceptor or adenosine) desensitizes receptor coupling to inhibition of adenylate cyclase and simultaneously potentiates the stimulating effects of various hormones on adenylate cyclase (for references see Thomas & Hoffman, 1987). Thus, it appears that prolonged exposure to agonists inhibiting adenylate cyclase increases the functional G_s/G_i ratio, whereas agonists stimulating adenylate cyclase (such as β-adrenoceptor agonists) decrease this ratio. Thus, it might well be that in the present study chronic treatment of patients with β_1-adrenoceptor antagonists may cause a reversal of a tonic decrease of G_s by the endogenous catecholamines resulting in enhanced G_s function.

The question remains why possible changes in the functional G_s/G_i ratio towards G_s induced by chronic β_1-adrenoceptor antagonist treatment may lead to an increased sensitivity of β_2-adrenoceptors, but not of β_1-adrenoceptors. At present, we do not know the answer and, hence, can only speculate on the mechanism: in the human heart β_2-adrenoceptors couple much more efficiently to adenylate cyclase than β_1-adrenoceptors (Brodde *et al.*, 1984; Gille *et al.*, 1985; Kaumann *et al.*, 1989; Bristow *et al.*, 1989). For example, procaterol increases human right atrial and ventricular adenylate cyclase activity solely through β_2-adrenoceptor stimulation by about 65 to 70% of the maximum activation induced by isoprenaline (Brodde *et al.*, 1984; 1989), although β_2-adrenoceptors represent only 30% of the total β-adrenoceptor number in the human heart (for references see Brodde, 1987; Jones *et al.*, 1989). As discussed above, long-term treatment with β_1-selective antagonists seems to sensitize the G_s-coupled β-adrenergic pathway in the human heart. As β_2-adrenoceptors are much more efficiently coupled to adenylate cyclase than β_1-adrenoceptors, it is quite plausible that such a sensitization enhances the contractile response to β_2-adrenoceptor stimulation. On the other hand as β_1-adrenoceptors obviously couple only weakly to adenylate cyclase, sensitization of this pathway might only marginally affect contractile responsiveness. However, such a mechanism can only be operative if one assumes that β_1- and β_2-adrenoceptors are located on the same cell. Future experiments have to show whether such a mechanism may be responsible for the surprising finding that chronic treatment with β_1-selective antagonists increases β_1-adrenoceptor density but simultaneously sensitizes β_2-adrenoceptor rather than β_1-adrenoceptor functional responsiveness in the human right atrium.

Sensitization of adenylate cyclase stimulating pathways in patients treated with β_1-selective antagonists makes it interesting to look at receptors which inhibit adenylate cyclase. The muscarinic M_2-receptor mediates inhibition of adenylate cyclase and negative inotropic effects in the human right atrium (Waelbroeck *et al.*, 1984; Deighton *et al.*, 1990). Our data demonstrate that patients chronically treated with β-blockers have fewer muscarinic receptors than control subjects. Similarly, preliminary data in rats also suggest decreased cardiac muscarinic receptors after chronic β-blocker treatment (Strasser *et al.*, 1988). The decrease in muscarinic receptor number in our patients was accompanied by a desensitization of inhibition of adenylate cyclase activity and of the negative inotropic effects in response to carbachol. Thus, number and function of muscarinic M_2-receptors are reduced in the atria of patients chronically treated with β_1-selective antagonists.

Three possibilities should be taken into account to explain the desensitization of muscarinic receptors in β-blocker-treated patients: (1) The β_1-selective adrenoceptor antagonists could have concomitant agonistic effects at muscarinic receptors. This is an extremely unlikely possibility, especially as the affinities of atenolol, metoprolol, and bisoprolol at muscarinic receptors are in the millimolar range (data not shown). (2) β-Blocker treatment could be associated with a reflex enhancement in vagal tone, which would desensitize muscarinic receptors. However, in healthy volunteers the β-adrenoceptor agonist isoprenaline increases vagal activity (Arnold & McDevitt, 1984) and it is very unlikely that β-adrenoceptor antagonists produce the same effects. (3) Muscarinic receptors

could be located on the same cells as β-adrenoceptors. If this were the case, the mechanisms which amplify cyclic AMP formation via increased G_s might further enhance adenylate cyclase activity by desensitizing muscarinic receptors which inhibit adenylate cyclase. Further studies in better defined systems such as cultured cells are necessary to test this hypothesis. Nevertheless, it is attractive because examples of receptor cross-talk have been described in other receptor systems in cultured cells (Fuxe & Agnati, 1985).

The present finding of decreased cardiac muscarinic receptor density and function and increased β_2-adrenoceptor but not β_1-adrenoceptor function following long-term treatment with β_1-adrenoceptor antagonists may be of importance for the therapeutic use of β-adrenoceptor antagonists. Firstly, when muscarinic receptor function decreases, the inhibitory influence of the vagus on cardiac β-adrenoceptor function is attenuated. Thus, such a reduction in vagal activity might exaggerate the symptoms of adrenergic hypersensitivity often observed after abrupt withdrawal of β-adrenoceptor antagonist treatment in patients suffering from hypertension or angina pectoris (Prichard *et al.*, 1983). Such withdrawal phenomena that were initially observed after sudden cessation of propranolol treatment (Prichard *et al.*, 1983) can also occur after sudden withdrawal of β_1-adrenoceptor antagonists: Walden *et al.* (1988) have recently shown, in healthy volunteers, that abrupt withdrawal of atenolol markedly increases the sensitivity of human cardiac β-adrenoceptors to isoprenaline-induced tachycardia, but not to exercise-induced tachycardia. Isoprenaline-induced tachycardia is a mixed cardiac β_1- and β_2-adrenoceptor-mediated effect, while exercise-induced tachycardia is a pure β_1-adrenoceptor- mediated effect (Brodde *et al.*, 1988; McDevitt, 1989; Motomura *et al.*, 1990). These results, therefore, support our present findings that chronic treatment of patients with β_1- selective antagonists enhances β_2-adrenoceptor, but not β_1-adrenoceptor sensitivity.

Secondly, in recent years patients with severe heart failure (and markedly reduced cardiac β-adrenoceptor density) have been beneficially treated with low doses of the β_1-selective antagonist metoprolol (Waagstein *et al.*, 1983; 1989; Heilbrunn *et al.*, 1989). The theoretical rationale for such treatment is that β-adrenoceptor antagonists can increase β-adrenoceptor density, and thus may restore the previously reduced β-adrenoceptor density in these patients (Fowler & Bristow, 1985). Assuming that a similar phenomenon occurs in patients with severe heart failure, i.e. metoprolol increases cardiac β_1-adrenoceptor density and simultaneously enhances β_2-adrenoceptor sensitivity, then the possibility that these patients be treated additionally with a β_2-adrenoceptor agonist should be considered. Such combined treatment with a β_1-adrenoceptor antagonist and a β_2-adrenoceptor agonist should lead to a recovery of cardiac β_1-adrenoceptors and simultaneously might improve contractility via sensitized β_2-adrenoceptors.

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References

ARNOLD, J.M.O. & McDEVITT, D.G. (1984). Vagal activity is increased during isoprenaline infusion in man. *Br. J. Clin. Pharmacol.*, **18**, 311–316.

BILSKI, A.J., HALLIDAY, S.E., FITZGERALD, J.D. & WALE, J.L. (1983). The pharmacology of a β_2-selective adrenoceptor antagonist (ICI 118,551). *J. Cardiovasc. Pharmacol.*, **5**, 430–437.

BRISTOW, M.R., HERSHBERGER, R.E., PORT, J.D., MINOBE, W. & RASMUSSEN, R. (1989). β_1- and β_2-adrenergic receptor-mediated adenylate cyclase stimulation in nonfailing and failing human ventricular myocardium. *Mol. Pharmacol.*, **35**, 295–303.

BRODDE, O.-E. (1986). Bisoprolol (EMD 33512), a highly selective β_1-adrenoceptor antagonist: in vitro and in vivo studies. *J. Cardiovasc. Pharmacol.*, **8** (Suppl. 11), S29–S35.

BRODDE, O.-E. (1987). Cardiac beta-adrenergic receptors. *ISI Atlas Sci.: Pharmacol.*, **1**, 107–112.

BRODDE, O.-E., DAUL, A., WELLSTEIN, A., PALM, D., MICHEL, M.C. & BECKERINGH, J.J. (1988). Differentiation of β_1- and β_2-adrenoceptor-mediated effects in humans. *Am. J. Physiol.*, **254**, H199–H206.

BRODDE, O.-E., O'HARA, N., ZERKOWSKI, H.-R. & ROHM, N. (1984). Human cardiac β-adrenoceptors: Both β_1- and β_2-adrenoceptors are functionally coupled to the adenylate cyclase in right atrium. *J. Cardiovasc. Pharmacol.*, **6**, 1184–1191.

BRODDE, O.-E., ZERKOWSKI, H.-R., BORST, H.G., MAIER, W. & MICHEL, M.C. (1989). Drug- and disease-induced changes of human cardiac β_1- and β_2-adrenoceptors. *Eur. Heart J.*, **10** (Suppl. B), 38–44.

DEIGHTON, N.M., MOTOMURA, S., BORQUEZ, D., ZERKOWSKI, H.-R., DOETSCH, N. & BRODDE, O.-E. (1990). Muscarinic cholinoceptors in the human heart: demonstration, subclassification, and distribution. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **341**, 14–21.

FELDMAN, A.M., CATES, A.E., VAEZEY, W.B., HERSHBERGER, R.E., BRISTOW, M.R., BAUGHMAN, K.L., BAUMGARTNER, W.A. & VAN DOP, C. (1988). Increase of the 40,000-mol wt pertussis toxin substrate (G protein) in the failing human heart. *J. Clin. Invest.*, **82**, 189–197.

FOWLER, M.B. & BRISTOW, M.R. (1985). Rationale for beta-adrenergic blocking drugs in cardiomyopathy. *Am. J. Cardiol.*, **55**, 120D–124D.

FUXE, K. & AGNATI, L.F. (1985). Receptor-receptor interactions in the central nervous system. A new integrative mechanism in synapses. *Med. Res. Rev.*, **5**, 441–482.

GILLE, E., LEMOINE, H., EHLE, B. & KAUMANN, A.J. (1985). The affinity of (−)-propranolol for β_1- and β_2-adrenoceptors of human heart. Differential antagonism of the positive inotropic effects and adenylate cyclase stimulation by (−)-noradrenaline and (−)-adrenaline. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **331**, 60–70.

GIRALDO, E., MARTOS, F., GOMEZ, A., GARCIA, A., VIGANO, M.A., LADINSKY, H. & SANCHEZ DE LA CUESTA, F. (1988). Characterization of muscarinic receptor subtypes in human tissues. *Life Sci.*, **43**, 1507–1515.

HALL, J.A., KAUMANN, A.J. & BROWN, M.J. (1989). Increased β_2 adrenoceptor coupling to inotropic effects in human atrial myocardium due to atenolol treatment. *Br. J. Pharmacol. Proc. Suppl.*, **97**, 391P.

HALL, J.A., KAUMANN, A.J., WELLS, F.C. & BROWN, M.J. (1988). β_2-Adrenoceptor mediated inotropic responses of human atria: receptor subtype regulation by atenolol. *Br. J. Pharmacol. Proc. Suppl.*, **93**, 116P.

HEILBRUNN, S.M., SHAH, P., BRISTOW, M.R., VALANTINE, H.A., GINSBURG, R. & FOWLER, M.B. (1989). Increased β-receptor density and improved hemodynamic response to catecholamine stimulation during long-term metoprolol therapy in heart failure from dilated cardiomyopathy. *Circulation*, **79**, 483–490.

IKEZONO, K., MICHEL, M.C., ZERKOWSKI, H.-R., BECKERINGH, J.J. & BRODDE, O.-E. (1987). The role of cyclic AMP in the positive inotropic effect mediated by β_1- and β_2-adrenoceptors in isolated human right atrium. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **335**, 561–566.

JONES, C.R., MOLENAAR, P. & SUMMERS, R.J. (1989). New views of human cardiac β-adrenoceptors. *J. Mol. Cell. Cardiol.*, **21**, 519–535.

KAUMANN, A.J., HALL, J.A., MURRAY, K.J., WELLS, F.C. & BROWN, M.J. (1989). A comparison of the effects of adrenaline and noradrenaline on human heart: the role of β_1- and β_2-adrenoceptors in the stimulation of adenylate cyclase and contractile force. *Eur. Heart J.*, **10** (Suppl B), 29–37.

KAUMANN, A.J. & LEMOINE, H. (1987). β_2-Adrenoceptor-mediated positive inotropic effect of adrenaline in human ventricular myocardium. Quantitative discrepancies with binding and adenylate cyclase stimulation. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **335**, 403–411.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurements with the Folin phenol reagent. *J.*

Biol. Chem., **193**, 265–275.

McDEVITT, D.G. (1979). Adrenoceptor blocking drugs: clinical pharmacology and therapeutic use. *Drugs*, **17**, 267–288.

McDEVITT, D.G. (1989). In vivo studies on the function of cardiac β-adrenoceptors in man. *Eur. Heart J.*, **10** (Suppl. B), 22–28.

McPHERSON, G.A. (1985). Analysis of radioligand binding experiments: a collection of computer programs for the IBM PC. *J. Pharmacol. Meth.*, **14**, 213–228.

MICHEL, M.C., PINGSMANN, A., BECKERINGH, J.J., ZERKOWSKI, H.-R., DOETSCH, N. & BRODDE, O.-E. (1988). Selective regulation of β_1- and β_2-adrenoceptors in the human heart by chronic β-adrenoceptor antagonist treatment. *Br. J. Pharmacol.*, **94**, 685–692.

MOTOMURA, S., ZERKOWSKI, H.-R., DAUL, A. & BRODDE, O.-E. (1990). On the physiologic role of beta-2 adrenoceptors in the human heart: In vitro and in vivo studies. *Am. Heart J.*, **119**, 608–619.

NEUMANN, J., SCHMITZ, W., SCHOLZ, H., VON MEYERINCK, L., DÖRING, V. & KALMAR, P. (1988). Increase in myocardial G_i-proteins in heart failure. *Lancet*, **ii**, 936–937.

PRICHARD, B.N.C., TOMLINSON, B., WALDEN, R.J. & BHATTACHARJEE, P. (1983). The β-adrenergic blockade withdrawal phenomenon. *J. Cardiovasc. Pharmacol.*, **5** (Suppl. 1), S56–S62.

REITHMANN, C., GIERSCHIK, P., SIDIROPOULOS, D., WERDAN, K. & JAKOBS, K.H. (1989). Mechanism of noradrenaline-induced heterologous desensitization of adenylate cyclase stimulation in rat heart muscle cells: increase in the levels of inhibitory G-protein α-subunits. *Eur. J. Pharmacol.*, **172**, 211–221.

SCATCHARD, G. (1949). The attraction of proteins for small molecules and ions. *Ann. New York Acad. Sci.*, **51**, 660–672.

SCRIABINE, A. (1979). β-Adrenoceptor blocking drugs in hypertension. *Annu. Rev. Pharmacol. Toxicol.*, **19**, 269–284.

STRASSER, R.H., BREHM, B. & MARQUETANT, R. (1988). Interaction of adenylate cyclase coupled receptors: chronic β-blockade transregulates inhibitory receptor-systems. *Eur. Heart J.*, **9** (Suppl. 1), 116 (abstract).

THOMAS, J.M. & HOFFMAN, B.B. (1987). Adenylate cyclase supersensitivity: a general means of cellular adaptation to inhibitory agonists? *Trends Pharmacol. Sci.*, **8**, 308–311.

VAN ROSSUM, J.M. (1963). Cumulative dose-response curves II: Technique for the making of dose-response curves in isolated organs and the evaluation of drug parameters. *Arch. Int. Pharmacodyn. Ther.*, **143**, 299–330.

WAAGSTEIN, F., CAIDAHL, K., WALLENTIN, I., BERGH, C.-H. & HJALMARSON, A. (1989). Long-term β-blockade in dilated cardiomyopathy. Effects of short-term and long-term metoprolol treatment followed by withdrawal and readministration of metoprolol. *Circulation*, **80**, 551–563.

WAAGSTEIN, F., HJALMARSON, A., SWEDBERG, K. & WALLENTIN, I. (1983). Beta-blockers in dilated cardiomyopathy: they work. *Eur. Heart J.*, **4**, 173–178.

WAELBROECK, M., ROBBERECHT, P. & CHRISTOPHE, J. (1984). A comparison between muscarinic receptor occupancy, adenylate cyclase inhibition and inotropic responses in human heart. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **325**, 170–175.

WALDEN, R.J., TOMLINSON, B., GRAHAM, B.R., LIU, J.B. & PRICHARD, B.N.C. (1988). The withdrawal syndrome: atenolol and bopindolol in healthy volunteers. *Br. J. Clin. Pharmacol.*, **25**, 657P (abstr.).

ZERKOWSKI, H.-R., IKEZONO, K., ROHM, N., REIDEMEISTER, J.CHR. & BRODDE, O.-E. (1986). Human myocardial β-adrenoceptors: demonstration of both β_1- and β_2-adrenoceptors mediating contractile responses to β-agonists on the isolated right atrium. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **332**, 143–147.

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Positive inotropic effect of porcine left ventricular extract on canine ventricular muscle

#†S. Navaratnam, †T. Chau, #M. Agbanyo, #†*D. Bose & [1]#†J.C. Khatter

#Department of Internal Medicine, †Pharmacology & Therapeutics and *Anesthesia, University of Manitoba, Winnipeg, Manitoba, Canada, R3E OZ2

1 We previously isolated an extract from porcine left ventricle that possessed digitalis-like properties such as inhibition of cardiac and kidney Na^+, K^+-ATPase, displacement of [^{3}H]-ouabain from its binding sites and cross reactivity with digoxin antibodies. The extract also had a positive inotropic effect on the guinea-pig heart.

2 In the present study the positive inotropic response of the extract was characterized in canine right ventricular trabeculae. Maximum inotropic response (501 ± 20%) was produced by 300 μl and the half maximal increase occurred with 125 μl of the extract.

3 Ouabagenin produced aftercontractions in rapidly paced trabeculae. Equipotent and even greater amounts of the extract did not produce aftercontractions.

4 The extract increased the amplitude of the delayed component (P2) of biphasic contractions produced by replacing about 92–96% of the external Ca with Sr. A smaller increase in the size of the early component (P1) was also seen.

5 The extract decreased post-rest potentiation after rest for 30 s and 2 min. After 8 min of rest, post-rest potentiation was converted to post-rest depression.

6 The extract (20 μl) produced a decrease in the amplitude of the post-rest rapid cooling contracture (RCC) at all rest intervals. The steady state RCC, although greater than that in the control muscle, was increased to a lesser extent than the size of the steady state electrically driven contractions.

7 It is suggested that the extract from porcine left ventricle produces a positive inotropic response by increasing the trans-sarcolemmal influx of Ca. It also has additional effect(s) on the sarcoplasmic reticulum in that it may facilitate the loss of Ca from the sarcoplasmic reticulum and/or inhibit the uptake of Ca by the organelle.

Introduction

Isolation of a digitalis-like substance (DLS) from biological materials such as plasma, cardiac tissue, placenta etc. has been attempted by many laboratories (Gruber *et al.*, 1980; Kelly *et al.*, 1985; Balzan *et al.*, 1986, Ebara *et al.*, 1986). In our laboratory, we isolated a crude fraction from porcine left ventricle, after extraction of lipids, chromatographic separation and desalting (Khatter *et al.*, 1986). The extract had digitalis-like properties such as inhibition of cardiac and kidney Na^+, K^+-ATPase dose-dependently, displacement of [^{3}H]-ouabain from its binding sites and cross reactivity with antibodies to digoxin (Khatter *et al.*, 1986; Agbanyo & Khatter 1990; Khatter *et al.*, 1990). We also investigated the effect of this extract on ^{86}Rb uptake, which is a representation of functional Na^+ pump activity. The extract inhibited the uptake of ^{86}Rb in a dose-dependent fashion, indicating that it inhibits the Na pump.

Even though many laboratories have been able to demonstrate the presence of digitalis-like substance(s) either immunologically or biochemically, the functional characterization of positive inotropism was not done in these studies (Gruber *et al.*, 1980; Kelly *et al.*, 1985; Balzan *et al.*, 1986; Ebara *et al.*, 1986). The crude extract obtained in our laboratory from porcine ventricle was consistently inotropic in guinea-pig isolated hearts (Khatter *et al.*, 1986). Furthermore, in our previous studies we also excluded the possibility of catecholamines being responsible for the inotropic effect because: (a) it was not blocked by a β-adrenoceptor antagonist, (b) the extract did not compete for [^{3}H]-(−)-dihydroalprenolol binding (Khatter *et al.*, 1986) and (c) in high performance liquid chromatography (h.p.l.c.) analysis it did not have similar peaks as those of adrenaline, noradrenaline or dopamine (Agbanyo & Khatter, 1990). Essentially the extract obtained from porcine left ventricle had contractile, immunological and biochemical properties like those of digitalis. The present study was undertaken to characterize further the positive inotropic response of the extract on canine ventricular trabeculae, in order to investigate its possible mechanism of action.

[1] Author for correspondence at: Section of Cardiology, Department of Medicine, GF 330, Health Sciences Centre, 700 William Ave., Winnipeg, Manitoba, Canada R3E OZ3.

Methods

Isolation of digitalis-like substance (DLS)-containing extract from porcine left ventricle

The detailed methodology for the isolation of the crude porcine ventricular extract has been described previously (Khatter *et al.*, 1986). A homogenate of the porcine left ventricle was obtained, lipids were extracted with methanol and diethyl ether and the fraction subjected to column chromatography (Sephadex G-25-80 fine bead) and desalting. The final extract from 150 g porcine left ventricle was neutralized to pH 7.4 with 1 M Tris and adjusted to a total volume of 5 ml.

Effect of DLS containing extract on canine right ventricular trabeculae

Experimental preparation Tissue preparation and tension measurements were carried out as described previously (Hryshko *et al.*, 1989a). Briefly, mongrel dogs of either sex (8–12 kg) were anaesthetized with sodium pentobarbitone (35 mg kg^{-1}, i.v.), hearts were rapidly excised and thin, free. running right ventricular trabeculae were dissected from the wall of the heart. Isolated muscle preparations were transferred to a 5 ml vertical bath containing oxygenated (95% O_2, 5% CO_2) Krebs Henseleit (KH) solution (in mM: NaCl 118,

KCl 4.7 $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.4, $NaHCO_3$ 25 and glucose 11) maintained at a pH of 7.4 and at a temperature of 37°C. Muscles were field stimulated by pulses delivered from a computer-controlled stimulus sequencer (Boyechko & Bose, 1982) with a frequency of 0.5 Hz and amplitude of 75–100% above threshold. Muscles were stretched to an optimal length for maximal isometric force production and isometric contractions were recorded with a force transducer (Grass FT-03C). Periods of rest ranging from 30–480 s were imposed on an otherwise constant stimulus pattern. Dose-response of the porcine extract was tested by addition of increasing amounts of the extract to the tissue bath in a cumulative manner.

Rapid cooling contracture (RCC) The apparatus employed to elicit rapid cooling contracture (RCC) has been described in detail elsewhere (Hryshko *et al.*, 1989b). Briefly, the muscle chamber consists of single bath and two reservoirs containing bathing medium at 37°C and −4°C connected in parallel to the bath. This allowed instantaneous (< 1 s) changing of bath solutions. RCCs were elicited by cooling the preparation from 37° to 0–2°C by changing the solution. RCCs were obtained in place of a normal electrical twitch (steady state RCC) or after variable periods of rest (post-rest RCC).

Biphasic contraction Biphasic contraction in canine trabeculae was produced as described by King & Bose (1983). Trabeculae were equilibrated in KH solution by stimulating at 0.5 Hz for 1 hour. This was followed by replacement of solution with SrKH (identical composition to KH except that it contained 2.5 mM Sr^{2+} instead of $CaCl_2$), which produced a series of changes in the shape of contractions. After 10–20 min in SrKH, the two components designated as phase 1 (P1) and phase 2 (P2) had approximately equal tensions. The biphasic contraction was maintained by the addition of 0.1–0.2 mM Ca^{2+} to the external medium containing 2.5 mM Sr^{2+}.

Drugs

Ouabagenin and sotalol were obtained from Sigma Chemical Co. and solutions were made in distilled deionized water. All chemicals for physiological solutions were reagent grade.

Statistics

Statistical significance was determined with Student's *t* test. The level of significance chosen for all experiments was $P < 0.05$. Data have been expressed as mean ± s.e.mean.

Results

Positive inotropic effect of extract

The dose-response relationship of the positive inotropic effect of the extract was studied by cumulative addition. Figure 1 shows the typical fast (1a and b) and slow (1c and d) recording of tension development in canine trabeculae. Addition of 50 µl of the extract produced a small initial negative inotropic effect followed by a marked positive inotropic response within a few seconds (Figure 1c). The maximum positive inotropic response was seen 2–3 min after addition of the extract. The log dose-response plot is shown in Figure 2 which clearly illustrates a sigmoid dose-response relationship with the maximum increase in developed tension of 501 ± 20%. The half maximal increase in developed tension (ED_{50}) was produced by 125 µl of the extract. Under similar experimental conditions, the dose-response relationship of ouabagenin was also studied. Maximum inotropic effect was produced by 10^{-6} M ouabagenin. Higher concentrations of ouabagenin caused aftercontractions, increase in diastolic tension and decrease in active tension. The positive inotropic effect of the extract is expressed as a percentage of the maximum response to ouabagenin in Figure 2b. A volume of 100 µl of the extract was approximately equipotent to 10^{-6} M ouabagenin and 300–350 µl extract produced 300% greater inotropy compared to that produced by a maximum concentration of ouabagenin. In addition to the increase in developed tension, the duration of contraction was also prolonged in the presence of the extract (Figure 1a). The prolongation of the total contraction duration was caused by the prolonged time to peak tension as well as by a slowing of relaxation. The prolongation of contraction duration was dose-dependent and the maximum prolongation of contraction occurred with a similar dose (200–350 µl) to that producing near maximum increase in developed tension. The duration of contraction was unchanged in the presence of ouabagenin (Figure 1b).

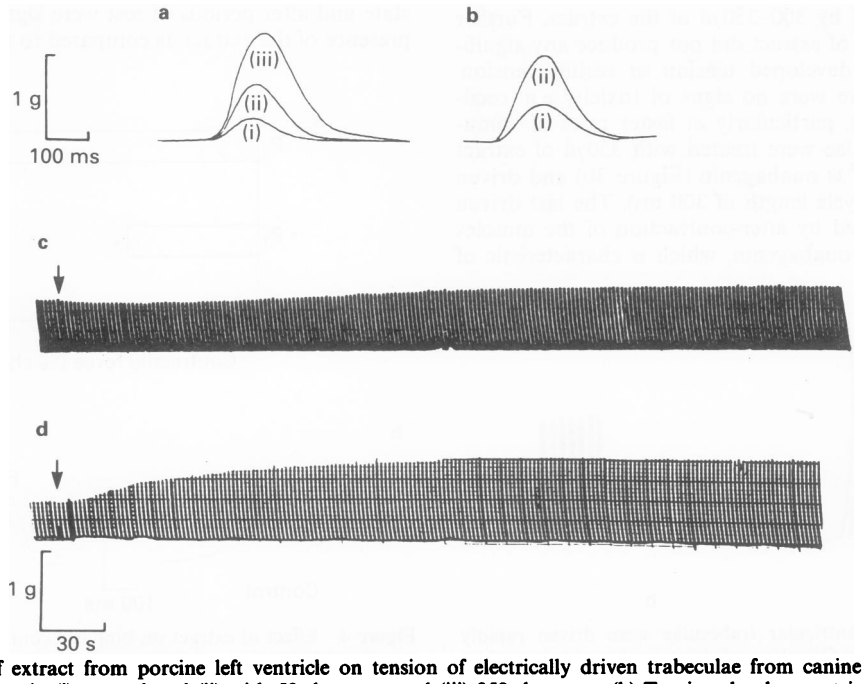

Figure 1 Effect of extract from porcine left ventricle on tension of electrically driven trabeculae from canine right ventricle. (a) Tension development in (i) control and (ii) with 50 µl extract and (iii) 350 µl extract. (b) Tension development in (i) control and (ii) ouabagenin 10^{-6} M; (c) and (d) show the time course of positive inotropy in response to the extract (5 µl) and ouabagenin (10^{-6} M) respectively at slow recording speed.

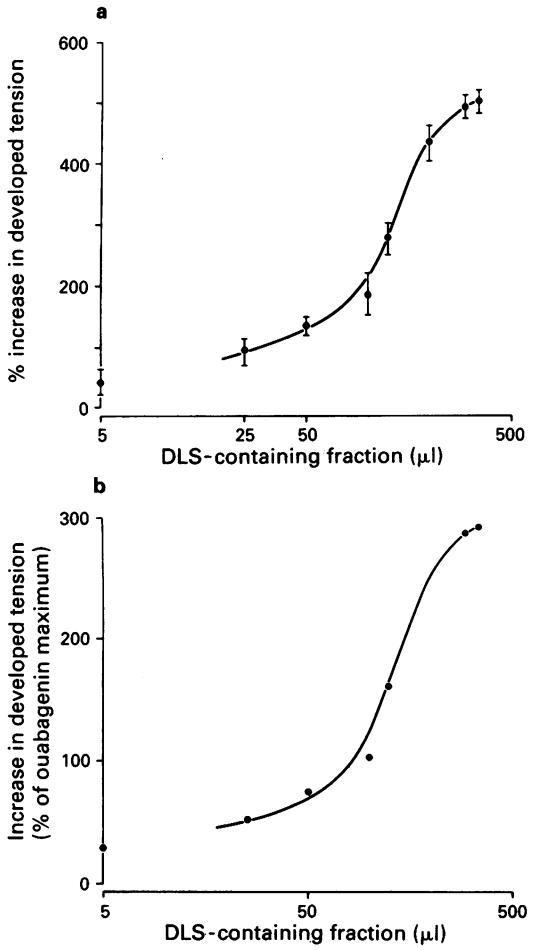

Figure 2 (a) Cumulative log dose-effect of the extract on the contractile force of canine trabeculae. (b) Cumulative log-dose response to the extract as a percentage of the maximum positive inotropic response to ouabagenin (10^{-6} M). Results are shown as mean of 5 experiments; s.e.mean indicated by vertical bars in (a).

Absence of digitalis-like toxicity

As demonstrated in Figure 2, the maximum inotropic response was produced by 300–350 μl of the extract. Further increase in the amount of extract did not produce any significant change in either developed tension or resting tension. More importantly, there were no signs of toxicity e.g. oscillatory aftercontractions, particularly at faster rates of stimulation. Isolated trabeculae were treated with 350 μl of extract (Figure 3a) or 5×10^{-6} M ouabagenin (Figure 3b) and driven at a faster rate (basic cycle length of 300 ms). The last driven contraction was followed by after-contraction of the muscles that were treated with ouabagenin, which is characteristic of digitalis toxicity. However, the muscles that were treated with the extract did not manifest these aftercontractions.

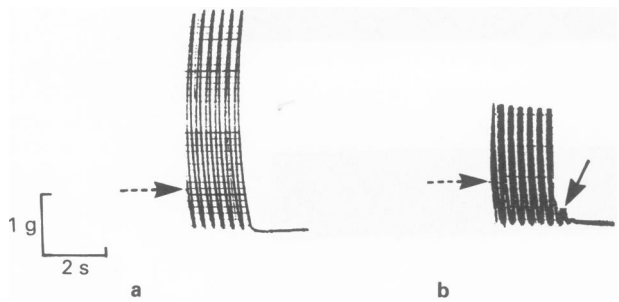

Figure 3 Canine right ventricular trabeculae were driven rapidly (basic cycle length 300 ms). Cessation of drive in the presence of (a) 350 μl of extract and (b) 5×10^{-6} M ouabagenin. Arrow with dashed line depicts active tension developed prior to addition of the agent. Solid arrow shows aftercontraction in the presence of ouabagenin.

Biphasic response

The biphasic contractions were produced by replacing 92–96% of calcium in the bathing solution with strontium. These conditions produced a prolonged contraction with two distinct phasic components, the early component P1 and the late component P2. Addition of 20–40 μl of extract in the presence of 10^{-5} M sotalol (a β-adrenoceptor antagonist) produced a substantial increase in the amplitude of the latter component P2 and a smaller increase in P1 (Figure 4b). The pooled data from 5–6 experiments are illustrated in Figure 4a. It demonstrates that the extract produces significant increase in the amplitude of P1 and P2 but the effect on P2 is greater than that on P1.

Rest potentiation

Figure 5 shows the contractile response of canine isolated right ventricular trabeculae to 30 s, 2 and 8 min rest periods. The control muscle (Figure 5a) shows post-rest potentiation after all three rest intervals. Addition of DLS containing extract produced rest depression after 8 min rest and the amplitudes of post-rest contraction after 30 s and 2 min were smaller than that of control muscle.

Rapid cooling contracture (RCC)

The typical response of the isolated trabeculae to rapid cooling is illustrated in Figure 6. The control muscle shows a progressive decrease in the amplitude of the cooling contracture with increasing duration of rest prior to cooling (Figure 6a). In the presence of extract (200 μl) (Figure 6b), there was a decrease in the amplitude of the post-rest RCC at all rest durations. The steady state RCC after the addition of the extract was greater than that in the control muscle. However, its size when expressed as a percentage of the preceding steady state contraction, was decreased compared to control steady state RCC similarly expressed. The pooled data from 4–5 experiments are depicted in Figure 7. It can be clearly seen that the amplitudes of rapid cooling contracture at steady state and after periods of rest were significantly smaller in the presence of the extract as compared to control muscle.

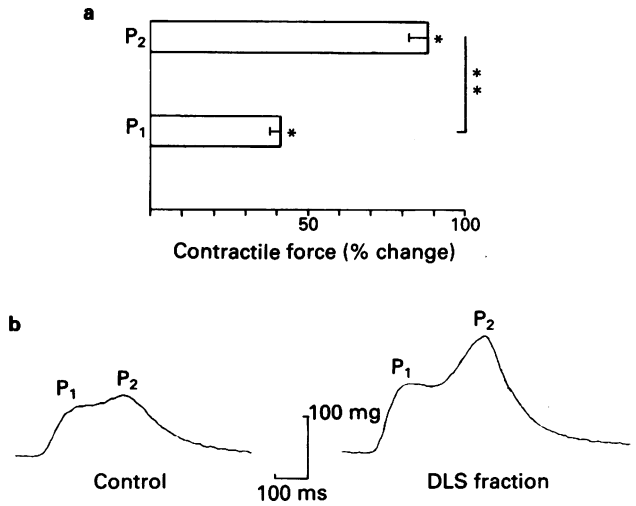

Figure 4 Effect of extract on biphasic contraction produced by substituting 92–96% external Ca by Sr. (a) Pooled data from 5 experiments showing the effect of 20 μl of extract on the two components (P1 and P2) of the biphasic contraction. (b) Effect of extract on biphasic contraction showing the predominant effect on P1.

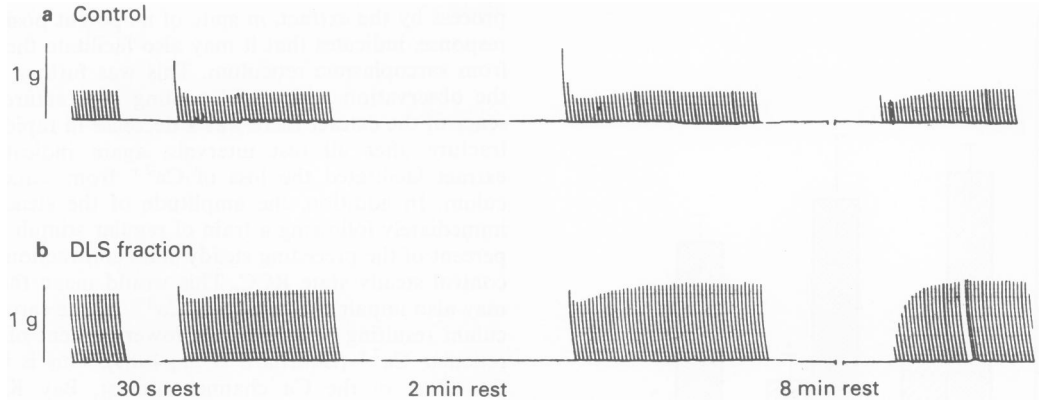

Figure 5 Typical response of canine ventricular trabeculae to varying periods of rest in an untreated muscle (a) and after the treatment with extract (b).

Discussion

The present study was undertaken in order to characterize the positive inotropic effect of DLS-containing extract from porcine left ventricle. In agreement with our previous data (Khatter *et al.*, 1986), the present results confirmed that the extract is as potent a positive inotropic agent in the dog as it is on guinea-pig heart. As described earlier, the extract is an inhibitor of the Na pump and displaces [^{3}H]-ouabain from its high affinity binding sites (Khatter *et al.*, 1986; Agbanyo & Khatter, 1990). Hence it is reasonable to hypothesize that the inotropic response may also be produced by the inhibition of Na^+, K^+-ATPase and subsequent effect on Na^+-Ca^{2+} exchange and elevation of intracellular Ca^{2+}. In support of our hypothesis, the studies on biphasic contraction have indicated that the extract primarily increases the delayed slow component (P2), which is caused by divalent cation entry across sarcolemma (King & Bose, 1983). The extract also increased the amplitude of the early component (although to a smaller extent than P2). This indicates an increase in the release of activator cation from the sarcoplasmic reticulum. The latter effect may in fact be a secondary response to increased sarcolemmal Ca^{2+} influx. Cardiac glycosides which cause inotropy via an effect on Na^+-Ca^{2+} exchange also increase P2 (King, 1982). However, unlike cardiac glycosides which either decrease or do not change the duration of contraction, the extract significantly prolonged it. This was due to the prolonged time to peak tension and half maximal relaxation. The duration of contraction is determined by the duration of sarcolemmal Ca^{2+} influx, sarcoplasmic reticular Ca^{2+} release as well as Ca^{2+} re-uptake (Langer, 1973). Hence if the extract increases the influx of Ca^{2+} via voltage-gated calcium channels, it may prolong the duration of the action potential in a similar manner to a calcium channel agonist (January *et al.*, 1988) and thus may also prolong the contraction duration. Alternatively, the extract may increase the influx of Ca^{2+} in a manner similar to digitalis but may also have an additional effect on the sarcoplasmic reticulum that may be responsible for the prolongation of contraction.

Aftercontractions after rapid pacing of the heart are characteristic of digitalis toxicity (Ferrier & Moe, 1973) and are caused by the oscillatory release of Ca^{2+} during diastole from the Ca^{2+} overloaded sarcoplasmic reticulum (Tsien *et al.*, 1978). The absence of digitalis-like toxicity even with the maximum dose of DLS-containing extract would indicate that either the increased trans-sarcolemmal influx of Ca^{2+} by a digitalis-like mechanism is matched by a concomitant increase in efflux, or the extract has an additional effect which protects the cardiac tissue from calcium overload. In any event, the absence of Ca^{2+} overload type toxicity would suggest that the extract may have better inotropic characteristics than digitalis.

In the present study we also investigated the effect of the extract on post-rest potentiation and rapid cooling contracture, which provide an estimate of the functional capacity of sarcoplasmic reticulum to store and release Ca^{2+} (Hryshko *et al.*, 1989a,b). In the presence of extract, there was a reduction in the extent of post-rest potentiation after 30 s and 2 min rest and a conversion of post-rest potentiation to post-rest depression after rest for 8 min. Much experimental evidence suggests that post-rest potentiation is due to the redistribution of calcium within the sarcoplasmic reticulum during diastole (Bers, 1985; Bridge, 1986; Bers *et al.*, 1987; Bose *et al.*, 1988)

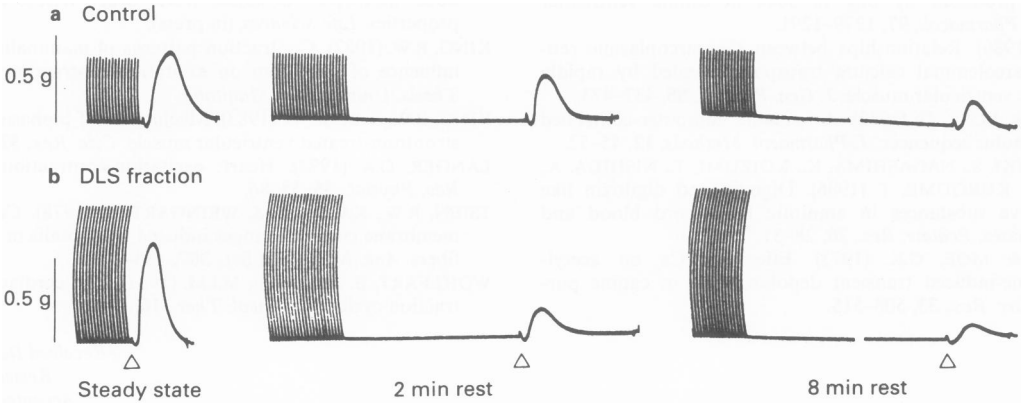

Figure 6 Typical response of canine trabeculae to rapid cooling from 37°C to 0°C in less than a second. The rapid cooling contractures were obtained in place of a regularly driven beat or after 2 and 8 min of rest period. The arrows under the individual recordings represent the time of initiation of cooling. (a) Represents the control tracing and (b) tracing after administration of extract from porcine left ventricle.

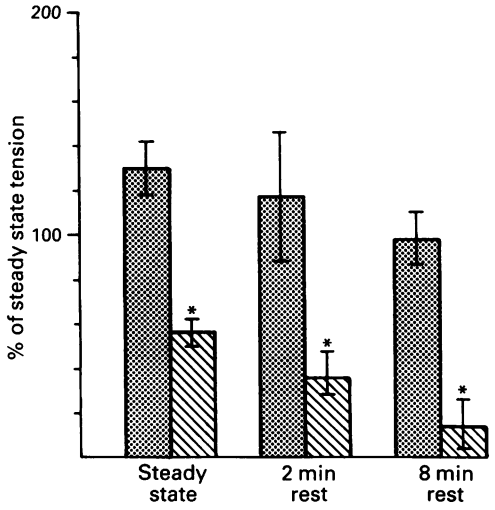

Figure 7 Pooled data from 4 experiments demonstrating the effect of extract on rapid cooling contracture during steady state and after 2 and 8 min of rest. *Significantly different from appropriate control value ($P < 0.05$).

and the progressive reduction in the post-rest contraction amplitude with increasing duration of rest is due to the continuous but slow loss of calcium from the sarcoplasmic reticulum during diastole (Wohlfart & Noble 1982; Bose *et al.*, 1988). The present observation of enhancement of the latter process by the extract, in spite of its potent positive inotropic response, indicates that it may also facilitate the loss of Ca^{2+} from sarcoplasmic reticulum. This was further supported by the observation with rapid cooling contracture. In the presence of the extract there was a decrease in rapid cooling contracture after all rest intervals, again indicating that the extract facilitated the loss of Ca^{2+} from sarcoplasmic reticulum. In addition, the amplitude of the steady state RCC immediately following a train of regular stimuli was a smaller percent of the preceding steady state contraction compared to control steady state RCC. This would mean that the extract may also impair the uptake of Ca^{2+} by the sarcoplasmic reticulum resulting in a relatively lower content of sarcoplasmic reticular Ca^{2+} (Bouchard *et al.*, 1989). This is in contrast to the effect of the Ca channel agonist, Bay K 8644 which increases the amplitude of steady state RCC even though it, like the extract, decreases the size of the RCC after various durations of rest.

In summary, the present study indicates that the DLS-containing extract from porcine left ventricle produces a positive inotropic response by increasing the influx of sarcolemmal Ca^{2+}. It also has additional effects on the sarcoplasmic reticulum which may facilitate the loss and/or inhibit the uptake of Ca^{2+} and which may also be responsible for the prolonged contraction duration. Electrophysiological studies to define the effect of the extract on transmembrane action potential and on whole cell ionic current(s), however, are necessary to elucidate further its mechanism of action.

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References

AGBANYO, M. & KHATTER, J.C. (1990). Purification and characterization of endogenous digitalis-like substance (DLS) from pig heart. *Research Commun. Chem. Pathol. Pharmacol.*, **68**, 41–54.

BALZAN, S., GHIONE, S., CLERICO, A. & MONTALI, K. (1986). Correlation between endogenous digoxin-like immunoreactivity and ^{3}H-ouabain displacement on erythrocyte membranes in extracts of human plasma. *Clin. Biochem.*, **19**, 311–314.

BERS, D.M. (1985). Ca influx and sarcoplasmic reticulum Ca release in cardiac muscle activation during post-rest recovery. *Am. J. Physiol.*, **248**, H366–H381.

BERS, D.M., BRIDGE, J.H.B. & MACLEOD, K.T. (1987). The mechanism of ryanodine action in cardiac muscle assessed with calcium-sensitive microelectrode and rapid cooling contracture. *Can. J. Physiol. Pharmacol.*, **65**, 610–618.

BOSE, D., KOBAYASHI, T., BOUCHARD, R.A. & HRYSHKO, L.V. (1988). Scattered light intensity fluctuations in the canine ventricular myocardium: correlation with inotropic drug effect. *Can. J. Physiol. Pharmacol.*, **66**, 1232–1238.

BOUCHARD, R.A., HRYSHKO, L.V., SAHA, J.K. & BOSE, D. (1989). Effects of caffeine and ryanodine on depression of post-rest tension development produced by Bay K 8644 in canine ventricular muscle. *Br. J. Pharmacol.*, **97**, 1279–1291.

BRIDGE, J.H.B. (1986). Relationships between the sarcoplasmic reticulum and sarcolemmal calcium transport revealed by rapidly cooling rabbit ventricular muscle. *J. Gen. Physiol.*, **88**, 437–473.

BOYECHKO, G. & BOSE, D. (1982). A versatile computer-controlled biological stimulus sequencer. *J. Pharmacol. Methods*, **12**, 45–52.

EBARA, H., SUZUKI, S., NAGASHIMA, K., KOIZUMI, T., NISHIDA, A., KANBE, Y. & KUROUME, T. (1986). Digoxin and digitoxin like immunoreactive substances in amniotic fluid, cord blood and serum of neonates. *Pediatr. Res.*, **20**, 28–31.

FERRIER, G.R. & MOE, G.K. (1973). Effect of Ca on acetyl-strophanthidine-induced transient depolarization in canine purkinje tissue. *Circ. Res.*, **33**, 508–515.

GRUBER, R.A., WHITAKER, J.M. & BUCKALEV, V.M. (1980). Endogenous digitalis-like substance in plasma of volume-expanded dogs. *Nature*, **287**, 7430–7480.

HRYSHKO, L.V., BOUCHARD, R.A., CHAU, T. & BOSE, D. (1989a). Inhibition of rest-potentiation in canine ventricular muscle by the calcium channel agonist BAY K 8644: comparison with caffine. *Am. J. Physiol.*, **257**, H399–H406.

HRYSHKO, L.V., KOBAYASHI, T. & BOSE, D. (1989b). Possible inhibition of canine ventricular sarcoplasmic reticulum by BAY K 8644. *Am. J. Physiol.*, **257**, H407–H414.

JANUARY, C.T., RIDDLE, J.M., & SALATA, J.J. (1988). A model for early afterdepolarization: Inductions with the calcium agonist BAY K 8644. *Circ. Res.*, **62**, 562–571.

KELLY, R.A., O'HARA, D.S, CANESSA, M.L., MITCH, W.E. & SMITH, T.W. (1985). Characterization of digitalis-like factors in human plasma. *J. Biol. Chem.*, **260**, 11396–11406.

KHATTER, J.C., AGBANYO, M. & HOESCHEN, R.J. (1986). Endogenous digitalis-like substance in pig left ventricle. *Life Sciences*, **39**, 2483–2492.

KHATTER, J.C., AGBANYO, M. & NAVARATNAM, S. (1990). Endogenous inotropic substance from heart tissue has digitalis-like properties. *Life Sciences*, (in press).

KING, B.W. (1982). Contraction patterns of mammalian myocardium: influence of strontium on excitation-contraction coupling. *Ph.D. Thesis, University of Manitoba.*

KING, B.W. & BOSE, D. (1983). Mechanism of biphasic contractions in strontium-treated ventricular muscle. *Circ. Res.*, **52**, 65–75.

LANGER, G.A. (1973). Heart: excitation-contraction coupling. *Ann. Rev. Physiol.*, **35**, 55–86.

TSIEN, R.W., KASS, R.S. & WEINGART, R. (1978). Calcium ions and membrane current changes induced by digitalis in cardiac purkinje fibers. *Ann. N.Y. Acad Sci.*, **307**, 483–490.

WOHLFART, B. & NOBLE, M.I.M. (1982). The cardiac excitation contraction cycle. *Pharmacol. Ther.*, **16**, 1–43.

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Pharmacological studies of human erectile tissue: characteristics of spontaneous contractions and alterations in α-adrenoceptor responsiveness with age and disease in isolated tissues

[1]George J. Christ, *Saul Maayani, Mira Valcic & Arnold Melman

Department of Urology, Montefiore Medical Center of the Albert Einstein College of Medicine, Bronx, NY 10467, and *Departments of Anesthesiology and Pharmacology, Mount Sinai School of Medicine, CUNY, NY 10029, U.S.A.

1 The pathophysiology of impotence related to vascular smooth muscle dysfunction in the male corpus cavernosum was studied on human isolated erectile tissue (HET). Studies were conducted on 140 sections of HET obtained from 38 male patients undergoing surgery for implantation of penile prostheses to correct underlying erectile dysfunction.

2 Spontaneous myotonic oscillations were characteristic of greater than 90% of all HET preparations at 37°C. These spontaneous oscillations were markedly attenuated by indomethacin, BW755C, nifedipine, removal of extracellular Ca^{2+}, or lower temperatures ($\leqslant 32°C$), but were not sensitive to inhibition by atropine, phentolamine or tetrodotoxin. Our data suggest that the oscillations may, at least in part, result from the generation and/or release of a stable cyclo-oxygenase product and a consequent increase in transmembrane Ca^{2+} influx.

3 The phenylephrine-induced contractions in HET may be reliably assayed up to 24 h after surgical removal, without significant alterations in the EC_{50}, maximum response (E_{max}) or slope index of the steady-state concentration-response curve to phenylephrine.

4 The competitive and surmountable nature of the antagonism of phenylephrine-induced contractions by prazosin and yohimbine allowed calculation of antagonist dissociation constants. The calculated pK_b values for prazosin and yohimbine, respectively, were 9.47 ± 0.49 and 5.54 ± 0.22. The rank order of agonist potency in HET was: noradrenaline = phenylephrine $\gg$ clonidine. These data indicate the presence of a population of membrane receptors that are predominantly of the α_1-adrenoceptor subtype.

5 The entire patient population was stratified on a decennial basis into five age groups, and each age group was subsequently subdivided into diabetic and nondiabetic diagnostic categories. With respect to the steady-state phenylephrine concentration-response curves, a Winer two-factor analysis of variance revealed a significant effect of age on the calculated pEC_{50} value, as well as a significant age-diagnosis interaction. A *post hoc* statistical analysis for unpaired samples yielded significant differences between pEC_{50} values for diabetic and nondiabetic patients in age groups 41–50 and 61–70 years. In addition, a Winer two-factor analysis of variance also detected a significant effect of age on the calculated E_{max} value.

6 In conclusion, our studies demonstrate that spontaneous contractions in HET are likely to be mediated by the generation and release of a stable cyclo-oxygenase product. Furthermore, the results of both agonist and antagonist studies are consistent with the presence of a homogeneous α_1-adrenoceptor population. Lastly, the responsiveness of isolated HET to phenylephrine was shown to be altered by both age and disease.

Introduction

Alterations in tissue responsiveness to endogenous substances may play a role in the aetiology of a variety of vascular disease states. For example, such alterations are among the contributing factors to the vascular complications of diabetes mellitus (Brody & Dixon; 1964; Fortes *et al.*, 1983; Factor *et al.*, 1984; White & Carrier, 1986) and perhaps essential hypertension, coronary vasospasm, Raynaud's syndrome (van Zwieten, 1987; Vanhoutte, 1987; Hollenberg, 1987) and male impotentia as well (see below). However, despite recently demonstrated interspecies differences in vascular responsiveness to endogenous substances (Malomvolgyi *et al.*, 1988), relatively little work has focused on the study of vascular pathophysiology utilizing human isolated tissue preparations (Docherty, 1987; Godfraind *et al.*, 1988).

The human erectile tissue (HET) preparation has been used by several laboratories to study the pharmacology of human vascular smooth muscle (Adaikan & Karim, 1981; Fovaeus *et al.*, 1987; Heaton, 1989; Saenz de Tejada *et al.*, 1989a,b; Christ *et al.*, 1989). HET is a readily available smooth muscle preparation obtained from the vascular sinusoids of the male corpus cavernosum. The therapeutic success of intracorporal pharmacotherapy in the treatment of erectile dysfunction has highlighted the central role of corporal smooth muscle in the erectile process and has revealed that relaxation of this tissue is impaired in a large proportion of impotent men (Melman *et al.*, 1981; Juenemann *et al.*, 1986; Lue & Tanagho, 1987; Christ *et al.*, 1989; Saenz de Tejeda *et al.*, 1989a). Thus, isolated HET is a natural preparation for the study of potential age- and disease-related alterations in erectile function, and perhaps other human vascular smooth muscle disorders as well.

The current preponderance of evidence, both *in vitro* and *in vivo*, indicates that maintenance of the flaccid penile state is, at least in part, related to release of noradrenaline from sympathetic nerves and contraction of corporal smooth muscle subsequent to activation of a homogeneous postsynaptic α-adrenoceptor population (Adaikan & Karim, 1981; Hedlund & Andersson, 1985a; Saenz de Tejada *et al.*, 1989a,b). Although binding studies in membrane preparations of HET also suggest the presence of a single population of α-adrenoceptors (Levin & Wein, 1980), a conclusive pharmacological classification of the receptor that mediates

[1] Author for correspondence.

phenylephrine- and noradrenaline-induced contractions at the intact tissue level, as either the α_1- or α_2-adrenoceptor subtype, is still lacking. Additionally, there are no rigorous descriptions of the characteristics of the spontaneous contractile activity commonly observed in HET *in vitro*. This paper addresses these issues, details the conditions under which isolated HET may be reliably used to study some aspects of the pharmacology of human vascular smooth muscle and demonstrates that the characteristics of phenylephrine-induced steady-state contractions in HET are both age- and pathology-dependent.

Methods

Tissue preparation

Four to eight sections of human corpus cavernosal tissue were obtained from each of 38 patients undergoing surgery for implantation of penile prostheses. The patients age ranged from 31 to 84 years, and this population consisted of three broad diagnostic groups; (1) psychogenic impotence, (2) impotence due to organic dysfunction (e.g. diabetes, Peyronie's disease, vasculogenic), and (3) impotence secondary to pharmacotherapy (e.g., β-adrenoceptor blockade).

Sections of HET approximately 2 × 2 × 6 mm in size, were suspended between two small fish hooks in 20 ml organ chambers containing Krebs-Henseleit buffer of the following composition (mM): NaCl 110, KCl 4.80, $CaCl_2$ 2.5, $MgSO_4$ 1.20, KH_2PO_4 1.20, $NaHCO_3$ 25.0 and dextrose 11.0, in glass distilled water. Organ chambers were maintained at 37 ± 1°C and were continuously aerated with 95% O_2 and 5% CO_2 to maintain pH = 7.4 ± 0.1. The sections of HET were initially suspended at 2–3 g basal tension, washed periodically with fresh buffer, and allowed to stabilize at 37°C over a period of 90–120 min. When a stable resting tension was attained, the tissues were set to 2 g basal tension.

Tissues were primed twice (at 37°C) by the addition of 10 μM phenylephrine to the organ bath. Contractions were measured isometrically with a Grass Force Displacement Transducer (Model FT-03), and recorded on a Grass Polygraph (Model 7D). After a steady-state contraction was achieved, the drug was removed from the tissue by replacing the contents of the bath at least 3 times with fresh buffer.

Construction of concentration-response curves (CRCs) and receptor classification studies

For these studies, the organ bath temperature was lowered to 32°C and concentration-response curves to phenylephrine, noradrenaline or both were constructed by the cumulative addition of drug at half log unit increments. In the α-adrenoceptor classification studies, a control concentration-response curve to phenylephrine was first obtained, and then the tissues were washed with fresh buffer several times and allowed to relax to baseline tension over the next 60–90 min. When a stable baseline tension was reached, either prazosin (0.5 nM) or yohimbine (3–100 μM) was added for 30 min before construction of a second concentration-response curve on the same tissue.

Stratification of the patient population

For the purposes of statistical analysis, the patient population was stratified by decade into 5 age groups (see Results), with each age group subsequently subdivided into two distinct diagnostic groups: (1) those patients whose impotence was associated with diabetes mellitus (both insulin-dependent and non-insulin-dependent diabetics were grouped together) and (2) those patients with impotence associated with organic manifestations other than diabetes (see above) or who were diagnosed as having psychogenic impotence.

Data analysis

The magnitude of the spontaneous oscillations was calculated from the index recently proposed by Katusic *et al.*, (1988). Briefly, rhythmic activity was quantified by eye, as the product of the mean contractile amplitude and the frequency during a 10 min period.

Concentration-response curve data were analyzed on an IBM compatible computer using the RS/1 software package (BBN; Cambridge, MA). Briefly, concentration-response curve data were fitted to a simple logistic equation of the form:

$$E = E_{max}/\{1 + (EC_{50}/[D]^{n_H}\} \quad (1)$$

where E is the observed effect in g of tension, [D] is the molar concentration of drug, E_{max} is the calculated maximal effect, EC_{50} is the concentration of agonist necessary for one half E_{max}, and n_H is the slope factor. EC_{50} values are expressed as the negative logarithm of the geometric mean (pEC_{50}) ± s.e.mean, while all other logistic parameters are expressed as the arithmetic mean ± s.e.mean.

Since Chi-Square analysis revealed that the slope of the Schild plot (Arunlakshana & Schild, 1959) for yohimbine antagonism of the phenylephrine-induced contraction in HET was not significantly different from unity, and that prazosin antagonism of the phenylephrine-induced contraction was also consistent with competitive and surmountable antagonism, antagonist dissociation constants were determined from Equation 2:

$$pK_b = \frac{\log[B]}{DR - 1} \quad (2)$$

where B is the molar concentration of antagonist, DR (dose ratio) is the ratio of the EC_{50} doses before and after addition of antagonist to the organ bath and pK_b is the antagonist dissociation constant, expressed as the negative logarithm of the geometric mean ± s.e.mean.

Statistical analysis

All statistical calculations were performed with Apple Macintosh-based Statview II software. A Winer two-factor analysis of variance for balanced samples was used to assess the effects of age and disease on the logistic parameters E_{max} and pEC_{50}. When a significant effect of age was detected, a *post hoc* Scheffe test was used for all paired comparisons among age groups. When a significant age-diabetes interaction was detected, a *post hoc* Scheffe test was also used for all paired comparisons among age groups within each diagnostic category and Student's *t* test for unrelated samples was used for comparison between diagnostic categories within each age group.

Phenylephrine, noradrenaline, prazosin, yohimbine, phentolamine, atropine, propranolol, ethyleneglycol-*bis*-N,N,N,N-tetraacetate (EGTA), tetrodotoxin, indomethacin, hydrocortisone and clonidine were obtained from Sigma Chemical Company (St Louis, MO, U.S.A.). Nifedipine and Bay K 8644(1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3-pyridinecarboxylic acid methyl ester) were obtained from Research Biochemicals Incorporated (RBI, Natick MA, U.S.A.), cocaine was obtained from the Montefiore Hospital Pharmacy, and BW755C (3-amino-1-[*m*-(trifluoromethyl)-phenyl]-2-pyrazoline) was the generous gift of Dr Michal Schwartzman; other compounds were of analytical grade.

Results

Characteristics of the spontaneous myotonic oscillations

During the initial tissue equilibration period at 37°C (90–120 min, see Methods), spontaneous myotonic oscillations

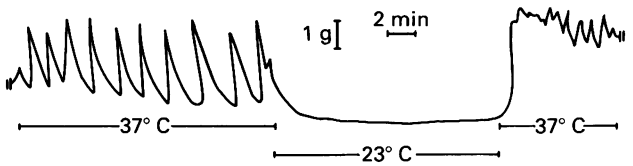

Figure 1 Representative tracing of an original polygraph recording, illustrating the temperature-sensitivity of the spontaneous myotonic oscillations in human isolated erectile tissue. Note changes in frequency and amplitude of the oscillations.

occurred in greater than 90% of all erectile tissue preparations from the same patient (Figure 1). The frequency and amplitude of the spontaneous contractions exhibited substantial variation among tissue sections taken from different patients as well as among individual tissue sections taken from the same patient. For example, attempts to quantitate the spontaneous oscillations using an index recently proposed by Katusic *et al.* (1988), revealed that the spontaneous activity varied over time by as much as 40% in the same HET preparation, while exhibiting greater than 10 fold variation among 8 different HET preparations from the same patient. Therefore, to permit pharmacological studies in this preparation, it was necessary to characterize some aspects of this oscillatory behaviour.

The observed spontaneous oscillatory contractions can be distinguished from sustained phenylephrine-induced contractions based on variety of criteria, as listed in Table 1. For example, the appearance of spontaneous oscillatory activity was inversely related to the ambient temperature; that is, oscillations were much less likely to occur at 32°C (not shown) than 37°C and were completely eliminated at 23°C (Figure 1). Figure 2a illustrates that chelation of extracellular Ca^{2+} by cumulative addition of EGTA caused a gradual cessation of spontaneous activity. Similarly, replacement of the Krebs buffer with Ca^{2+}-free/2 mM EGTA buffer also terminated spontaneous activity (Figure 2b). Addition of 0.1 μM nifedipine either prevented the appearance of the spontaneous myotonic oscillations or abolished those that were present (Figure 3), while the addition of 0.1 μM Bay K 8644 accelerated the appearance of oscillatory contractions in previously quiescent tissues, or amplified pre-existing oscillatory activity (Figure 3). The effects of nifedipine and Bay K 8644 were mutually antagonistic, regardless of their order of addition.

Addition of 10 μM indomethacin significantly attenuated the spontaneous activity in tissues taken from 7 out of 8 patients, as follows: it abolished the oscillations in 4 subjects, and decreased the amplitude and/or frequency of the spontaneous contractions in the HET preparations of 3 other patients, with no apparent effect on only 1 subject. BW755C (10–50 μM) caused a complete cessation of the spontaneous contractions in all tissues taken from 3 patients. Atropine (1 μM, 1 subject), phentolamine (1 μM, 1 subject), and tetrodotoxin (5 μM, 4

Table 1 Some discriminators of human erectile tissue tone generation

	Steady-state contraction phenylephrine (10 μM)	*Spontaneous oscillations*
Extracellular Ca^{2+} dependence	Yes	Yes
Nifedipine sensitivity (10 nM)	No	Yes
Bay K 8644 sensitivity (10 nM)	No	Yes
Temperature sensitivity	No	Yes
Prazosin or phentolamine sensitivity (1 μM)	Yes	No
Indomethacin sensitivity (10 μM)	No	Yes

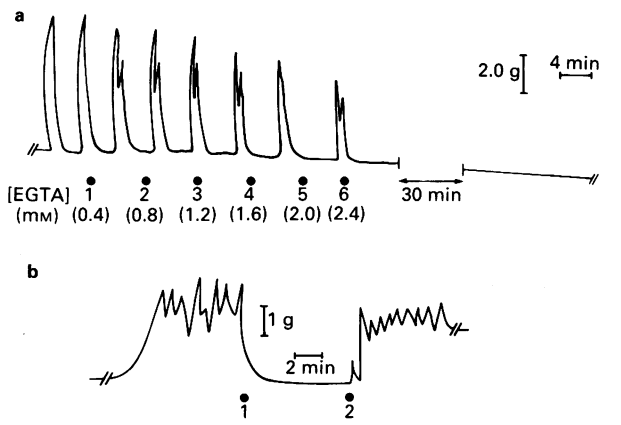

Figure 2 Dependence of spontaneous myotonic oscillations on extracellular $[Ca^{2+}]$. Illustrated are representative tracings of original polygraph recordings from two different human isolated erectile tissue preparations maintained at 37°C. Similar results were obtained in tissues from several other patients (not shown). Points 1–6 in (a) represent the cumulative addition of EGTA in 0.4 mM aliquots; note that the oscillations were restored upon re-addition of extracellular Ca^{2+} (not shown). In (b), (1) = Ca^{2+}-free/EGTA buffer; (2) = 2.5 mM Ca^{2+}. Also, note the difference in scale between (a) and (b). All concentrations refer to the final concentration in the organ bath.

subjects), had no effect on spontaneous activity. Furthermore, on a number of occasions we demonstrated that one can immediately induce oscillations in a quiescent tissue by exposing it to the buffer removed from the organ bath of an oscillating tissue.

Concentration-response curves to α_1-adrenoceptor agonists and assessment of response stability in the isolated HET preparation

Addition of phenylephrine at concentrations lower than 1 μM frequently induced oscillatory contractions at 37°C, thus complicating analysis of the concentration-response curve at concentrations below the EC_{50}. Addition of higher concentrations of phenylephrine elicited sustained tonic contractions. By lowering the bath temperature to 32°C we were able to obtain complete concentration-response curves to phenylephrine that were generally free from spontaneous activity (Figure 4).

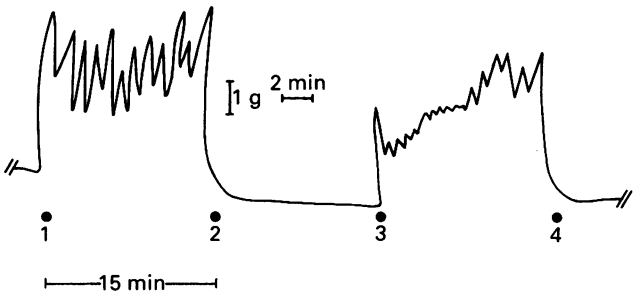

Figure 3 The effects of the dihydropyridines nifedipine and Bay K 8644 on spontaneous activity in human isolated erectile tissue (HET) and their mutual reversibility. Shown is a representative tracing of an original polygraph recording of an HET preparation maintained as 37°C, prior to the appearance of oscillatory activity. All concentrations represent the final free concentration in the organ bath. At point (1) addition of Bay K 8644 (0.1 μM) to a previously quiescent tissue elicited oscillatory activity; (2) = Ca^{2+}-free EGTA buffer which abolished oscillatory activity; (3) owing to the high lipophilicity of the dihydropyridines, they are very difficult to wash out of the tissue, thus re-addition of Ca^{2+} (2.5 mM) to the organ bath caused immediate oscillatory activity due to the presence of a residual amount of Bay K 8644; (4), the addition of nifedipine (0.1 μM) reversed the effects of Bay K 8644, and abolished spontaneous activity. Note change in time scale.

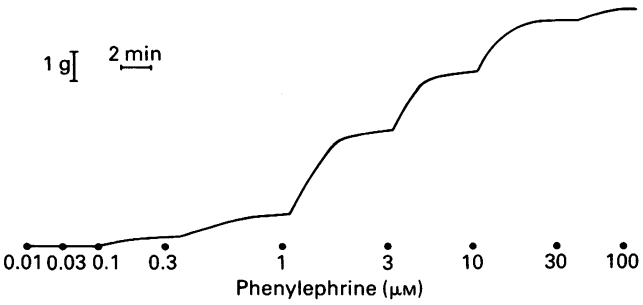

Figure 4 Representative tracing of the graded contractile response of human isolated erectile tissue to phenylephrine at 32°C. Note the absence of oscillations and the stability of the phenylephrine-induced contractile response.

By this method, concentration-response curves to phenylephrine were constructed on all 20 tissues from 5 patients at both 4 h and 24 h after dissection. A paired statistical analysis of the E_{max}, EC_{50} and slope factor values at 4 and 24 h revealed no significant time-dependent alterations of these parameters (Table 2). In addition, the maximal response to 10 μM phenylephrine was stable for up to 30 min without decay at both time intervals. However, despite the similarity in tissue size (see Methods), the calculated E_{max} values varied substantially among tissue sections taken from the same patient; 3 fold differences in maximal contraction were not uncommon.

Classification of the α-adrenoceptor subtype mediating contraction of HET

Antagonist studies Prazosin (0.5 nM) and yohimbine (3–100 μM) both produced rightward, parallel and surmountable shifts of the phenylephrine concentration-response curve that were consistent with simple competitive binding (Figure 5); while the pK_b value for yohimbine was calculated from the Schild equation (Arunlakshana & Schild, 1959), the pK_b value of prazosin was calculated using Equation 2 (see Methods). The mean pK_b values were 5.54 ± 0.22 (2.88 μM, $n = 8$ tissues from 3 patients) for yohimbine, and 9.47 ± 0.49 (0.34 nM, $n = 5$ tissues from 4 patients) for prazosin. These pK_b values are similar to those reported for antagonist inhibition of α-adrenoceptor responses in other isolated tissue preparations (Timmermans & Van Zwieten, 1982; Minneman & Abel, 1987; Bevan *et al.*, 1989) and reflect the predominance of the α_1-adrenoceptor subtype in HET.

Agonist studies Further receptor classification studies were performed with three agonists; phenylephrine, noradrenaline, and clonidine. Cumulative addition of phenylephrine (Figure 4) or noradrenaline (in the presence of metabolic inhibitors and tissue uptake blockers, see Methods) elicited graded contractile responses in HET. Computer fits of Equation 1 to observed concentration-response curve data yielded mean E_{max}, pEC_{50} and slope factor values of 5.36 ± 0.17, 5.94 ± 0.03 (1.15 μM) and 1.00 ± 0.02, respectively, for phenylephrine (158 tissue preparations from 39 patients), and 4.40 ± 0.50, 6.10 ± 0.08 (0.79 μM) and 0.97 ± 0.05 respectively for noradrenaline (14 tissue preparations from 5 patients). Paired statistical analysis of the concentration-response curve parameters for phenylephrine and noradrenaline on the same

Table 2 Time-dependent effects on phenylephrine-induced contraction

	E_{max} (g)	*Slope* pEC_{50}	(n_H)
Day 1 (4 h)	5.3 ± 0.47	6.06 ± 0.13	0.95 ± 0.05
Day 2 (24 h)	4.9 ± 0.56	5.86 ± 0.09	0.99 ± 0.07

The pEC_{50} values are given as the geometric mean ± s.e.mean, while all other values represent the arithmetic mean ± s.e.mean. The sample size (n) for phenylephrine is 20 tissue preparations from 5 patients. Paired analysis revealed no significant differences between Day 1 and Day 2 for any of the parameters derived from computer fits of the logistic equation to phenylephrine concentration-response curve data.

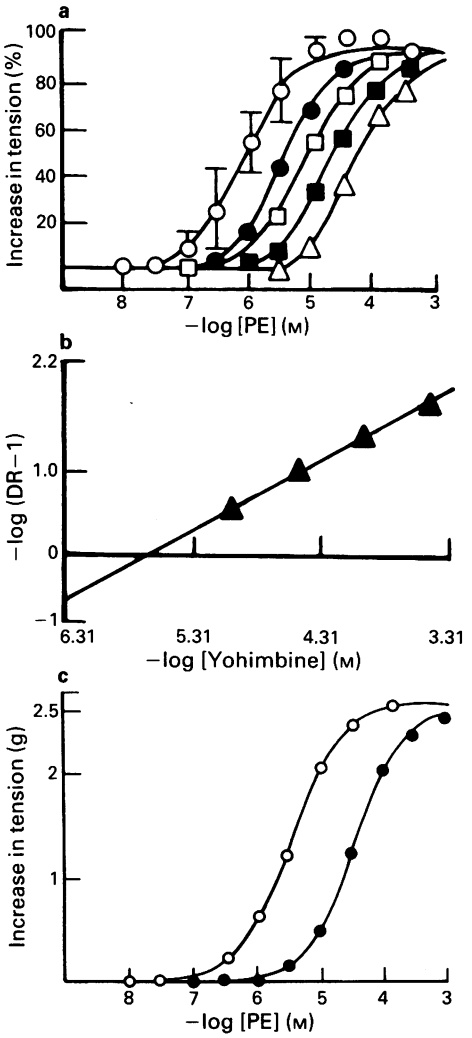

Figure 5 Characterization of the α-adrenoceptor subtype which mediates phenylephrine-induced contraction in human isolated erectile tissue. (a) Concentration-response curves to phenylephrine were normalized as a percentage of the maximal observed response to phenylephrine in each of four different tissues from the same patient. The control curve (○) represents the mean value (with s.e.mean shown by vertical bars) for all four tissues. Each tissue was then preincubated with a single concentration of yohimbine; 3 (●), 10 (□), 30 (■), or 100 (△) μM respectively, before repeating the concentration-response curve to phenylephrine. All concentration-response curves were computer-fitted to the logistic equation. (b) Schild plot of the concentration-response curve data from (a). The slope of the Schild plot was not different from unity, therefore, the slope was constrained to unity, and the K_b value was calculated from the Schild equation (K_b = 1.99 μM; pK_b = 5.70). (c) Representative computer-fitted curves to observed concentration-response curve data in the absence (○) and presence (●) of prazosin (0.5 nM). The prazosin K_b value was calculated using Equation 2 (see Methods) (K_b = 50 pM; pK_b = 10.3). Notice the complete surmountability and the parallel rightward shift in the phenylephrine concentration-response curve produced by both yohimbine and prazosin. All concentration-response curve data were collected at 32°C.

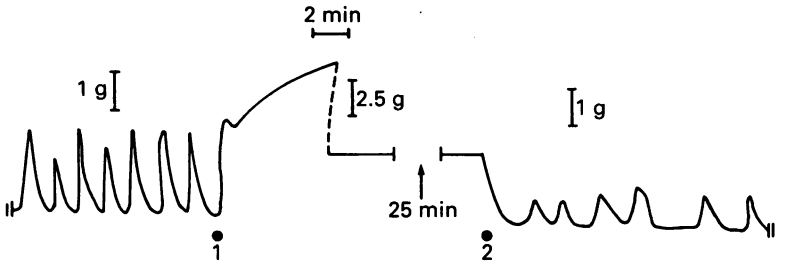

Figure 6 Reversible 'masking' of spontaneous contractions of human isolated erectile tissue (HET) by phenylephrine. The tonic contraction to 10 μM phenylephrine (1) reversibly 'masks' characteristic spontaneous activity. Shown is a representative tracing of an original polygraph recording taken from an HET preparation maintained at 37°C. Note the change in scale after the addition of phenylephrine and the change in the characteristics of the spontaneous activity upon removal of phenylephrine from the organ bath (2).

tissues revealed no significant differences. Furthermore, the contractile response to clonidine was variable and much smaller than that to phenylephrine. In addition, there was no detectable response to clonidine in the presence of 1 μM prazosin.

Effect of age and diagnostic category on the phenylephrine-induced steady-state response

The effects of the subject age and diagnostic category on the phenylephrine concentration-response curve parameters described above were evaluated. For this analysis, the entire patient population was stratified on a decennial basis, and each age group was subsequently subdivided into diabetic and nondiabetic diagnostic categories, respectively (see Methods). The value of the slope parameter (n_H) was not significantly different from unity (as determined by Chi-Square analysis) and thus was not included in this analysis. However, a Winer two-factor analysis of variance revealed a significant effect of age on the calculated pEC_{50} value ($P < 0.01$), as well as a significant age-diagnosis interaction ($P < 0.03$), but failed to detect any effect of diagnosis alone ($P = 0.43$). A *post hoc* Scheffe test revealed significant differences in the pEC_{50} values among the 5 age groups (Figure 7a). Simple linear regression analysis of pEC_{50} versus age for the diabetic tissues illustrates that the pEC_{50} value increases significantly with increasing age (correlation coefficient, $r = 0.82$, Figure 7b). There was no significant correlation between pEC_{50} and age for the nondiabetic tissues ($r = 0.06$). *Post hoc* comparisons of pEC_{50} values between the diabetic and nondiabetic categories within each age group revealed significant differences between the 2 diagnostic categories in age groups 41–50 and 61–70 (Table 3). In addition, paired comparisons among the 5 age groups within each diagnostic category revealed significant differences in pEC_{50} values for both diabetic and nondiabetic tissues (Table 3).

With respect to E_{max}, a Winer two-factor analysis of variance revealed a significant effect of age ($P < 0.01$), but no effect of diagnosis ($P = 0.86$), and no age-diagnosis interaction ($P < 0.06$). A *post hoc* Scheffe test yielded significant differences among the 5 age groups in the calculated E_{max} values (Figure 8b). The linear regression analysis shown in Figure 8b demonstrates that increases in E_{max} were significantly correlated with increasing age ($r = 0.85$).

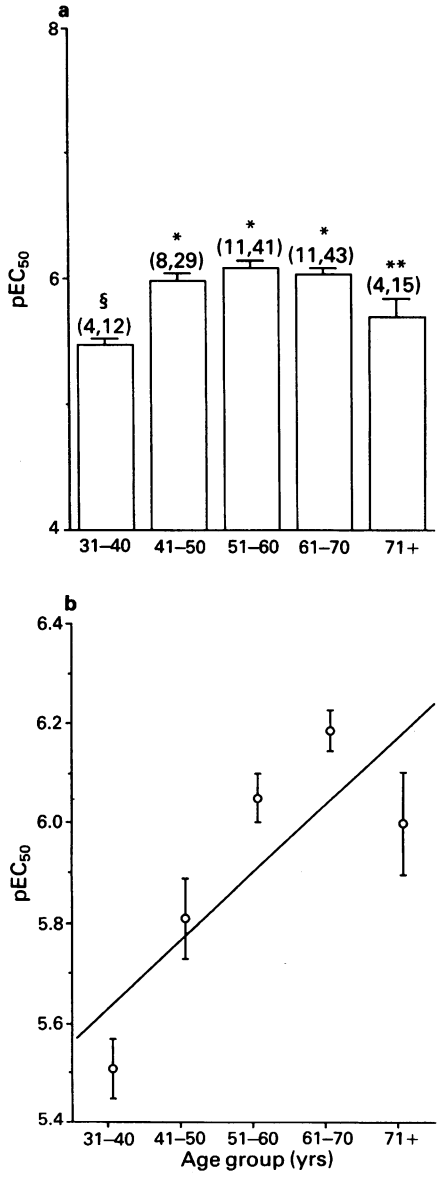

Figure 7 Effect of age and diagnosis on the pEC_{50} value. (a) Depicts the mean calculated pEC_{50} (negative logarithm of the geometric mean) values for each age group: *denotes significant difference from age group 31–40, $P < 0.05$; **denotes significant difference from age group 61–70, $P < 0.05$. (b) Depicts a simple linear regression of the mean pEC_{50} value versus age for the diabetic tissues and shows that the pEC_{50} is strongly positively correlated with age (correlation coefficient, $r = 0.82$). All values represent the mean with s.e.mean shown by vertical bars. In (a), §number to the left of comma denotes the number of subjects, while the number to the right of the comma denotes the total number of tissues; the number of subjects and tissues is the same for (a) and (b).

Discussion

One of the most noteworthy features of the isolated HET preparation is the characteristic myotonic automaticity observed at 37°C (Figure 1, Saenz de Tejada *et al.*, 1989b). The spontaneous contractions described here exhibited considerable variation with respect to both their amplitude and frequency (between patients, as well as among tissue sections taken from the same patient), and thus eluded attempts at a

Table 3 Effects of age and disease on the sensitivity of phenylephrine-induced contractions in human isolated erectile tissue

	Age group (years)				
pEC_{50}	31–40	41–50	51–60	61–70	71+
Diabetic	5.50 ± 0.06 (1,4)[a]	5.80[e] ± 0.08 (3,10)	6.04[c] ± 0.06 (6,24)	6.18[c] ± 0.06 (3,14)	5.99 ± 0.10 (1,4)
Nondiabetic	5.46 ± 0.09 (2,8)	6.07[b,c] ± 0.06 (5,19)	6.13[c,d] ± 0.11 (5,17)	5.96[b] ± 0.07 (8,29)	5.59 ± 0.18 (3,11)

[a] The number in parentheses to the left of the comma denotes the number of patients in each category, while the number to the right of the comma denotes the total number of tissues. [b] Denotes statistically significant difference from corresponding diabetic value within the same age group. [c] Denotes statistically significant difference from age group 31–40 within the same diagnostic category. [d] Denotes statistically significant difference from age group 71+ for the nondiabetic category. [e] Denotes statistically significant difference from age group 61–70 for the diabetic category. Statistical significance was determined as $P < 0.05$. pEC_{50} represents the negative logarithm of the geometric mean ± s.e.mean; where the s.e.mean is based on the total number of tissues.

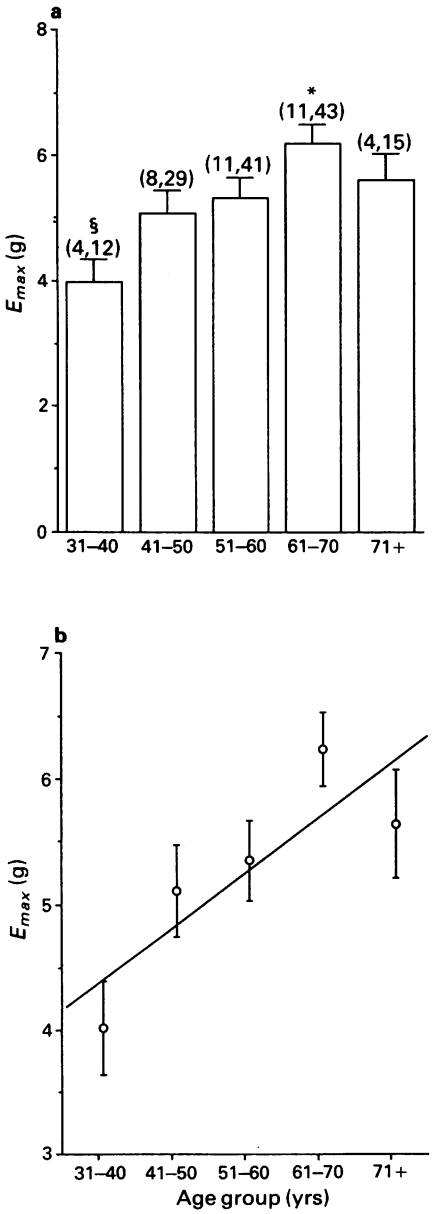

Figure 8 Effect of age on the phenylephrine E_{max}. (a) Depicts the mean E_{max} values for each age group: *denotes significant difference from age group 31–40, $P < 0.05$. (*b*) Depicts a simple linear regression of the mean E_{max} value versus age and shows that E_{max} is strongly positively correlated with age (correlation coefficient, $r = 0.85$). All values represent the mean with s.e.mean shown by vertical bars. In (a), §number to the left of comma denotes the number of subjects while the number to the right of the comma denotes the total number of tissues; the number of subjects and tissues is the same for (a) and (b).

quantitative description (see Results). Despite these limitations, our studies have revealed many interesting qualitative features of the spontaneous contractions. The fact that one can immediately induce oscillations in a quiescent tissue by exposing it to the buffer removed from the organ bath of an oscillating tissue strongly suggests that the oscillations are, at least in part, related to the generation and release of a stable endogenous substance.

The failure of atropine and phentolamine to inhibit spontaneous contractions demonstrates that the source of these contractions is not a rhythmic release of either endogenous acetylcholine or noradrenaline. In contrast, the inhibition of the spontaneous oscillations by either indomethacin or BW755C indicates that the oscillations may be mediated by products of the cyclo-oxygenase pathway, as we have previously suggested (Melman *et al.*, 1986) and has been shown in human isolated saphenous vein (Schoeffter & Godfraind, 1989). In fact, prostaglandin synthesis has been demonstrated in HET *in vitro* (Roy *et al.*, 1984), and addition of prostaglandin $F_{2\alpha}$ to HET actually induces rhythmic contractile activity (unpublished observations; Roy *et al.*, 1984; Hedlund & Andersson, 1985b). The properties of the spontaneous myotonic oscillations in HET described here are similar to those reported for phentolamine-induced oscillations in the guinea-pig bladder detrusor muscle (Satake *et al.*, 1984), as well as KCl-induced oscillations in the canine basilar artery (Vanhoutte, 1988). In the basilar artery, it was suggested that the rhythmic pattern of contraction was due to activation of endothelial cyclo-oxygenase with the subsequent release of vasoconstrictor prostaglandins and of prostacyclin; the latter of which exerts an inhibitory feedback on the activated cyclo-oxygenase, thus ensuring rhythmic contractions. Since electron microscopy has demonstrated the presence of endothelial cells in the HET preparation (unpublished observations), and other laboratories have shown this endothelium to be functionally active (Saenz de Tejada *et al.*, 1989a), a similar mechanism to that suggested for canine basilar artery may be responsible for generation of spontaneous activity in HET.

Previous studies concerning the classification of the α-adrenoceptor subtype mediating contraction in HET have relied primarily on the rank order of agonist potency, displacement of agonist concentration-response curves by a single concentration of antagonist (without calculation of antagonist affinity) and the relaxation of precontracted HET by selective α-adrenoceptor antagonists (Adaikan & Karim, 1981; Hedlund & Andersson, 1985a; Saenz de Tejada *et al.*, 1989b). Our studies include pK_b values calculated from antagonist studies under well established equilibrium conditions (Figure 5, see Results) and demonstrate that the phenylephrine- and noradrenaline-induced contractions in isolated HET are mediated by activation of predominantly α_1- and not α_2-adrenoceptors. In addition, we demonstrated a rank order of agonist potency consistent with the presence of a predominantly α_1-adrenoceptor population; i.e., noradrenaline = phenylephrine $\gg$ clonidine.

Pharmacological studies on human isolated vascular smooth muscle tissues represent an initial step toward understanding mechanisms of contraction and relaxation *in vivo* and, ultimately, toward characterization of alterations in vascular smooth muscle function related to age and pathology. At present, we are using the steady-state logistic parameters as an index for detecting fundamental alterations in the pharmacology of vascular smooth muscle with age or disease. This is because a significant difference in any one of the logistic parameters, whether among age groups or between diabetic and nondiabetic patients within the same age group, reflects a fundamental difference in the phenylephrine concentration-response relationship.

Our data demonstrate that the pEC_{50} was subject to significant variation with age (Figure 7a) and that, for the diabetic tissues but not the nondiabetic tissues, pEC_{50} was positively correlated with age (Figure 7b). These age- and pathology-dependent characteristics result in significant differences in the pEC_{50} between diabetic and nondiabetic tissues in certain age groups (Table 3). In addition, we demonstrated that the maximal phenylephrine-induced contraction in HET (E_{max}) is also positively correlated with age. Despite this strong correlation, a statistically significant difference in the mean E_{max} values among the 5 age groups was found only between age groups 31–40 and 61–70.

In conclusion, our studies demonstrate that the spontaneous contractile activity observed in HET is likely to be mediated by the generation and release of a stable cyclooxygenase product. Furthermore, the results of steady-state studies with both agonists and antagonists are consistent with the presence of a homogeneous α_1-adrenoceptor population. Lastly, the responsiveness of isolated HET to phenylephrine was shown to be altered by both age and disease.

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References

ADAIKAN, P.G. & KARIM, S.M.M. (1981). Adrenoceptors in the human penis. *J. Auton. Pharmacol.*, **1**, 199–203.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BEVAN, J.A., BEVAN, R.D. & SHREEVE, S.M. (1989). Variable receptor affinity hypothesis. *FASEB. J.*, **3**, 1696–1704.

BRODY, M.J. & DIXON, R.L. (1964). Vascular reactivity in experimental diabetes mellitus. *Circ. Res.*, **14**, 494–501.

CHRIST, G.J., VALCIC, M., MAAYANI, S. & MELMAN, A. (1989). Kinetic studies of contraction in human erectile tissue (HET) and rabbit aortic rings in vitro: modulation by papaverine and the dihydropyridine analog nifedipine. *Int. J. Impotence Res.*, **1**, 1–10.

DOCHERTY, J.R. (1987). The use of the human saphenous vein in pharmacology. *Trends. Pharmacol. Sci.*, **8**, 358–361.

FACTOR, S.M., MINASE, T., CHO, S., CAPASSO, J.M. & SONNENBLICK, E.H. (1984). Coronary microvascular abnormalities in the hypertensive-diabetic rat. *Am. J. Pathol.*, **116**, 9–20.

FORTES, Z.B., GARCIA LEME, J. & SCIVOLETTO, R. (1983). Influence of diabetes on the reactivity of mesenteric microvessels to histamine, bradykinin and acetylcholine. *Br. J. Pharmacol.*, **78**, 39–48.

FOVAEUS, M., ANDERSSON, K.E. & HEDLUND, H. (1987). Effects of some calcium channel blockers on isolated human penile erectile tissues. *J. Urol.*, **138**, 1267–1272.

GODFRAIND, T., MOREL, N. & WIBO, M. (1988). Tissue specificity of dihydropyridine-type calcium antagonists in human isolated tissues. *Trends. Pharmacol. Sci.*, **9**, 37–39.

HEATON, J.P.W. (1989). Synthetic nitrovasodilators are effective, in vitro, in relaxing penile tissue from impotent men: the findings and their implications. *Can. J. Physiol. Pharmacol.*, **67**, 78–81.

HEDLUND, H. & ANDERSSON, K.E. (1985a). Comparison of the responses to drugs acting on adrenoceptors and muscarinic receptors in human isolated corpus cavernosum and cavernous artery. *J. Auton. Pharmacol.*, **5**, 81–88.

HEDLUND, H. & ANDERSSON, K.E. (1985b). Contraction and relaxation induced by some prostanoids in isolated human penile erectile tissue and cavernous artery. *J. Urol.*, 134, 1245–1250.

HOLLENBERG, N.K. (1987). Collateral arterial tree and response to serotonin. *J. Cardiovasc. Pharmacol.*, **10**, S35–S38.

JUENEMANN, K.P., LUE, T.F., FOURNIER, G.R.F. & TANAGHO, E.A. (1986). Hemodynamics of papaverine and phentolamine-induced penile erection. *J. Urol.*, **136**, 158–161.

KATUSIC, Z.S., SHEPHERD, J.T. & VANHOUTTE, P.M. (1988). Potassium-induced endothelium-dependent rhythmic activity in the canine basilar artery. *J. Cardiovasc. Pharmacol.*, **12**, 37–41.

LEVIN, R.M. & WEIN, A.J. (1980). Adrenergic alpha-receptors outnumber beta-receptors in human penile corpus cavernosum. *Invest. Urol.*, **18**, 225–226.

LUE, T.F. & TANAGHO, E.A. (1987). Physiology of erection and pharmacological management of impotence. *J. Urol.*, **137**, 829–836.

MALOMVOLGYI, B., HADHAZY, P. & MAGYAR, K. (1988). Relaxation by prostacyclin (PGI_2) of human, dog and rabbit femoral artery strips. Interspecies difference. *Biomed. Biochim. Acta*, **47**, S125–S128.

MELMAN, A., BRESSLER, R.S., HENRY, D.P. & MACADOO, V.K. (1981). Ultrastructure of human penile erectile tissue in patients with abnormal norepinephrine content. *Invest. Urol.*, **19**, 46–50.

MELMAN, A., MAAYANI, S. & SCHWARTZMAN, M. (1986). Prostaglandin synthesis as a putative biochemical correlate of spontaneous myotonic oscillations in the isolated human penile erectile tissue. *Sex. Funct.*, **135**, A, 1028.

MINNEMAN, K.P. & ABEL, P.W. (1987). Relationship of alpha-1 adrenergic receptor occupancy to tissue response. *The Alpha-1 Adrenergic Receptors*. ed. Ruffolo, R.R., Jr. pp. 267–324. Clifton, N.J.: Humana Press.

ROY, A.C., TAN, S.M., KOTTEGODA, S.R. & RATNAM, S.S. (1984). Ability of human corpora cavernosa muscle to generate prostaglandins and thromboxanes in vitro. *IRCS. Med. Sci.*, **12**, 608–609.

SAENZ DE TEJADA, I., GOLDSTEIN, I., AZADZOI, K., KRANE, R.J. & COHEN, R.A. (1989a). Impaired neurogenic and endothelium-mediated relaxation of penile smooth muscle from diabetic men with impotence. *N. Engl. J. Med.*, **320**, 1025–1030.

SAENZ DE TEJADA, I., KIM, N., LAGAN, I., KRANE, R.J. & GOLDSTEIN, I. (1989b). Regulation of adrenergic activity in penile corpus cavernosum. *J. Urol.*, **142**, 1117–1121.

SATAKE, N., SHIBATA, S. & UEDA, S. (1984). Phentolamine-induced rhythmic contractions in bladder detrusor muscle of guinea-pig. *Br. J. Pharmacol.*, **83**, 965–971.

SCHOEFFTER, P. & GODFRAIND, T. (1989). Spontaneous rhythmic contractions of human saphenous veins from old subjects are sensitive to cyclooxygenase inhibitors. *Experientia*, **45**, 459–461.

TIMMERMANS, P.B.M.W.M. & VAN ZWIETEN, P.A. (1982). α_2-adrenoceptors: Classification, localization, mechanisms, and targets for drugs. *J. Med. Chem.*, **25**, 1389–1401.

VANHOUTTE, P.M. (1987). Cardiovascular effects of serotonin. *J. Cardiovasc. Pharmacol.*, **10**, suppl. 3, S8–S11.

VANHOUTTE, P.M. (1988). Endothelium-dependent contractions in veins and arteries. In *Relaxing and Contracting Factors*. p. 30. Clifton, N.J.: Humana Press.

VAN ZWEITEN, P.A. (1987). Pathophysiological relevance of serotonin. *J. Cardiovasc. Pharmacol.*, **10**, Suppl. 3, S19–S25.

WHITE, R.E. & CARRIER, G.O. (1986). Supersensitivity and endothelium dependency of histamine-induced relaxation in mesenteric arteries isolated from diabetic rats. *Pharmacology*, **33**, 34–38.

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Evidence for BK_1 bradykinin-receptor-mediated prostaglandin formation in osteoblasts and subsequent enhancement of bone resorption

[1]Östen Ljunggren & Ulf H. Lerner

Department of Oral Pathology, University of Umeå, S-901 87 Umeå, Sweden

1 The effects of the BK_1 bradykinin (BK)-receptor agonist des-Arg^9-BK on bone resorption and prostaglandin formation in osteoblasts have been studied.

2 Des-Arg^9-BK (1 μM) stimulated the release of ^{45}Ca from prelabelled neonatal mouse calvarial bones and the formation of prostaglandin E_2 (PGE_2) in calvarial bones. The stimulatory effect on bone resorption and PGE_2 formation could be totally inhibited by indomethacin, flurbiprofen and hydrocortisone.

3 The BK_1 receptor antagonist des-Arg^9-Leu^8-BK (10 μM) inhibited des-Arg^9-BK (0.01–0.1 μM)-induced release of ^{45}Ca from prelabelled neonatal mouse calvarial bones, while leaving BK (0.1–1 μM)-induced ^{45}Ca release unaffected.

4 In isolated osteoblast-like cells from neonatal mouse calvarial bones, des-Arg^9-BK (1 μM) induced a slowly developing increase in PGE_2 formation that was significantly different from untreated controls after 24 h. Treatment with BK caused a rapid burst (within minutes) of PGE_2 formation.

5 Des-Arg^9-Leu^8-BK (10 μM) selectively inhibited des-Arg^9-BK (1 μM)-induced PGE_2 and prostacyclin formation in isolated osteoblast-like cells incubated for 72 h. Des-Arg^9-Leu^8-BK did not affect BK and Lys-BK (1 μM)-induced PGE_2 and prostacyclin formation in isolated osteoblast-like cells incubated for 72 h.

6 These data indicate that osteoblasts are equipped with BK_1-receptors mediating enhanced prostaglandin formation and subsequent bone resorption.

Introduction

We have previously reported that the inflammatory mediator bradykinin (BK) is capable of stimulating bone resorption *in vitro* (Gustafson & Lerner, 1983). The stimulatory effect of BK on bone resorption is abolished by several inhibitors of prostaglandin formation, suggesting that the effect on bone resorption is mediated via enhanced endogenous prostaglandin formation in bone tissue (Gustafson & Lerner, 1983; Lerner *et al.*, 1987). This view is compatible with the observations that prostaglandin E_2 (PGE_2) and prostacyclin are potent stimulators of bone resorption *in vitro* (reviewed in Raisz & Martin, 1984). Furthermore, BK stimulates prostaglandin formation in isolated osteoblast-like cells from neonatal mouse calvarial bones and in the cloned murine osteoblastic cell lineage MC3T3-E1 (Lerner *et al.*, 1989), as well as in isolated human osteoblast-like cells (Ljunggren *et al.*, 1990). Based upon these findings, we have proposed that BK may serve as a mediator of the inflammatory induced bone resorption seen in areas of chronic inflammatory processes, e.g. rheumatoid arthritis, periodontitis and osteomyelitis.

There are at least two subtypes of BK receptors, the BK_1- and BK_2 receptors (Regoli & Barabé, 1980). These receptors have been pharmacologically characterized by using different kinin analogues with agonistic and antagonistic properties (Regoli & Barabé, 1980; Vavrek & Stewart, 1985). By comparing the rank order potency of such analogues, we have recently found that osteoblasts are equipped with BK_2-receptors mediating prostaglandin formation (Ljunggren & Lerner, 1987). However, we have also found that the BK_1-receptor agonist des-Arg^9-BK enhances bone resorption *in vitro* (Lerner *et al.*, 1987). The aim of this study was therefore to determine whether des-Arg^9-BK induced bone resorption was due to a partial BK_2 agonistic effect, or if there may exist a BK_1-receptor-mediated mechanism involved in inflammatory induced bone resorption.

[1] Author for correspondence.

Methods

Measurement of bone resorption

Bone resorption was assessed by measuring the release of ^{45}Ca from neonatal mouse calvarial bones, prelabelled with 1.5 μCi ^{45}Ca 4 days prior to dissection (Reynolds, 1976; Lerner, 1987). The calvarial bones were dissected into four fragments and only the posterior 2/3 of the parietal bones were used. The sensitivity in the neonatal mouse calvarial bone resorption assay is the same whether we use calvarial halves or calvarial quarters (data not shown). The samples were preincubated for 24 h in CMRL 1066 culture medium containing indomethacin (1 μM). This was done in order to reduce basal ^{45}Ca release in untreated controls (Lerner, 1987). The bones were then thoroughly rinsed and subsequently incubated for 72 h in CMRL 1066 containing test substances or vehicle. After 72 h incubation, ^{45}Ca-release was determined by analyzing the amount of radioactivity in the media, using liquid scintillation. The bones were dissolved in HCl and the remaining radioactivity was measured. The results were expressed as the percentage release of initial ^{45}Ca (Lerner, 1987).

Isolation and culture of osteoblast-like cells from mouse calvarial bones

Calvarial bones from 2–3 days-old mice were dissected out and bone cells were isolated by sequential enzymatic digestion (Boonekamp *et al.*, 1984). The calvarial bones were transferred to Ehrlenmeyer flasks in a shaking water bath (37°C), treated with EDTA (0.4 mM) in phosphate buffered saline (PBS) for 3 × 10 min, washed with PBS and subsequently treated with bacterial collagenase (180 u ml^{-1}) in PBS without EDTA for 4 × 10 min. The cells from the two last digestion fluids were collected, centrifuged, resuspended in Alpha Modification of Eagle's Medium (α-MEM) with 10% Foetal Calf Serum (FCS), and seeded in 25 cm^2 tissue culture flasks. When confluent, the cells were detached with trypsin (1 mg ml^{-1}) and reseeded in

2 cm^2 multiwell culture dishes. Only cells from passages 1–2 were used in the experiments. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Determination of PGE_2 formation in calvarial bones

Calvarial bones were dissected, preincubated with indomethacin and rinsed as described above. The bones were thereafter incubated in CMRL 1066 with test substances or vehicle for 72 h. Samples from the incubation media were withdrawn, acidified to pH 3.5 and stored at −20°C. The amount of PGE_2 released from the calvarial bones into the medium, during the incubation, was then analyzed as described below.

Determination of PGE_2 and 6-keto-$PGF_{1\alpha}$ production in osteoblasts

Prior to the experiments, the cell layers were rinsed twice with prewarmed Tyrode solution and preincubated for 30 min in α-MEM without FCS. The preincubation media were then removed and fresh medium with test substances or vehicle was added (500 μl/well). At different time points, as stated in legends, the media were removed, acidified to pH 3.5 with HCl, frozen and stored at −20°C. The amounts of PGE_2 and prostacyclin (as assessed by analysis of the stable breakdown product 6-keto-$PGF_{1\alpha}$) in the media were analyzed with commercially available Radio Immuno Assay (RIA)-kits with [^{125}I]-PGE_2 and [^{125}I]-6-keto-$PGF_{1\alpha}$ as tracers. After the experiments, the cells were detached with trypsin and counted in a haemocytometer.

Statistics

Statistical evaluation of the data was performed by use of Student's *t* test for unpaired samples.

Materials

Hydrocortisone, BK, Lys-BK, des-Arg^9-BK, des-Arg^9-Leu^8-BK and serum albumin were from Sigma Chemical Co., St. Louis, MO, U.S.A.; CMRL 1066 medium, α-MEM and FCS from Flow Laboratories, Irvine, Scotland; bacterial collagenase (clostridium, type 1) from Wortington Biochemical, Freehold, NJ, U.S.A.; $^{45}CaCl_2$ (specific activity 20–30 Ci g^{-1}), PGE_2 and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), RIA-kits from New England Nuclear Chemicals, Dreieich, West Germany; multiwell plastic culture dishes from Costar, Cambridge, Mass, U.S.A. Flurbiprofen was kindly provided by Astra, Södertälje, Sweden. Indomethacin was a kind gift from Merck, Sharp and Dohme, Haarlem, The Netherlands.

Results

Des-Arg^9-BK (1 μM) and BK (1 μM) stimulated the release of prelabelled ^{45}Ca from neonatal mouse calvarial bones during 72 h incubation (Table 1). In addition, BK and des-Arg^9-BK (1 μM) also enhanced the formation of PGE_2 in the calvarial bones (Table 1). Addition of indomethacin (1 μM) to the incubation medium totally inhibited BK and des-Arg^9-BK-induced formation of PGE_2 and release of prelabelled ^{45}Ca (Table 1). Two additional, structurally different, inhibitors of prostaglandin synthesis flurbiprofen (1 μM) and hydrocortisone (1 μM) also totally abolished des-Arg^9-BK-induced formation of PGE_2 and release of prelabelled ^{45}Ca from calvarial bones incubated for 72 h (Table 1).

Des-Arg^9-BK, dose-dependently, stimulated ^{45}Ca release from prelabelled neonatal mouse calvarial bones cultured for 72 h (Figure 1). The lowest concentration of des-Arg^9-BK causing a statistically significant effect was 10 nM and a maximal stimulatory effect (130% of controls) was seen at 1 μM (mean from 5 experiments; Figure 1). The BK_1-receptor antagonist des-Arg^9-Leu^8-BK (10 μM) totally inhibited the effect of des-Arg^9-BK at low concentrations (0.01–0.1 μM), and significantly reduced the effect of 1 μM des-Arg^9-BK (Figure 1). Des-Arg^9-Leu^8-BK had no effect by itself on ^{45}Ca release from prelabelled calvarial bones (Figure 1).

BK (0.1 and 1 μM)-induced release of ^{45}Ca from prelabelled calvarial bones was not sigificantly affected by des-Arg^9-Leu^8-BK (10 μM; Table 2).

In isolated osteoblast-like cells from neonatal mice, BK (1 μM), but not des-Arg^9-BK (1 μM), induced a rapid formation of PGE_2 (within minutes). An elevated amount of PGE_2 in the incubation media, withdrawn from cells treated with BK, was sustained for 48 h (Figure 2). In these long term incubations, a stimulatory effect of des-Arg^9-BK (1 μM) on the formation of PGE_2 developed slowly during culture, causing a significant stimulation after 24 h (Figure 2). The degree of stimulation at 24 and 48 h, however, was substantially less than that obtained with BK (Figure 2).

During 48 h incubation of osteoblast-like cells, the BK_1 receptor antagonist des-Arg^9-Leu^8-BK (10 μM) significantly inhibited des-Arg^9-BK (1 μM)-induced PGE_2 and 6-keto-$PGF_{1\alpha}$ synthesis (Table 3). BK (1 μM) and Lys-BK (1 μM)-induced formation of PGE_2 and 6-keto-$PGF_{1\alpha}$ in 48 h incu-

Table 1 Effect of bradykinin (BK) and des-Arg^9-BK on prostaglandin E_2 (PGE_2)-formation and ^{45}Ca-release in neonatal mouse calvarial bones

Agonist	*Conc.* (μM)	*Inhibitor*	*Conc.* (μM)	*PGE_2* (pg/bone)	*^{45}Ca* (%-release)
—	—	—	—	72.5 ± 9.8	27.1 ± 1.1
—	—	Indomethacin	1	14.8 ± 1.5[a]	24.6 ± 0.6
—	—	Flurbiprofen	1	12.5 ± 1.8[a]	25.0 ± 0.9
—	—	Hydrocortisone	1	38.8 ± 9.8	29.3 ± 1.6
BK	1	—	—	783.3 ± 46.3[a]	51.0 ± 2.3[a]
BK	1	Indomethacin	1	18.3 ± 5.5[a,b]	28.4 ± 0.6[b]
des-Arg^9-BK	1	—	—	301.0 ± 40.5[a]	41.3 ± 3.2[a]
des-Arg^9-BK	1	Indomethacin	1	12.8 ± 2.0[a,c]	28.2 ± 1.5[c]
des-Arg^9-BK	1	Flurbiprofen	1	22.3 ± 3.3[a,c]	30.0 ± 1.0[c]
des-Arg^9-BK	1	Hydrocortisone	1	37.5 ± 5.5[a,c]	33.8 ± 1.2[c]

Prelabelled neonatal mouse calvarial bones were dissected into four fragments using only the posterior two thirds of the parietal bones. After 24 h preincubation with indomethacin (1 μM) the samples were rinsed and incubated for 72 h in CMRL 1066 with test substances or vehicle. After the incubation, the amount of ^{45}Ca released into the medium was calculated by liquid scintillation and the amount of PGE_2 released to the medium was calculated by RIA.

The values are means ± s.e.mean for 5–6 samples.

[a] Significantly different from untreated controls ($P < 0.01$).

[b] Significantly different from BK-treated bones ($P < 0.01$).

[c] Significantly different from des-Arg^9-BK-treated bones ($P < 0.01$).

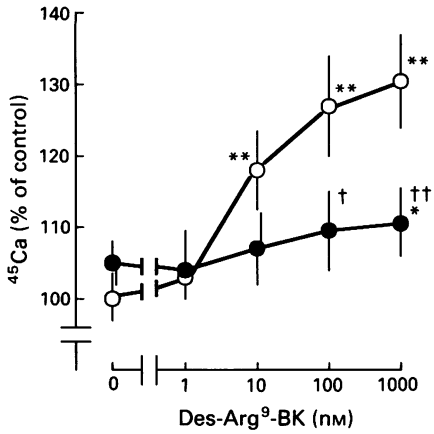

Figure 1 Effect of the BK_1 antagonist des-Arg^9-Leu^8-BK on des-Arg^9-BK-induced bone resorption. Prelabelled neonatal mouse calvarial bones were dissected into four fragments using only the posterior two thirds of the parietal bones. The fragments were preincubated in indomethacin (1 μM) for 24 h. Thereafter the bones were rinsed and incubated for 72 h submerged in 2.5 ml CMRL 1066 culture medium with test substances or vehicle. The amount of ^{45}Ca released into the culture media was analysed by liquid scintillation. (○) Des-Arg^9-BK; (●) des-Arg^9-BK + des-Arg^9-Leu^8-BK (10 μM). Values represent pooled data (means with ± s.e.mean shown by vertical lines) from 5 separate experiment with 5–7 bone fragments in each group. The data are presented as % of control with the control values set as 100%. Basal resorption in the 5 experiments (means ± s.e.mean) were 19.40 ± 1.97%; 25.92 ± 1.50%; 33.34 ± 1.90%; 34.71 ± 2.88% 41.18 ± 1.44%. Significantly different from untreated controls: * $P < 0.05$; ** $P < 0.01$. Significantly different from des-Arg^9-BK: † $P < 0.05$; †† $P < 0.01$.

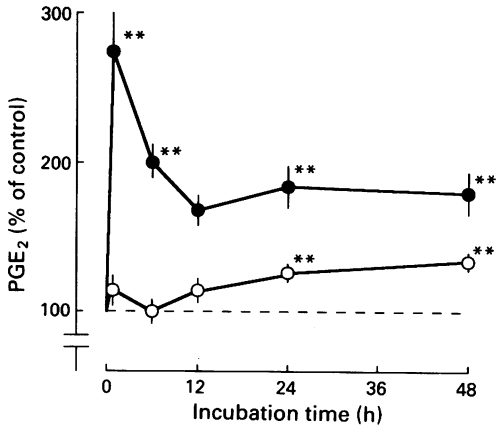

Figure 2 Long term time course of bradykinin (BK) and des-Arg^9-BK-induced prostaglandin E_2 (PGE_2) formation in isolated osteoblast-like cells. Osteoblast-like cells were isolated from neonatal mouse calvarial bones by sequential enzymatic digestion with bacterial collagenase. The cells were seeded in multiwell culture dishes. Prior to the experiments, the cell layer were rinsed and the cells were subsequently incubated in α-MEM containing 1% FCS with or without kinins. At different time points, the media were withdrawn and acidified to pH 3.5 and frozen. The cells were detached with trypsin and counted in a haemocytometer. PGE_2 in the incubation media was analyzed by RIA. Symbols represent pooled data from three different experiments and are expressed as % of control with the control values set at 100%. The mean amount of PGE_2 ± s.e.mean in the control wells (pg/1000 cells) after 48 h was 2.27 ± 0.18 (55,000 cells/cm^2), 0.90 ± 0.01 (62,000 cells/cm^2) and 15.52 ± 0.86 (28,800 cells/cm^2). (●) BK (1 μM); (○) des-Arg^9-BK (1 μM). Significantly different from untreated controls ** $P < 0.01$.

bation of osteoblast-like cells was not affected by des-Arg^9-Leu^8-BK (10 μM; Table 3); nor were the basal levels of PGE_2 and 6-keto-$PGF_{1\alpha}$ affected by des-Arg^9-Leu^8-BK.

Discussion

Prolonged incubation with the BK_1 receptor agonist des-Arg^9-BK stimulated prostaglandin formation in isolated osteoblast-like cells from neonatal mouse calvarial bones. This was not due to a weak agonistic effect on BK_2-receptors since the BK_1 antagonist des-Arg^9-Leu^8-BK, in long term incubations, selectively inhibited des-Arg^9-BK induced prostaglandin synthesis whereas BK and Lys-BK induced prostaglandin synthesis was unaffected. Kinetic analysis revealed that the stimulatory effect of BK on PGE_2 synthesis was seen very rapidly (within minutes) whereas that of des-Arg^9-BK was observed first after a time lag of 24 h. The decreasing treated-versus-control ratio for BK-treated cells between 2–6 h (Figure 2) was due to a slow increase in PGE_2 formation in the control cells and not to decreasing amounts of PGE_2 in the culture media. The selective inhibitory action of des-Arg^9-Leu^8-BK and the different time courses indicate the presence of both BK_1 and BK_2 receptors in osteoblasts linked to prostanoid formation. This is further supported by our finding that both BK and des-Arg^9-BK are stimulators of ^{45}Ca release from prelabelled neonatal mouse calvarial bones *in vitro*, by a mechanism that was abolished by agents that inhibit prostaglandin synthesis (e.g. indomethacin and hydrocortisone). In addition, des-Arg^9-Leu^8-BK inhibited des-Arg^9-BK- but not BK-induced ^{45}Ca release from the calvarial bones. Thus, the data obtained in neonatal mouse calvarial bones are compatible with the data obtained in the isolated osteoblast-like cells.

Although the effect of BK in most cells is due to an action on BK_2 receptors (Regoli & Barabé, 1980), osteoblasts are not the only cell type which express BK_1 receptors. Thus it has been reported that this BK receptor subtype is present in isolated arterial vessels (Churchill & Ward, 1986; Guimaraes *et al.*, 1986; Rhaleb *et al.*, 1989). In addition there are reports showing that the BK_1 receptor is induced and expressed by the cells due to tissue damage or exposure to various substances e.g. lipopolysaccharides and interleukin-1β (IL-1) (Regoli *et al.*, 1978; 1981; Barabé *et al.*, 1982; Deblois *et al.*, 1988). It is therefore possible that the isolated osteoblasts synthesize and express BK_1 receptors due to damage during the isolation and culture procedures or due to an autocrine regulation by IL-1 synthesized during culture. Such a mechanism is supported by the lag period in the kinetics of des-Arg^9-BK induced formation of prostanoids. The view, however, that the presence of BK_1 receptors is only due to induction, has been challenged by data obtained with different preparations of iso-

Table 2 Effect of des-Arg^9-Leu^8-BK on BK-induced ^{45}Ca release from prelabelled neonatal mouse calvarial bones

		^{45}Ca-release (% of control)	
Agonist	*Conc.* (μM)	−des-Arg^9-Leu^8-BK	+des-Arg^9-Leu^8-BK
—	—	100.0 ± 3.9	106.1 ± 4.3
BK	0.1	128.6 ± 8.9[a]	149.2 ± 12.2[a]
BK	1.0	160.1 ± 13.9[a]	145.7 ± 9.5[a]

Calvarial bones from neonatal mice were dissected into four fragments using only the posterior two thirds of the parietal bones. After 24 h preincubation with indomethacin (1 μM) the samples were rinsed with Tyrode solution and subsequently incubated for 72 h in CMRL 1066 with test substances or vehicle. Thereafter the % release of ^{45}Ca into the culture media was analysed by liquid scintillation. Values represent pooled data from 3 different experiments and are expressed as % of control with the ^{45}Ca release in untreated controls set as 100%. The basal ^{45}Ca % release in untreated controls (mean ± s.e.mean) for the 3 experiments were 41.18 ± 1.44 ($n = 5$); 25.92 ± 1.51 ($n = 6$); 19.40 ± 1.97 ($n = 6$).
Values represent means ± s.e.mean for 17 samples from three different experiments.
[a] Significantly different from untreated controls ($P < 0.01$).

Table 3 Effect of the BK_1 receptor antagonist des-Arg^9-Leu^8-BK on BK, Lys-BK and des-Arg^9-BK induced prostaglandin formation in isolated osteoblast-like cells

Agonist	*Conc.* (μM)	*Addition*	*Conc.* (μM)	*Prostaglandin formation* (pg/1000 cells) PGE_2	*6-keto-$PGF_{1\alpha}$*
—	—	—	—	57.2 ± 9.6	7.6 ± 0.4
—	—	des-Arg^9-Leu^8-BK	10	47.7 ± 6.6	7.1 ± 0.3
BK	1	—	—	158.4 ± 4.6[a]	22.3 ± 0.7[a]
BK	1	des-Arg^9-Leu^8-BK	10	153.3 ± 8.4[a]	20.8 ± 1.3[a]
Lys-BK	1	—	—	129.5 ± 21.6[a]	20.3 ± 1.3[a]
Lys-BK	1	des-Arg^9-Leu^8-BK	10	136.4 ± 2.9[a]	21.2 ± 1.2[a]
des-Arg^9-BK	1	—	—	108.8 ± 12.1[a]	12.9 ± 2.3[a]
des-Arg^9-BK	1	des-Arg^9-Leu^8-BK	10	64.3 ± 6.4[b]	10.1 ± 0.7[c,d]

Osteoblast-like cells were insolated from neonatal mouse calvarial bones by sequential enzymatic digestion. Prior to the experiment, the cell layers were rinsed with Tyrode solution and subsequently incubated in α-MEM containing 1% FCS with kinins or vehicle for 48 h. After the experiment, the incubation media were withdrawn, acidified to pH 3.5 and stored at −20°C. The amounts of prostaglandin E_2 (PGE_2) and 6-keto-$PGF_{1\alpha}$ in the culture media were analysed with RIA.
Values represent means ± s.e.mean for 6 wells.
[a] Significantly different from untreated controls ($P < 0.01$).
[b] Significantly different from des-Arg^9-BK ($P < 0.01$).
[c] Significantly different from untreated controls ($P < 0.05$).
[d] Significantly different from des-Arg^9-BK (0.05).

lated arterial vessels indicating that BK_1 receptors in these organs are not generated *de novo*, but present from the beginning of the isolation (Churchill & Ward, 1986; Guimaraes *et al.*, 1986; Rhaleb *et al.* 1989). The recent report that the osteoblastic osteosarcoma cell line ROS 17/2.8 is equipped with BK_1 receptors specifically binding des-Arg^9-BK (Santora, 1989), indicates that BK_1 receptors may be a constituent feature of osteoblasts.

BK can stimulate prostaglandin biosynthesis in various organs including kidney, heart, lung, blood vessels, spleen and uterus (for rev. see Nasjletti & Malik, 1979). In these organs the prostaglandin response has been observed only after stimulation with BK and analogues with affinity for the BK_2 receptor. Bone tissue is the only tissue, to our knowledge, where it has been directly shown that BK_1 receptors mediate enhanced prostaglandin synthesis. Indirect evidence, however, that the BK_1 receptor may stimulate prostaglandin synthesis has been reported from studies on the rabbit mesenteric and dog renal arteries, where the relaxant effects of des-Arg^9-BK can be blocked by indomethacin (Churchill & Ward, 1986; Rhaleb *et al.*, 1989).

In conclusion, our data indicate that both BK_1 and BK_2 receptors on osteoblasts are mediating an increase in prostaglandin synthesis. The large differences in time course of the response to specific agonists for the two receptors indicate that different signal transducing systems are linked between receptor occupancy and prostaglandin synthesis. It is very interesting to note that, like our observations on prostaglandin biosynthesis, the effect of des-Arg^9-BK on relaxation of dog renal arteries is considerably slower than that caused by BK (Rhaleb *et al.*, 1989). Our data, together with those of others (Regoli *et al.*, 1981; Deblois *et al.*, 1988) may suggest that in areas adjacent to inflammatory processes, bone tissue may become more sensitive to kinins due to upregulation of the number of BK_1 receptors in addition to the already present BK_2 receptors.

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References

BOONEKAMP, P.M., HEKKELMAN, J.W., HAMILTON, J.W., COHN, D.V. & JILKA, R.L. (1984). Effect of culture on the hormone responsiveness of bone cells isolated by an improved sequential digestion procedure. *Proc. Kon. Ned. Akad. Wet.*, **B87**, 371–382.

BARABÉ, J., BABIUK, C. & REGOLI, D. (1982). Binding of [^{3}H]des-arg^9-BK to rabbit anterior mesenteric vein. *Can. J. Pharmacol.*, **60**, 1551–1555.

CHURCHILL, L. & WARD, P.E. (1986). Relaxation of isolated mesenteric arteries by des-arg^9-bradykinin stimulation of B1 receptors. *Eur. J. Pharmacol.*, **130**, 11–18.

DEBLOIS, D., BOUTHILLIER, J. & MARCEAU, F. (1988). Effect of glucocorticoids, monokines and growth factors on the spontaneously developing responses of the rabbit isolated aorta to des-Arg^9-bradykinin. *Br. J. Pharmacol.*, **93**, 969–977.

GUIMARAES, J.A., VIEIRA, M.A., EICH, E. & MAACK, T. (1986). Effects of kinins in the isolated perfused rat kidney and evidence for the presence of renal B1 receptors. *Adv. Exp. Med. Biol.*, **198**, part A, 559–562.

GUSTAFSON, G.T. & LERNER, U. (1983). Bradykinin stimulates bone resorption and lysosomal enzyme release in cultured mouse calvaria. *Biochem. J.*, **219**, 329–332.

LERNER, U.H. (1987). Modification of the culture technique for mouse calvarial bone improve the responsiveness to stimulators of bone resorption. *J. Bone Min. Res.*, **2**, 375–383.

LERNER, U., JONES, I.L. & GUSTAFSON, G.T. (1987). Bradykinin – a new potential mediator of inflammatory induced bone resorption. *Arthr. Rheum.*, **30**, 530–540.

LERNER, U.H., RANSJÖ, M. & LJUNGGREN, Ö. (1989). Bradykinin stimulates production of prostaglandin E_2 and prostacyclin in murine osteoblasts. *Bone and Mineral.*, **5**, 139–154.

LJUNGGREN, Ö. & LERNER, U. (1987). Bradykinin induced PGE_2 production in isolated osteoblasts is mediated via B2 bradykinin receptors. In *Calcium Regulation and Bone Metabolism.* ed. Cohn, D.V., Martin, T.J. & Meunier, P.J. p. 651 (abstr.). Amsterdam, Elsevier.

LJUNGGREN, Ö, ROSENQUIST, J., RANSJÖ, M. & LERNER, U.H. (1990). Bradykinin stimulates prostaglandin E_2 formation in isolated human osteoblast-like cells. *Biosc. Rep.*, **10**, 121–126.

NASJLETTI, A. & MALIK, K.U. (1979). Relationships between the kallikrein-kinin and prostaglandin systems. *Life Sci.*, **25**, 99–110.

RAISZ, L.G. & MARTIN, T.J. (1984). Prostaglandins in bone and mineral metabolism. In *Bone and Mineral Research*, Annual 2. ed.

Peck, W.A. pp. 286–310. Amsterdam: Elsevier.

REGOLI, D., MARCEAU, F. & BARABÉ, J. (1978). De novo formation of vascular receptors for bradykinin. *Can. J. Pharmacol.*, **56**, 674–677.

REGOLI, D. & BARABÉ, J. (1980). Pharmacology of bradykinin and related kinins. *Pharmacol. Rev.*, **32**, 1–46.

REGOLI, D.C., MARCEAU, F. & LAVIGNE, J. (1981). Induction of B1-receptors for kinins in the rabbit by a bacterial lipopolysaccharide. *Eur. J. Pharmacol.*, **71**, 105–115.

REYNOLDS, J.J. (1976). Organ cultures of bone: studies on the physiology and the pathology of resorption. In *Organ Culture in Biomedical Research*. ed. Balls, M. & Monnickendam, M. pp. 355–366. Cambridge: University Press.

RHALEB, N., DION, S., BARABÉ, J., ROUISSI, N., JUKIC, D., DRAPEAU, G. & REGOLI, D. (1989). Receptors for kinins in isolated arterial vessels of dogs. *Eur. J. Pharmacol.*, **162**, 419–427.

SANTORA II, A.C. (1989). Des-arg^9-bradykinin stimulation of ROS 17/2.8 rat osteosarcoma adenosine 3',5'-cyclic monophosphate content: receptor specificity. *J. Bone Min. Res.*, **4**, suppl. 1, S332 (abstr.).

VAVREK, R.J. & STEWART, J.M. (1985). Competitive antagonists of bradykinin. *Peptides*, **6**, 161–164.

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The effectiveness of α_2-adrenoceptor activation increases from the distal to the proximal part of the veins of canine limbs

Serafim Guimaraes & Jose P. Nunes

Department of Pharmacology, Faculty of Medicine, 4200-Porto, Portugal

1 The effectiveness of α_1- and α_2-adrenoceptor activation was compared at different levels of the saphenous and cephalic vein of the dog *in vitro*.

2 Helically cut strips were used to determine concentration-response curves to phenylephrine, noradrenaline, UK-14,304 (5-bromo-6-(imidazoline-2-ylamino)-quinoxaline) and B-HT 920 (2-amino-6-allyl-5,6,7,8-tetra-hydro-4H-(thiazo)-4,5-d-azepine). The effect of prazosin and yohimbine on these curves was also studied.

3 At the distal level, the maximum response to UK-14,304 amounted to 33 and 50% of those to noradrenaline in the saphenous and cephalic vein, respectively, while at the proximal level the maximum response to UK-14,304 amounted to 72 and 78% of those to noradrenaline, in the saphenous and cephalic vein, respectively.

4 In both vessels, the results obtained with B-HT 920 were very similar to those for UK-14,304.

5 The pD_2 values for UK-14,304 – which were identical at the three levels of both vessels – and the pA_2 values for the antagonism exerted by either prazosin or yohimbine against the responses to UK-14,304 indicate that the α_2-adrenoceptors are identical at the different levels of both vessels.

6 These results show that the effectiveness of α_2-adrenoceptor stimulation increases from the distal to the proximal regions of canine limb veins. Apparently, this is due to a greater density of α_2-adrenoceptors in the proximal regions.

7 Yohimbine is much more potent against phenylephrine distally than proximally in both vessels. However, after 30 nM phenoxybenzamine – a concentration which eliminates the vast majority of α_1-adrenoceptors without affecting α_2-adrenoceptors – yohimbine became equally potent at both levels, suggesting that the difference existing before phenoxybenzamine depended on α_1-adrenoceptors. Hence it is concluded that α_1-adrenoceptors in distal and proximal portions may differ.

Introduction

The existence of two subgroups of postjunctional α-adrenoceptors on blood vessels was postulated on the basis of results obtained in experiments performed *in vivo* (Docherty *et al.*, 1979; Drew & Whiting, 1979; Timmermans *et al.*, 1979; McGrath *et al.*, 1982). In contrast to the ease with which confirmatory evidence for the existence of these two subgroups of α-adrenoceptors has been accumulating from experiments carried out *in vivo*, it is relatively difficult to demonstrate postsynaptic α_2-adrenoceptors *in vitro*. It is now well established that although postjunctional α_2-adrenoceptors are consistently found in many isolated veins of different species (De Mey & Vanhoutte, 1981; Shepperson & Langer, 1981; Constantine *et al.*, 1982; Shogi *et al.*, 1983; Guimaraes *et al.*, 1983; 1987) they are very scarce or simply are not detected in arteries (Starke & Docherty, 1982; Hamed *et al.*, 1983; Skärby & Andersson, 1984; Polonia *et al.*, 1985). However, there are exceptions to this (Flavahan *et al.*, 1987; Templeton *et al.*, 1989; Dunn *et al.*, 1989).

Recently, in perfusion experiments with distal segments of canine saphenous vein it was observed that they responded poorly or did not respond at all to α_2-adrenoceptor agonists applied to either the intima or the adventitia (Nunes, De la Lande & Guimaraes, unpublished results). This unexpected finding prompted us to compare the effectiveness of α_2-adrenoceptor activation at different levels of the canine saphenous vein.

Methods

Mongrel dogs, 12–20 kg in weight, of either sex, were anaesthetized with pentobarbitone sodium (30 mg kg^{-1} injected in the forelimb) and segments of the saphenous and the cephalic vein trunks of about 25 mm in length were obtained from the distal, intermediate or proximal parts of the veins (Figure 1). After removal of veins, the animals were killed by an overdose of pentobarbitone sodium (100 mg kg^{-1}). The distance between the three segments was of about 5 cm each. From each segment a pair of helically cut strips (of about 2.5 × 25 mm) were prepared and suspended in a 20 ml bath containing Krebs-Henseleit solution (Guimaraes & Osswald, 1969); the endothelium was removed by gentle rubbing of the intimal surface with paper moistened in Krebs solution. After this procedure, a ring of 0.1–0.2 mm length was removed from

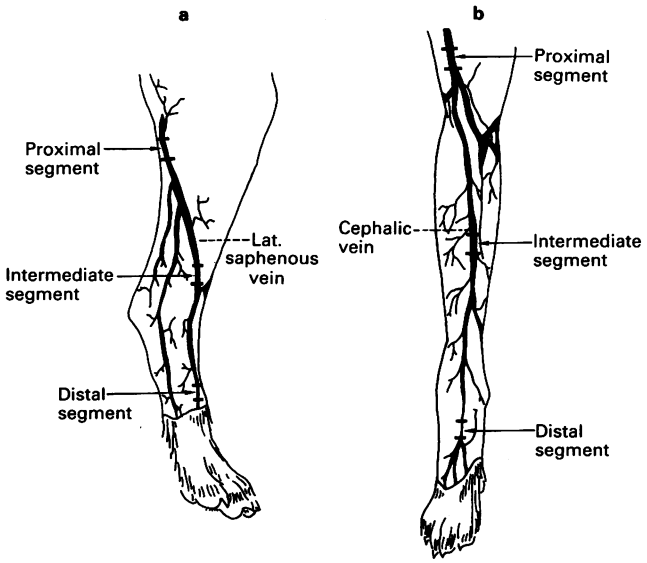

Figure 1 (a) Lateral saphenous vein trunk of the right hindleg, lateral aspect. (b) Cephalic vein trunk of the right antebrachium, cranial aspect. The arrows indicate the sites from where the vein segments were removed.

each vein segment to confirm by microscopy the removal of the endothelium. The bath solution was kept at 37°C and bubbled with 95% O_2 and 5% CO_2. The strips were mounted between a stationary tissue holder and a transducer and isometric contractions were recorded as changes in grams of tension on a Harvard Universal Oscillograph.

Each strip was allowed to stabilize for 1 h under a basal tension of 2 g before experimentation. The bath solution was replaced several times during the equilibration period. Concentration-response curves to phenylephrine, noradrenaline, UK-14,304 (5-bromo-6-(imidazoline-2-ylamino)-quinoxaline) and B-HT 920 (2-amino-6-allyl-5,6,7,8-tetrahydro-4H-(thiazo)-4,5-d-azepine) were determined by the method of stepwise cumulative additions of the agonist in the presence of cocaine (12 μM), to inhibit neuronal uptake (Trendelenburg, 1966), hydrocortisone (40 μM) to inhibit extraneuronal uptake (Iversen & Salt, 1970; Guimaraes *et al.*, 1978), and propranolol (1 μM) to block β-adrenoceptors (Black *et al.*, 1965).

The concentration of agonist in the bathing solution was increased 3 fold at each step, with each addition being made only after the response to the previous addition had attained a maximal level and remained steady. Two concentration-response curves were determined per strip. In experiments aimed at determining pD_2 values, two different agonists were used per strip, the order of administration being reversed in the paired strip; at the end of each experiment a maximum response was obtained to 300 μM noradrenaline, which was expressed in $g\,g^{-1}$ tissue. For the estimation of pA_2 values, two concentration-response curves were determined for one agonist per strip: one in the absence and the other in the presence of the antagonist, which was added 30 min before the determination of the second concentration-response curve. To detect changes in the sensitivity of the strips, time control concentration-response curves were determined for each agonist. Compensations were made whenever changes in sensitivity occurred with time. The use of high concentrations of UK-14,304 was accompanied by a quick development of desensitization (only to itself). Hence, only one concentration-response curve was determined for each of two paired strips: one in the presence and the other in the absence of the antagonist.

In some experiments, the effect of yohimbine was studied after the strips had been exposed to phenoxybenzamine (to eliminate α_1-adrenoceptors). In these experiments, the strips were exposed to 30 or 100 nM phenoxybenzamine for 40 min and washed for 45 min before the first concentration-response curve was determined.

After completion of a concentration-response curve, drugs were washed out from the preparation. In all the experiments, propranolol, cocaine and hydrocortisone were added to the bath 30 min before the addition of the agonist and were left in the bath throughout the experiment.

In some experiments, nearly maximal responses to 150 mM potassium chloride (KCl) were also determined. This concentration was indicated by preliminary experiments in which 50, 100, 150 and 200 mM KCl were used. In the vast majority of these experiments, the lowest concentration eliciting the maximal response was 150 mM. No osmotic compensation was allowed for when KCl was used.

The pD_2 values represent the negative logarithm of the molar concentration of the agonist required to obtain 50% of the maximal effect of noradrenaline.

To study the antagonism by prazosin or yohimbine, pA_2 values were calculated according to the method of Van Rossum (1963) from the equation: $pA_2 = pA_x + \log(x - 1)$ in which x represents the factor of the shift of the concentration-response curve to the right and pA_x the negative logarithm of the molar concentration of the antagonist which caused this shift. As usually three different concentrations of each antagonist were used, slopes of Schild plots were calculated as well. To keep the selectivity of their antagonistic actions, both prazosin and yohimbine were used in concentrations as low as possible: for prazosin, 10^{-8}, 3×10^{-8} and 10^{-7} M against phenylephrine and 10^{-6}, 3×10^{-6} and 10^{-5} M against UK-14,304, while for yohimbine the concentrations used were 10^{-8}, 3×10^{-8} and 10^{-7} M against UK-14,304 and phenylephrine (distal vein), and 10^{-7}, 3×10^{-7} and 10^{-6} M against phenylephrine (proximal vein).

The results are presented as arithmetic means with their standard errors (s.e.). Differences between means were compared by Student's t test. For multiple comparison between pairs of means, the Tukey-Kramer method was used (Sokal & Rohlf, 1981). A P value of 0.05 or less was considered significant.

Drugs

The drugs used were: B-HT 920 (2-amino-6-allyl-5,6,7,8-tetrahydro-4H-(thiazo)-4,5-d-azepine) (Boehringer, Ingelheim, F.R.G.), cocaine hydrochloride (Uquipa, Lisboa, Portugal), hydrocortisone 21-phosphate (Sigma, St. Louis, MD, U.S.A.), (−)-noradrenaline bitartrate (Koch-Ligh, Haverhill, U.K.), phenoxybenzamine hydrochloride (Smith, Kline and French, Philadelphia, PA, U.S.A.), (−)-phenylephrine hydrochloride (Boehringer Sohn, Mannheim, F.R.G.), potassium chloride (Sigma), prazosin hydrochloride (Pfizer, Seixal, Portugal), (±)-propranolol hydrochloride (ICI, Macclesfield, U.K.), rauwolscine hydrochloride (Roth, Karlsruhe, F.R.G.), UK-14,304 (5-bromo-6-(imidazoline)-2-ylamino)-quinoxaline) (Pfizer), yohimbine hydrochloride (Sigma).

Results

Saphenous vein

Noradrenaline, phenylephrine, UK-14,304 and B-HT 920 caused concentration-dependent contractions of the strips obtained from distal, intermediate and proximal parts of the saphenous vein trunk. The maximal responses to noradrenaline and phenylephrine were identical at the three levels of the vein trunk (Figure 2 and Table 1). However, noradrenaline was approximately 15 times more potent than phenylephrine (Figure 2 and Table 2). Potassium chloride (KCl) (150 mM) and the selective α_2-adrenoceptor agonists UK-14,304 and B-HT 920 caused maximal contractions which were smaller than those induced by noradrenaline. When expressed as % of the maximal response to noradrenaline, the maximal contraction to UK-14,304 reached 33.2 ± 3.7, 58.4 ± 3.7 and $72.1 \pm 5.4\%$ ($n = 10$), that to B-HT 920 27.5 ± 2.9, 51.6 ± 5.6 and $58.3 \pm 6.3\%$ ($n = 5$) and that to KCl 71.2 ± 7.6, 70.3 ± 6.1 and $73.6 \pm 6.3\%$ ($n = 5$) at the distal, intermediate and proximal levels, respectively (Figure 2 and Table 1).

Thus, maximal activation of the adrenoceptors with UK-14,304 and B-HT 920 results in markedly less contraction of the distal portion of the saphenous vein trunk than does

Table 1 Tension developed in saphenous vein with maximal contraction by different agonists

	Maximal contraction (in $g\,g^{-1}$ tissue)			
Agonist	*Distal*	*Intermediate*	*Proximal*	n
Noradrenaline	304 ± 22	327 ± 20	318 ± 12	10
Phenylephrine	296 ± 16	339 ± 18	320 ± 35	5
UK-14,304	102 ± 6*	190 ± 12	228 ± 16	10
B-HT 920	82 ± 9*	168 ± 18	183 ± 20	5
KCl	215 ± 23	216 ± 19	232 ± 20	5

The mean weights of the strips were: 33, 38 and 40 mg (from the distal, intermediate and proximal regions, respectively).
* Significant difference between the value for the distal and that for the respective proximal portion, $P < 0.05$.

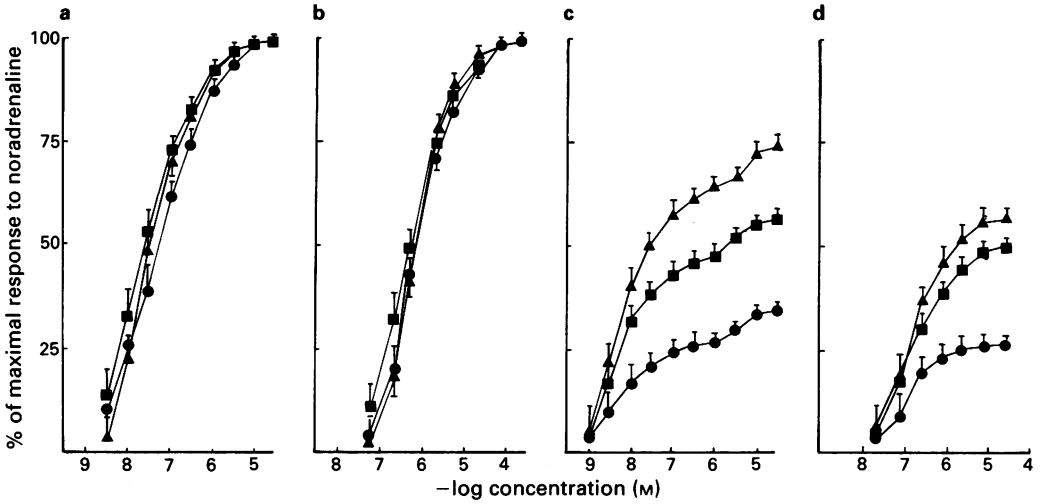

Figure 2 Concentration-response curves in dog saphenous vein to (a) noradrenaline, (b) phenylephrine, (c) UK-14,304 and (d) B-HT920 obtained at three different levels of the vessel: proximal (▲), intermediate (■) and distal (●). Each point represents the mean and vertical lines show s.e.mean.

activation of adrenoceptors of the proximal portion of this vein trunk.

The effect of phenylephrine on the responses to UK-14,304 and vice versa was also checked. Neither sub-effective (up to 10 nM phenylephrine; up to 500 pM UK-14,304) nor effective concentrations of one of these agonists enhanced or antagonized the responses to the other.

The pA_2 values for the antagonism exerted by prazosin and yohimbine on the effects of phenylephrine and UK-14,304 were determined only for the distal and the proximal segments

Table 2 pD_2 values for the contractile responses caused by different agonists on three different regions of the dog saphenous and cephalic vein trunks: distal, intermediate, and proximal (see Methods)

Agonist		*pD_2* *Distal*	*Intermediate*	*Proximal*	n
Phenylephrine	(Saphenous)	6.04 ± 0.04	6.26 ± 0.13	6.36 ± 0.06	5
	(Cephalic)	6.10 ± 0.07	6.21 ± 0.08	6.27 ± 0.05	4
Noradrenaline	(Saphenous)	7.29 ± 0.04	7.39 ± 0.08	7.42 ± 0.07	5
	(Cephalic)	7.18 ± 0.03	7.21 ± 0.07	7.25 ± 0.06	4
UK-14,304	(Saphenous)	7.99 ± 0.10	8.10 ± 0.05	8.12 ± 0.01	7
	(Cephalic)	8.25 ± 0.03	8.20 ± 0.01	8.08 ± 0.06	4
B-HT 920	(Saphenous)	6.82 ± 0.05	6.75 ± 0.03	6.72 ± 0.06	5
	(Cephalic)	7.01 ± 0.09	6.97 ± 0.08	6.89 ± 0.07	3

$pD_2 = -\log EC_{50}$. Data shown are means ± s.e.

Table 3 pA_2 values and slopes of the Schild regressions for prazosin and yohimbine against the agonists phenylephrine and UK-14,304 in canine saphenous and cephalic veins

Antagonist	*Agonist*	*Vein and segment used*		*pA_2*	*Slope*	n
Prazosin	Phenylephrine	Distal	Saphenous	7.65 ± 0.07	0.99 ± 0.14	5
			Cephalic	7.62 ± 0.04	0.88 ± 0.21	4
		Proximal	Saphenous	7.40 ± 0.05	0.96 ± 0.23	5
			Cephalic	7.48 ± 0.06	1.08 ± 0.20	4
	UK-14,304	Distal	Saphenous	6.02 ± 0.06	1.03 ± 0.15	5
			Cephalic	5.98 ± 0.07	0.90 ± 0.17	4
		Proximal	Saphenous	5.93 ± 0.04	1.17 ± 0.17	5
			Cephalic	6.08 ± 0.05	1.06 ± 0.22	4
Yohimbine	Phenylephrine	Distal	Saphenous	8.14 ± 0.04	1.14 ± 0.14	4
			Cephalic	8.27 ± 0.18	1.06 ± 0.18	5
		Proximal	Saphenous	6.90 ± 0.05*	0.92 ± 0.20	7
			Cephalic	7.17 ± 0.16*	1.12 ± 0.13	5
	UK-14,304	Distal	Saphenous	8.79 ± 0.03	1.07 ± 0.19	5
			Cephalic	8.82 ± 0.03	1.19 ± 0.18	4
		Proximal	Saphenous	8.88 ± 0.01	0.94 ± 0.21	5
			Cephalic	8.93 ± 0.02	0.98 ± 0.18	4

* Significantly different from the value for the respective distal portion ($P < 0.01$; paired experiments). The slopes of Schild regressions were not significantly different from 1.

and are presented in Table 3. While the values for the antagonism exerted by yohimbine on the effects of UK-14,304 are similar at the distal and the proximal levels, those for the antagonism by yohimbine of phenylephrine are much higher at the distal than at the proximal level.

Since the selectivity of phenylephrine for α_1-adrenoceptors is not very high (phenylephrine is about 30 times more effective on α_1- than on α_2-adrenoceptors; Starke, 1981) some involvement of α_2-adrenoceptor activation may interfere with the pA_2 values for the antagonism of yohimbine against this amine. To avoid, as much as possible, α_2-adrenoceptor stimulation by phenylephrine, incomplete concentration-response curves to this amine were determined such that the highest concentration of phenylephrine used after yohimbine was never higher than 30 times the minimal effective concentration before yohimbine (10 and 50 nM). The pA_2 values calculated under these conditions from the displacement of the concentration-response curve at the EC_{25} level, were 8.21 ± 0.18 ($n = 6$) at the distal and 7.36 ± 0.14 ($n = 6$) at the proximal levels ($P < 0.01$).

The pA_2 values for the antagonism by yohimbine of phenylephrine responses were also determined after 30 and 100 nM phenoxybenzamine. Very interestingly, after 40 min in 30 nM phenoxybenzamine, the maximum effect of phenylephrine reached $32.3 \pm 3.1\%$ ($n = 8$) of that obtained before phenoxybenzamine at distal and $60.2 \pm 5.4\%$ ($n = 8$) at proximal level, and the pD_2 values for phenylephrine were 5.21 ± 0.40 and 5.12 ± 0.51 ($n = 5$) at proximal and distal levels, respectively. Under the same conditions, the pA_2 values for yohimbine against phenylephrine were 8.47 ± 0.16 ($n = 6$) and 8.36 ± 0.14 ($n = 6$) at distal and proximal levels, respectively. After 100 nM phenoxybenzamine, it was impossible to compare pA_2 values, because at the distal level the response to phenylephrine was practically abolished in the majority of the strips; at the proximal level the maximum response to phenylephrine reached about 30% of that before phenoxybenzamine. The pA_2 value for prazosin vs UK-14,304 after phenoxybenzamine (30 nM) at proximal level was 6.01 ± 0.09 ($n = 6$).

The concentration-response curve for phenylephrine at distal level was displaced to the right by 10 and 30 nM rauwolscine (by factors of 2.5 ± 0.3 and 6.3 ± 0.7, respectively; $n = 6$), while at proximal level, it was not changed. In the presence of 30 nM rauwolscine, yohimbine (30 nM) displaced the concentration-response curves for phenylephrine at proximal and distal levels similarly (by factors of 5.2 ± 0.6 and 5.4 ± 0.7, respectively; $n = 6$) (Figure 3).

On vein strips of the same dogs, rauwolscine (30 nM) displaced the concentration-response curves for UK-14,304 to the right, equally at distal and proximal levels (by factors of 8.3 ± 1.1 and 9.5 ± 1.4, respectively; $n = 6$) (Figure 3).

Cephalic vein

The results obtained in the cephalic vein are very similar to those obtained in the saphenous one. All the agonists caused concentration-dependent contractions of the strips obtained from distal, intermediate and proximal regions of the cephalic vein trunk. As shown in Figure 4, noradrenaline and phenylephrine caused maximal contractions of the same magnitude, while UK-14,304, B-HT 920 and KCl caused maximal contractions which were sub-maximal when compared to those caused by noradrenaline.

The maximal contractions to UK-14,304 reached about 50.2 ± 4.8, 76.5 ± 8.1 and $78.1 \pm 6.7\%$ ($n = 10$) and those to B-HT 920 about 49.3 ± 5.1, 76.4 ± 6.8 and $75.7 \pm 7.3\%$ ($n = 4$) of those to noradrenaline at the distal, intermediate and proximal portions, respectively, while the maximal contractions to KCl reached about 69% both at proximal and distal levels (Figure 4 and Table 4).

These results show that the maximal activation of postjunctional α_2-adrenoceptors of the distal part of the cephalic vein generates a contraction that is much weaker than that induced by maximal activation of postjunctional α_2-adrenoceptors of the proximal part of this vein trunk.

Table 4 Tension developed in cephalic vein with maximal contraction by different agonists

	Maximal contraction (in $g\,g^{-1}$ tissue)			
Agonist	*Distal*	*Intermediate*	*Proximal*	n
Noradrenaline	286 ± 28	357 ± 31	342 ± 32	10
Phenylephrine	282 ± 39	363 ± 36	347 ± 39	5
UK-14,304	144 ± 11*	272 ± 21	267 ± 28	10
B-HT 920	139 ± 18*	260 ± 27	258 ± 49	4
KCl	200 ± 21	—	239 ± 24	5

The mean weights of the strips were: 28, 33 and 36 mg (from the distal, intermediate and proximal regions, respectively).
* Significant difference between the value for the distal and that for the respective proximal portion.

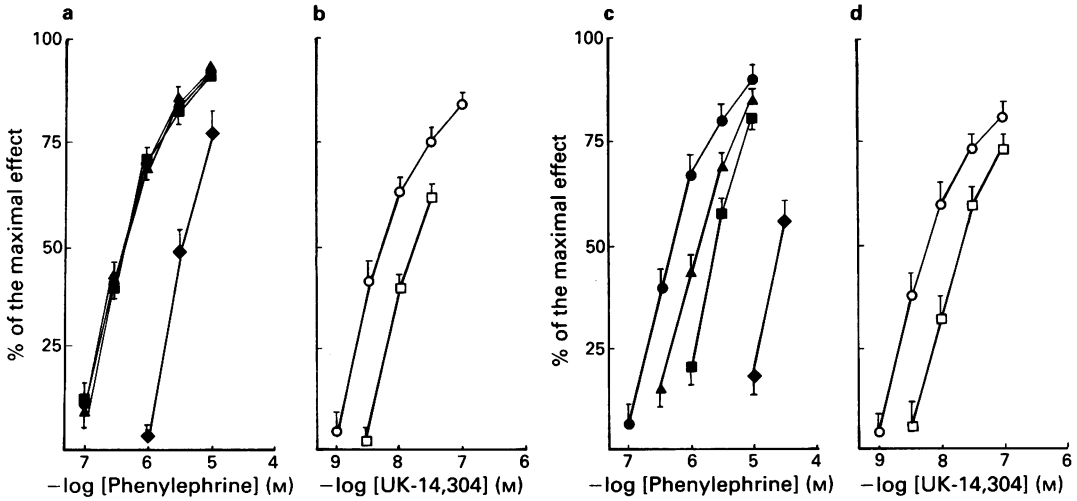

Figure 3 Concentration-response curves for phenylephrine (a and c) and UK-14,304 (b and d) at proximal (a and b) and distal (c and d) levels, in the absence and presence of the α_2-antagonists rauwolscine (Rauw) and yohimbine (Yoh). The responses to either agonist are expressed as % of its own maximum effect. In (a and c), (●) control, (▲) after 10 nM Rauw, (■) after 30 nM Rauw and (◆) after 30 nM Rauw + 30 nM Yoh; (b and d), (○) control, (□) after 30 nM Rauw. Each point represents the mean and vertical lines show s.e.mean.

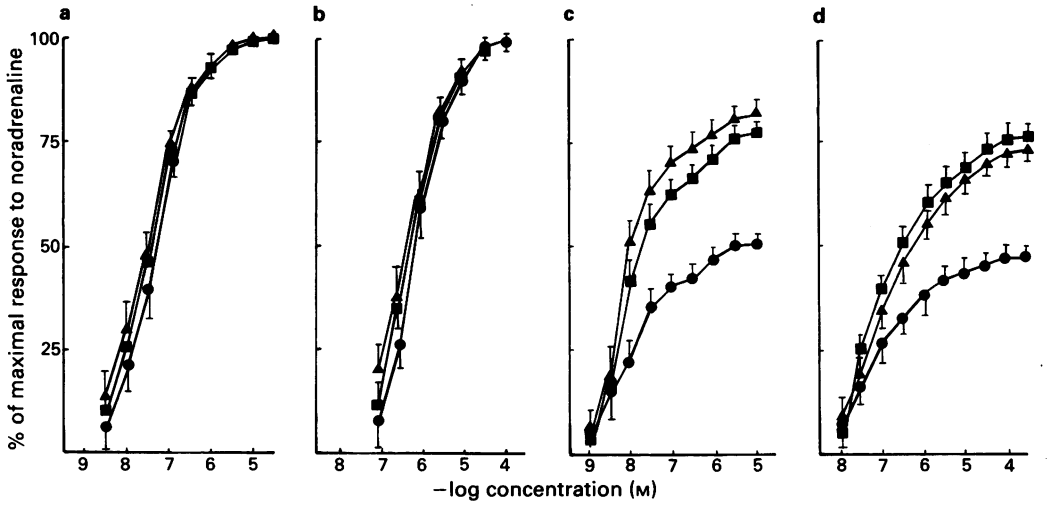

Figure 4 Dog cephalic vein. Concentration-response curves to (a) noradrenaline, (b) phenylephrine, (c) UK-14,304 and (d) B-HT 920 obtained at three different levels of the vessel: proximal (▲), intermediate (■), and distal (●). Each point represents the mean and vertical lines show s.e.mean.

The pA_2 values for the antagonism exerted by prazosin and yohimbine against the responses to phenylephrine and UK-14,304 were determined only for the distal and the proximal segments and are presented in Table 3. As in the saphenous vein, the pA_2 values for the antagonism by yohimbine of the effects of UK-14,304 are similar at the distal and proximal levels, while those for the antagonism by yohimbine of phenylephrine effects are higher at the distal than at the proximal level.

As for the saphenous vein, the pA_2 values for the antagonism exerted by yohimbine on phenylephrine responses after 30 nM phenoxybenzamine were not different at proximal and distal levels (8.39 ± 0.17 and 8.25 ± 0.12, $n = 6$, respectively).

Discussion

It is known that in several vessels where α_1- and α_2-adrenoceptors co-exist postsynaptically, α_2-adrenoceptor stimulation generates a low maximal response compared with α_1-adrenoceptor stimulation. This has been shown for the canine saphenous (Flavahan *et al.*, 1984; Fowler *et al.*, 1984; Guimaraes, 1986) and cephalic vein (Moura *et al.*, 1990) as for other vessels of the dog and other species (Starke & Docherty, 1982; Polonia *et al.*, 1985; Fontes Ribeiro & Macedo, 1986). The present results obtained in the saphenous and the cephalic veins of the dog agree well with these data. In fact, the maximal response to the selective α_2-adrenoceptor agonists UK-14,304 and B-HT 920 was smaller than the maximal responses to either noradrenaline or phenylephrine, thus allowing one to conclude that the maximal response to phenylephrine is most likely determined by α_1-adrenoceptor activation.

The present results further show that in both the canine saphenous and cephalic veins, the tension per gram of tissue developed by the maximal responses to α_2-adrenoceptor agonists increases from the distal to the proximal portions of those vessels, while those to noradrenaline, phenylephrine or KCl (activation not involving adrenoceptors) do not.

Interestingly, the maximal responses to phenylephrine, which were of the same magnitude at proximal and distal levels before phenoxybenzamine, became like those to α_2-adrenoceptor agonists (larger at proximal than at distal level) after phenoxybenzamine. Bearing in mind that after phenoxybenzamine (30 nM) the responses to phenylephrine are predominantly α_2-adrenoceptor-mediated (Guimaraes *et al.*, 1987), this finding clearly confirms that α_2-adrenoceptor stimulation is more effective at the proximal than at the distal level.

Since the maximal responses obtained in the intermediate portions of both the saphenous and cephalic veins are also intermediate between those obtained in the distal and proximal portions, one can conclude that the effectiveness of maximal α_2-adrenoceptor activation increases from the distal to the proximal segments of those veins of the canine limbs.

The differences in the α_2-adrenoceptor-mediated maximal responses observed in the present study may be the result of a number of factors: (1) a difference in the types of α_2-adrenoceptors involved in the responses, (2) a different interaction between α_1- and α_2-adrenoceptors along the vessel, (3) a different α_2-adrenoceptor density at the different levels, or (4) a difference in the α_2-adrenoceptor-response coupling characteristics along the vessel. The first hypothesis can easily be ruled out. The pD_2 values for the selective α_2-adrenoceptor agonists as well as the pA_2 describing interactions with selective α_2-adrenoceptor antagonists did not change significantly from region to region in each vessel, showing that receptors involved in responses to UK-14,304 and B-HT 920 are identical along the vessel. The same arguments are valid against the second hypothesis. Moreover, if there were a different interaction between α_1- and α_2-adrenoceptors along the vessel, the combination of α_1 and α_2-adrenoceptor agonists should be synergistic or antagonistic at one or other level and not additive along all the vessel, as was the case. Thus, the increase in the maximal response to α_2-adrenoceptor agonists in proximal compared with distal portions of both veins cannot be ascribed to some difference in the α_2-adrenoceptors involved or to a different interaction between α_1- and α_2-adrenoceptors.

Since the receptors may vary with respect to their density in various types of tissues, both at the central and peripheral levels (Timmermans & Van Zwieten, 1981), it is likely that the differences in the α_2-adrenoceptor-mediated maximal responses observed in the present study result from a difference in α_2-adrenoceptor density at the different levels. The abolition of the responses to phenylephrine at the distal level after 100 nM phenoxybenzamine, while a 30% maximal response still remained at the distal one, is highly suggestive of a lower density of α-adrenoceptors at the distal level. However, some difference in the receptor-response coupling characteristics along the vessel cannot be excluded as a causal factor.

In the last few years, evidence has accumulated in favour of the view that α_1-adrenoceptors do not have the same properties in all tissues. Analysis of α-adrenoceptor antagonists'

potencies in isolated vessels suggests that postjunctional α_1-adrenoceptors are heterogeneous (Flavahan & Vanhoutte, 1986; Minneman, 1988). In our experiments, which were carried out after inhibition of the different processes which remove the agonists from the α-adrenoceptor biophase, yohimbine was much more effective against the responses to phenylephrine at the distal than at the proximal level. The pA_2 values (8.14 at distal and 6.90 at proximal level in the saphenous, and 8.24 at distal and 7.17 at proximal level in the cephalic vein) show that yohimbine is 17 and 13 times more potent distally than proximally (in the saphenous and cephalic vein, respectively). Although the presence of α_2-adrenoceptors can disturb the analysis of α_1-adrenoceptor-mediated responses and vice versa in tissues where both subgroups coexist (McGrath *et al.*, 1989), these differences can hardly be explained on the basis of a different involvement of α_2-adrenoceptor activation at these two levels. In favour of the view that α_2-adrenoceptors are more involved in the responses to phenylephrine at the distal than at the proximal level – thus explaining the differential antagonism by yohimbine – it might be argued that in the presence of low concentrations of rauwolscine, yohimbine became equally potent against phenylephrine at both levels. But who knows what rauwolscine did in low concentrations? Did it antagonize classical α_2-adrenoceptors whose role is more prominent at the distal level or did it antagonize 'special' α_1-adrenoceptors existing at the distal level which are more sensitive to α_2-adrenoceptor antagonists than are the classical α_1-adrenoceptors? All the evidence obtained in the present investigation is in favour of the second hypothesis: firstly, if α_2-adrenoceptor activation had any involvement in this differential effect of yohimbine, yohimbine should have antagonized phenylephrine more effectively at the proximal than at the distal level – since α_2-adrenoceptor activation is more effective proximally than distally – and not the opposite; furthermore, UK-14,304, which is a highly selective α_2-adrenoceptor agonist, was equally antagonized at the distal and the proximal level by either yohimbine and rauwolscine. If phenylephrine acted on the same receptors, why wasn't it antagonized equally by these antagonists at those levels? Additionally, in the experiments carried out under conditions which did not favour α_2-adrenoceptor stimulation by phenylephrine – those in which incomplete concentration-response curves to phenylephrine were determined – yohimbine continued to be much more potent at distal than at proximal level, showing that the differential effect of yohimbine only depends on the α_1-adrenoceptor population. Some years ago, several authors using different experimental procedures, showed that in low concentrations, phenoxybenzamine inactivates α_1-adrenoceptors only: in the cat's spleen, Dubocovich & Langer (1974) demonstrated that the concentration of phenoxybenzamine required for the blockade of presynaptic α_2-adrenoceptors that regulate transmitter release is 30 times higher than that required for the blockade of postsynaptic α_1-adrenoceptors; in the pithed rat, Ruffolo *et al.* (1984) showed that $1\,mg\,kg^{-1}$ phenoxybenzamine will irreversibly inactivate approximately 84% of the α_1-adrenoceptors without affecting α_2-adrenoceptors; in the canine saphenous vein, one of the tissues used in the present study, it was shown that 30 nM phenoxybenzamine – a concentration which did not affect α_2-adrenoceptors – reduced the α_1-adrenoceptor population such that the responses to phenylephrine become predominantly α_2-adrenoceptor-mediated (Guimaraes *et al.*, 1987). With these data in mind, the antagonism of yohimbine against phenylephrine was also checked after 30 nM phenoxybenzamine, a concentration which eliminates the vast majority of α_1-adrenoceptors without affecting α_2-adrenoceptors. Under these conditions, yohimbine became equally potent at the proximal and the distal levels, showing that elimination of α_1-adrenoceptors removes the source for that differential antagonism by yohimbine. Hence, the difference in sensitivity to yohimbine of the responses to phenylephrine at the two levels may be due to a difference in the α_1-adrenoceptors involved.

In 1982, Ruffolo *et al.* demonstrated that in the rat aorta, α-adrenoceptors possess properties of both α_1- and α_2-adrenoceptors, since both prazosin and yohimbine are very potent against phenylephrine in this tissue. The α-adrenoceptors mediating the responses to phenylephrine in the distal portions of the veins of canine limbs seem to be like those described by these authors.

In summary, the present results suggest changes in both postjunctional α_1- and α_2-adrenoceptors along the canine saphenous and cephalic vein trunks: while α_2-adrenoceptors increase in density from the distal towards the proximal portion, α_1-adrenoceptors in distal and proximal portions may be of a different type.

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References

BLACK, J.W., DUNCAN, W.A.M. & SHANKS, R.G. (1965). Comparison of some properties of pronethanol and propranolol. *Br. J. Pharmacol.*, **25**, 577–591.

CONSTANTINE, J.W., LEBEL, W. & ARCHER, R. (1982). Functional postsynaptic α_2- but not α_1-adrenoceptors in dog saphenous vein exposed to phenoxybenzamine. *Eur. J. Pharmacol.*, **85**, 325–329.

DE MEY, J.C. & VANHOUTTE, P.M. (1981). Uneven distribution of postjunctional $alpha_1$- and $alpha_2$-like adrenoceptors in canine arterial and venous smooth muscle. *Circ. Res.*, **48**, 875–884.

DOCHERTY, J.R., MACDONALD, A. & McGRATH, J.C. (1979). Further sub-classification of α-adrenoceptors in the cardiovascular system, vas deferens and anococcygeus of the rat. *Br. J. Pharmacol.*, **67**, 421P–422P.

DREW, G.M. & WHITING, S.B. (1979). Evidence for two distinct types of postsynaptic α-adrenoceptor in vascular smooth muscle *in vivo*. *Br. J. Pharmacol.*, **67**, 207–215.

DUBOCOVICH, M.L. & LANGER, S.Z. (1974). Negative feedback regulation of noradrenaline release by nerve stimulation in the perfused cat's spleen: differences in potency of phenoxybenzamine in blocking the pre- and post-synaptic receptors. *J. Physiol.*, **237**, 505–519.

DUNN, W.R., McGRATH, J.C. & WILSON, V.G. (1989). Expression of functional postjunctional α_2-adrenoceptors in rabbit isolated distal saphenous artery – a permissive role for angiotensin II? *Br. J. Pharmacol.*, **96**, 259–261.

FLAVAHAN, N.A. & VANHOUTTE, P.M. (1986). α_1-Adrenoceptor subclassification in vascular smooth muscle. *Trends Pharmacol. Sci.*, **7**, 347–349.

FLAVAHAN, N.A., RIMELE, T.J., COOKE, J.P. & VANHOUTTE, P.M. (1984). Characterization of postjunctional $alpha_1$- and $alpha_2$-adrenoceptors activated by exogenous or nerve released norepinephrine in the canine saphenous vein. *J. Pharmacol. Exp. Ther.*, **230**, 699–705.

FLAVAHAN, N.A., COOKE, J.P., SHEPHERD, J.T. & VANHOUTTE, P.M. (1987). Human postjunctional $alpha_1$- and $alpha_2$-adrenoceptors: differential distribution in arteries of the limbs. *J. Pharmacol. Exp. Ther.*, **241**, 361–365.

FONTES RIBEIRO, C.A. & MACEDO, T.A. (1986). Pharmacological characterization of the postsynaptic α-adrenoceptors in human uterine artery. *J. Pharm. Pharmacol.*, **38**, 600–605.

FOWLER, P.J., GROUS, M., PRICE, W. & MATTHEWS, W.D. (1984). Pharmacological differentiation of postsynaptic alpha adrenoceptors in the dog saphenous vein. *J. Pharmacol. Exp. Ther.*, **229**, 712–718.

GUIMARAES, S. (1986). Postsynaptic α-adrenoceptors in blood vessels: discrepancies between results obtained *in vivo* and *in vitro*. In *New Aspects of the Role of Adrenoceptors in the Cardiovascular System*, ed. Grobecker, H., Philippu, A. & Starke, K., pp. 129–138. Berlin: Springer-Verlag.

GUIMARAES, S., BRANDAO, F. & PAIVA, M.Q. (1978). A study of the adrenoceptor-mediated feedback mechanism by using adrenaline as a false transmitter. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **305**, 185–188.

GUIMARAES, S. & OSSWALD, W. (1969). Adrenergic receptors in the veins of the dog. *Eur. J. Pharmacol.*, **5**, 133–140.

GUIMARAES, S., PAIVA, M.Q. & POLONIA, J.J. (1983). Alpha$_1$ and alpha$_2$-adrenoceptors of the dog saphenous vein and their relation to the sympathetic nerve terminals. *Progr. Neuropsychopharmacol.* (Abstracts) (Suppl.), 155.

GUIMARAES, S., PAIVA, M.Q. & MOURA, D. (1987). Alpha$_2$-adrenoceptor-mediated responses to so-called selective alpha$_1$-adrenoceptor agonists after partial blockade of alpha$_1$-adrenoceptors. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **335**, 397–402.

HAMED, A.T., JOHNSON, T.D., CHARLTON, K.G. & CLARKE, D.E. (1983). Pharmacological characterization of α-adrenoceptor subtypes in rat isolated thoracic aorta. *J. Auton. Pharmacol.*, **3**, 265–273.

IVERSEN, L.L. & SALT, P.J. (1970). Inhibition of cathecolamine uptake$_2$ by steroids in the isolated rat heart. *Br. J. Pharmacol.*, **40**, 528–530.

McGRATH, J.C., BROWN, C.M. & WILSON, V.G. (1989). Alpha-adrenoceptors: A critical review. *Med. Res. Rev.*, **9**, 407–533.

McGRATH, J.C., FLAVAHAN, N.A. & McKEAN, C.E. (1982). α_1- and α_2-adrenoceptor mediated pressor and chronotropic effects in rat and rabbit. *J. Cardiovasc. Pharmacol.*, **4**, S101–S107.

MINNEMAN, K.P. (1988). α_1-Adrenergic receptor subtypes, inositol phosphates, and sources of cell Ca^{2+}. *Pharmacol. Rev.*, **40**, 87–119.

MOURA, D.M., VAZ-DA-SILVA, M.J. & GUIMARAES, S. (1990). Alpha-adrenoceptor reserve in the canine cephalic vein. *Eur. J. Pharmacol.*, **179**, 9–16.

POLONIA, J.J., PAIVA, M.Q. & GUIMARAES, S. (1985). Pharmacological characterization of postsynaptic α-adrenoceptor subtypes in five different dog arteries *in vitro*. *J. Pharm. Pharmacol.*, **37**, 205–208.

RUFFOLO, R.R., Jr., MORGAN, E.L. & MESSICK, K. (1984). Possible relationship between receptor reserve and the differential antagonism of alpha$_1$- and alpha$_2$-adrenoceptor-mediated pressor responses by calcium channel antagonists in the pithed rat. *J. Pharmcol. Exp. Ther.*, **230**, 587–594.

RUFFOLO, R.R., Jr., WADDEL, J.E. & YADEN, E.C. (1982). Heterogeneity of postsynaptic alpha adrenergic receptors in mammalian aortas. *J. Pharmacol. Exp. Ther.*, **221**, 309–314.

SHEPPERSON, N.B. & LANGER, S.Z. (1981). The effects of the 2-aminotetrahydronaphtalene derivative M7 a selective α_2-adrenoceptor agonist *in vitro*. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **318**, 10–13.

SHOJI, T., TSURU, H. & SHIGEI, T. (1983). A regional difference in the distribution of postsynaptic alpha-adrenoceptor subtypes in canine veins. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **324**, 246–255.

SKARBY, T. & ANDERSSON, K.-E. (1984). Contraction-mediated α-adrenoceptors in isolated human omental, temporal and pial arteries. *J. Auton. Pharmacol.*, **4**, 219–229.

SOKAL, R.R. & ROHLF, F.J. (1981). *Biometry. The Principles and Practice of Statistics in Biological Research.* New York: Freeman and Company.

STARKE, K. (1981). α-Adrenoceptor sub-classification. *Rev. Physiol. Biochem. Pharmacol.*, **88**, 199–236.

STARKE, K. & DOCHERTY, J.R. (1982). Types and functions of peripheral α-adrenoceptors. *J. Cardiovasc. Pharmacol.*, **4**, S3–S7.

TEMPLETON, A.G.B., MACMILLAN, J., McGRATH, J.C., STOREY, N.D. & WILSON, V.G. (1989). Evidence for prazosin-resistant, rauwolscine-sensitive α-adrenoceptors mediating contractions in the isolated vascular bed of the rat tail. *Br. J. Pharmacol.*, **97**, 563–571.

TIMMERMANS, P.B.M.W.M., KWA, H.Y. & VAN ZWIETEN, P.A. (1979). Possible subdivision of postsynaptic α-adrenoceptors mediating pressor response in the pithed rat. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **310**, 189–193.

TIMMERMANS, P.B.M.W.M. & VAN ZWIETEN, P.A. (1981). The postsynaptic α_2-adrenoceptor. *J. Auton. Pharmacol.*, **1**, 171–183.

TRENDELENBERG, U. (1966). Mechanisms of supersensitivity and subsensitivity to sympathomimetic amines. *Pharmacol. Rev.*, **18**, 629–640.

VAN ROSSUM, J.M. (1963). Cumulative dose-response curves. Technique for the making of dose-response curves in isolated organs and the evaluation of drug parameters. *Arch. Int. Pharmacodyn.*, **143**, 299–330.

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Methoxyphenamine inhibits basal and histamine-induced nasal congestion in anaesthetized rats

W.A.K. Lau, [1]R.G. King & A.L.A. Boura

Department of Pharmacology, Monash University, Clayton, Victoria 3168, Australia

1 Nasal resistance in anaesthetized rats was assessed by measuring air overflow during ventilation of the nasal passages at constant pressure. Nasal basal resistance was reduced in a dose-dependent manner by methoxyphenamine hydrochloride (0.01–30 mg kg^{-1}, i.v.), pseudoephedrine hydrochloride (0.03–3 mg kg^{-1}, i.v.) and adrenaline bitartrate (0.01–3 μg kg^{-1}, i.v.). Both methoxyphenamine and pseudoephedrine were less potent and less efficacious than adrenaline but caused longer-lasting responses.

2 Nasal congestion induced by histamine (0.2% nebulised solution passed into the nasal passages for 15 s) was inhibited by i.v. administration of methoxyphenamine, pseudoephedrine, adrenaline, methoxamine or tyramine: the ID_{50}s against 0.2% histamine-induced nasal congestion were 1.16 (95% confidence limits; 0.5, 1.8) mg kg^{-1}, 0.25 (0.19, 0.33) mg kg^{-1}, 0.037 (0.018, 0.06) μg kg^{-1}, 8.12 (6.74, 9.65) μg kg^{-1} and 30.6 (26.1, 35.8) μg kg^{-1} respectively.

3 The inhibitory effects of both methoxyphenamine and tyramine on histamine-induced nasal congestion were reduced after administration of desmethylimipramine (0.1 and 1 mg kg^{-1}, i.v.) or prazosin (0.1 and 0.3 mg kg^{-1}, i.v.). Similarly, the inhibitory effects of methoxamine were reduced after prazosin (0.1 and 0.3 mg kg^{-1}).

4 These results indicate that methoxyphenamine (1 mg kg^{-1}, i.v.) inhibits histamine-induced nasal congestion in the rat. This action, at least in part, is probably indirect being mediated by release of neuronal noradrenaline which then acts on α_1-adrenoceptors.

Introduction

Methoxyphenamine is a sympathomimetic agent which causes bronchodilatation (Curry *et al.*, 1949; Grater, 1960) and is used in commercial preparations as a nasal decongestant. It is thought to be an indirectly acting sympathomimetic agent (Bowman & Rand, 1980). Nevertheless there have been few studies of the nasal decongestant properties of methoxyphenamine: Brooks *et al.* (1984) compared its effects with those of phenylpropanolamine and placebo as nasal decongestants in patients suffering from ragweed hay fever, but could demonstrate no activity for methoxyphenamine at the doses used. In laboratory animals the drug has not been examined for nasal decongestant properties. In the present study we therefore looked for evidence that methoxyphenamine could have nasal decongestant effects in anaesthetized rats, comparing its activity with that of the known decongestants adrenaline and pseudoephedrine and examining the mechanisms underlying any effects observed.

Methods

Male Wistar rats, weighing 300–450 g, were anaesthetized with pentobarbitone sodium (65 mg kg^{-1}, i.p.), supplements (10 mg kg^{-1}, i.v.) being given as required during the course of each experiment. The caudal trachea was cannulated and each animal was allowed to breathe spontaneously. Systemic blood pressure and heart rate were monitored from a cannulated femoral artery using a Statham (P23) pressure transducer triggering a cardiotachometer, both parameters being displayed on a Grass polygraph (79D). The femoral vein was cannulated for intravenous administration of drugs, each dose in a volume of 0.5 ml kg^{-1} body wt. All doses were flushed in with 0.2 ml saline (0.9% w/v NaCl).

Measurement of nasal airway resistance

The method used was a modification of that of Salem & Clemente (1972). A polyethylene cannula was inserted to a depth of 2–3 cm into the cephalic end of the trachea (Figure 1). The oesophagus was ligated and the buccal cavity blocked with alginate impression material thus ensuring that air could only pass through the nose and nasal passages. A lack of passage of air via the mouth was confirmed by use of a detergent solution over the mouth which bubbled if air was coming out. A modified Konzett & Rössler (1940) apparatus was used to ventilate the nasal cavities at a constant pressure (7–12 cmH_2O) with a respirator (Ugo Basile 7025) delivering humidified air (bubbled through water at 37°C) with a stroke volume of approximately 8 ml kg^{-1} (50 strokes min^{-1}). Changes in ventilation overflow (taken as a measure of changed intranasal resistance) were recorded with a bronchospasm transducer (Ugo Basile 7020) connected to a Ugo Basile unirecord (7050). Since the nasal cavities were ventilated at a constant pressure, decreased resistance of the nasal passages was reflected as a reduction in recorded air overflow.

Effects of drugs on nasal basal resistance

Measurements were made of the decreases in nasal ventilation overflow in response to 3–4 cumulative doses of methoxyphenamine (0.01–30 mg kg^{-1}, i.v.), adrenaline (0.01–3 μg kg^{-1}, i.v.) or pseudoephredrine (0.03–3 mg kg^{-1}). Doses were administered at intervals of approximately 15 min, each animal receiving in random order, either methoxyphenamine and adrenaline, or methoxyphenamine and pseudoephedrine.

Effects of drugs on histamine-induced nasal congestion

The method used was a modification of that of Misawa (1988). An ultrasonic nebuliser (DeVilbiss Pulmosonic; Middlesex, England) was placed in the respiratory circuit so that an aerosol passed into the nasal cavities with each stroke of the respirator (Figure 1). After an equilibration period of 15 min following surgical procedures, nebulised saline (0.9%) or histamine dihydrochloride (0.2% or 2%) was passed for 15 s into the nasal cavities, any change in ventilation overflow being measured. The ability of methoxyphenamine, adrenaline, pseudoephedrine, methoxamine or tyramine to inhibit histamine-induced congestion was determined by injecting intravenously a dose of drug 3 min after starting the 15 s

[1] Author for correspondence.

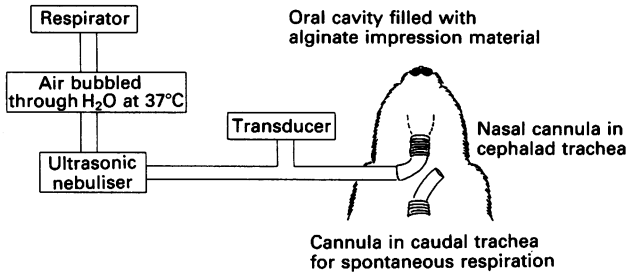

Figure 1 Diagram to show both the method of application of nebulised saline or histamine to nasal cavities of the rat and the measurement of nasal resistance.

administration of nebulised histamine. After responses to the drug had stabilized (approximately 2 min) a response to a higher dose of the drug was determined in the same fashion. Responses to 2–4 cumulative doses of each drug were measured in any one animal in this way.

When prazosin or desmethylimipramine were used, dose-response curves to methoxyphenamine (1–10 mg kg^{-1}) were obtained initially, and then repeated 10–15 min after each of two i.v. cumulative doses of prazosin, desmethylimipramine or after the vehicle (time controls). In the same manner, decongestant effects of the selective α_1-adrenoceptor agonist methoxamine (1–30 μg kg^{-1}) and the indirectly acting sympathomimetic amine tyramine (10–100 μg kg^{-1}) were tested and compared with the effect of methoxyphenamine in the anaesthetized rat.

Statistical analyses

Data represented graphically show means and standard errors. Significant differences between log dose-response curves were determined by linear regression analysis (Diem & Leutner, 1970). Values in parentheses are 95% confidence limits.

Drugs

The following drugs were used: methoxyphenamine hydrochloride (Upjohn), (−)-adrenaline bitartrate (Sigma), pseudoephedrine hydrochloride (Upjohn), histamine dihydrochloride (Sigma), prazosin hydrochloride (Sigma), desmethylimipramine hydrochloride (Sigma), methoxamine hydrochloride (Sigma) and tyramine monohydrochloride (Calbiochem). All, except the following, were dissolved in saline: prazosin was initially dissolved in glycerol and then diluted in 5% w/v glucose, and adrenaline in catecholamine diluent (NaCl 9 g l^{-1}; $NaH_2PO_4 \cdot 2H_2O$ 0.156 g l^{-1} and ascorbic acid 0.04 g l^{-1}). All drug doses are expressed in terms of the salt.

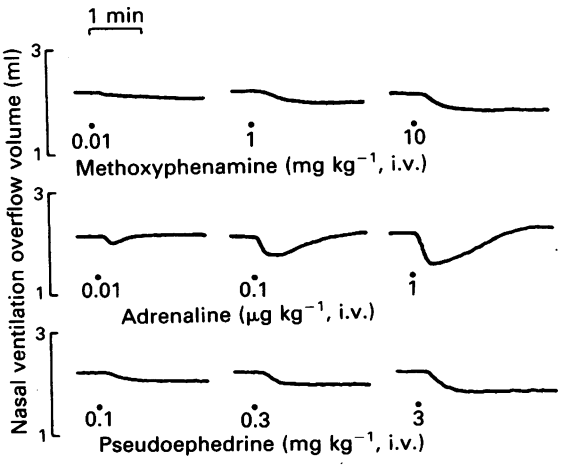

Figure 2 Tracings showing effects of methoxyphenamine, adrenaline and pseudoephedrine on nasal basal resistance in the anaesthetized rat.

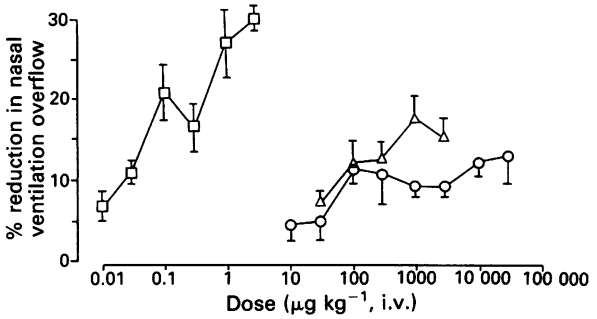

Figure 3 Effects of methoxyphenamine (○), adrenaline (□) and pseudoephedrine (△) on nasal basal resistance (n = 3–9). Values are given as the mean and the vertical bars show 1 s.e.mean or ± s.e.mean.

Results

Effects of methoxyphenamine, adrenaline or pseudoephedrine on nasal basal resistance

Administration of saline or catecholamine diluent (i.v.) did not alter nasal basal resistance in the anaesthetized rat. Figures 2 and 3 show the decongestant effects (indicated by decreases in ventilation overflow) caused after methoxyphenamine (0.01–30 mg kg^{-1}, i.v.), adrenaline (0.01–3 μg kg^{-1}, i.v.) or pseudoephedrine (0.03–3 mg kg^{-1}, i.v.). Adrenaline produced a dose-dependent decongestant effect which was relatively short-lasting (2–3 min). Both methoxyphenamine and pseudoephedrine were less potent and less efficacious than adrenaline, but produced longer-lasting responses (> 15 min) (Figure 2).

Effects of methoxyphenamine, adrenaline, pseudoephedrine, tyramine or methoxamine on histamine-induced nasal congestion

Nebulised saline passed for 15 s into the nasal cavities sometimes resulted in a slight increase in ventilation overflow

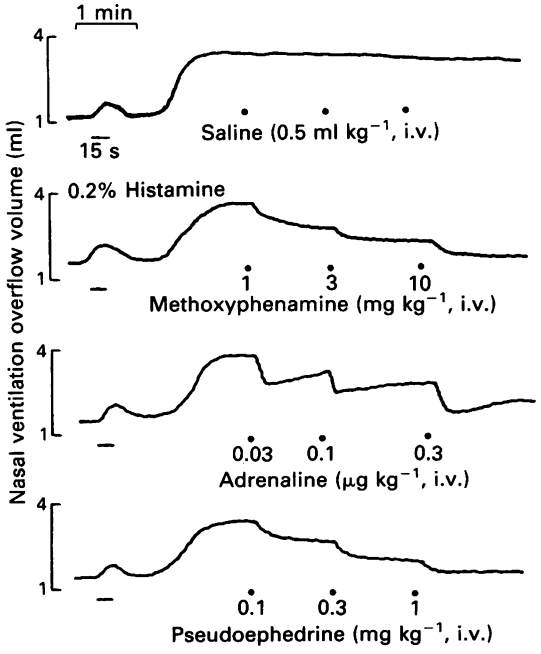

Figure 4 Tracings showing effects of saline, methoxyphenamine, adrenaline and pseudoephedrine on histamine-induced nasal congestion.

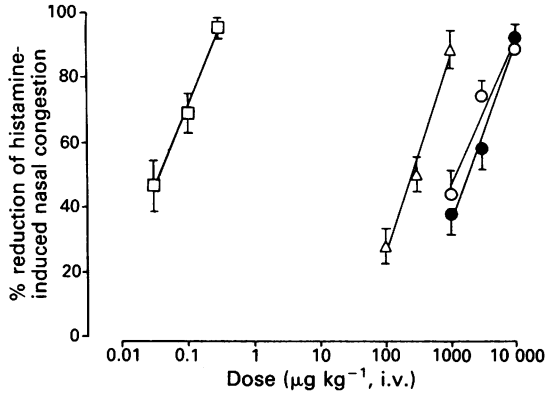

Figure 5 Effects of methoxyphenamine (○, ●), adrenaline (□) and pseudoephedrine (△) on histamine-induced nasal congestion ($n = 3$–7). Open symbols are with 0.2% histamine, closed symbols with 2% histamine. The points show the mean and the bars indicate 1 s.e.mean or ± s.e.mean.

which returned to the baseline level after ceasing administration. However, in contrast to this, an increase in ventilation overflow was observed 1–2 min after the start of a 15 s inhalation of histamine (0.2% or 2%) which persisted for 5–10 min thereafter (Figure 4). As shown in Figure 5, methoxyphenamine (1–10 mg kg^{-1}, i.v.), adrenaline (0.03–0.3 μg kg^{-1}, i.v.) or pseudoephedrine (0.1–1 mg kg^{-1}, i.v.) caused dose-dependent decreases in histamine-induced nasal resistance. The effects of methoxyphenamine and pseudoephedrine were again longer-lasting than adrenaline (Figure 4). Analysis of these data by linear regression showed that methoxyphenamine gave ID_{50}s of 1.16 (0.5, 1.8) and 1.9 (1.1, 2.7) mg kg^{-1} for inhibition of 0.2% and 2% histamine-induced nasal resistance respectively (Figure 5). This compares with ID_{50}s for adrenaline and pseudoephedrine of 0.037 (0.018, 0.06) μg kg^{-1} and 0.25 (0.19, 0.33) mg kg^{-1} (Figure 5) respectively (both for inhibition of 0.2% histamine-induced nasal congestion). As shown in Figures 6 and 7, both methoxamine (1–30 μg kg^{-1}) and tyramine (10–100 μg kg^{-1}) also inhibited 0.2% histamine-induced nasal congestion. The ID_{50}s were 8.12 (6.74, 9.65; $n = 5$) μg kg^{-1} and 30.6 (26.1, 35.8; $n = 6$) μg kg^{-1} respectively.

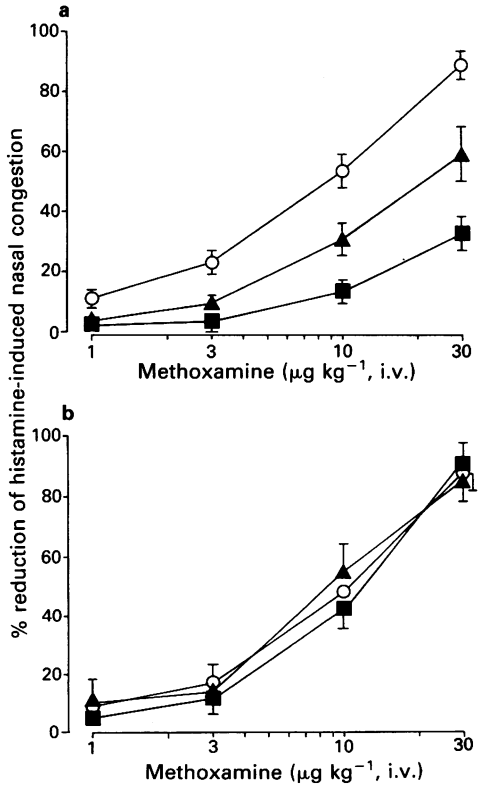

Figure 6 (a) Effects of prazosin (○, 0 mg kg^{-1}; ▲, 0.1 mg kg^{-1} and ■, 0.3 mg kg^{-1}) on inhibition by methoxamine of histamine-induced nasal congestion ($n = 4$). (b) Effects of desmethylimipramine (○, 0 mg kg^{-1}; ▲, 0.1 mg kg^{-1} and ■, 1 mg kg^{-1}) on inhibition by methoxamine of histamine-induced nasal congestion ($n = 4$). The points show the mean with the error bars indicating 1 s.e.mean or ± s.e.mean.

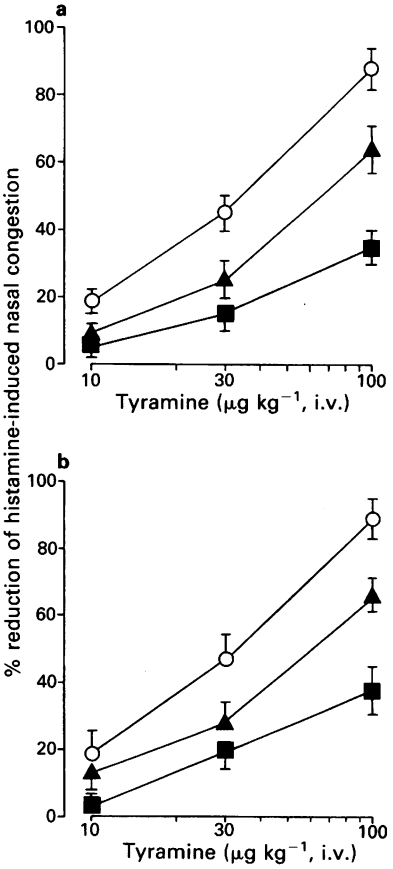

Figure 7 (a) Effects of prazosin (○, 0 mg kg^{-1}; ▲, 0.1 mg kg^{-1} and ■, 0.3 mg kg^{-1}) on inhibition by tyramine of histamine-induced nasal congestion ($n = 6$). (b) Effects of desmethylimipramine (○, 0 mg kg^{-1}; ▲, 0.1 mg kg^{-1} and ■, 1 mg kg^{-1}) on inhibition by tyramine of histamine-induced nasal congestion ($n = 4$). The points show the mean with the error bars indicating 1 s.e.mean or ± s.e.mean.

Effects of prazosin and desmethylimipramine on methoxyphenamine, methoxamine or tyramine-induced decrease of nasal resistance

Repeated dose-response curves obtained for agonist inhibition of histamine-induced nasal resistance did not vary significantly with time or with injection of vehicle at the same time intervals as antagonists. The test was performed over 2–3 h with 20 min intervals between dose-response curves for methoxyphenamine or methoxamine and a period of 30 min for tyramine. As shown in Figure 8a, prazosin (0.1 and 0.3 mg kg^{-1} i.v.) antagonized the inhibitory effects of methoxyphenamine on histamine-induced nasal resistance. For both doses of prazosin, there was a significant parallel shift of the log dose-response curve to methoxyphenamine, as assessed by linear regression analysis (Table 1). Similarly, as shown in Figure 8b, desmethylimipramine (0.1 and 1 mg kg^{-1}, i.v.) reduced the effects of methoxyphenamine on histamine-induced nasal resistance. Both doses of desmethylimipramine

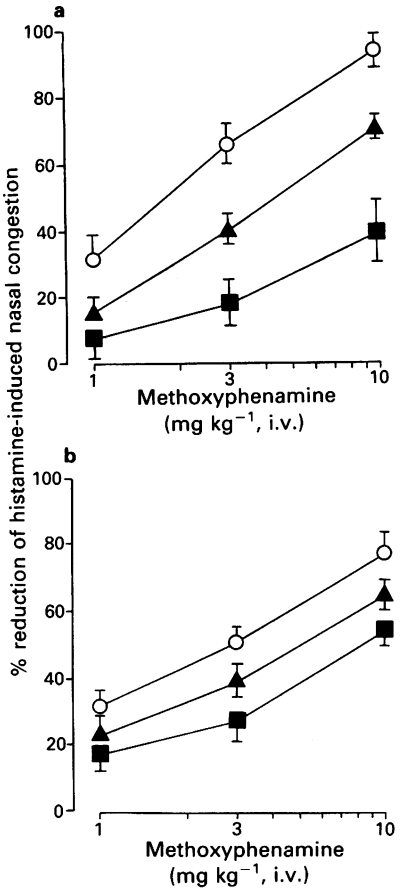

Figure 8 (a) Effects of prazosin (○, 0 mg kg^{-1}; ▲, 0.1 mg kg^{-1} and ■, 0.3 mg kg^{-1}) on inhibition by methoxyphenamine of histamine-induced nasal congestion ($n = 5$). (b) Effects of desmethylimipramine (○, 0 mg kg^{-1}; ▲, 0.1 mg kg^{-1} and ■, 1 mg kg^{-1}) on inhibition by methoxyphenamine of histamine-induced nasal congestion ($n = 5$). The points show the mean with the error bars indicating 1 s.e.mean or ± s.e.mean.

caused a significant parallel shift to the right of the log dose-response curve to methoxyphenamine (Table 1). Methoxamine (1–30 μg kg^{-1}) and tyramine (10–100 μg kg^{-1}) produced dose-dependent decreases of histamine-induced nasal congestion (Figures 6 and 7). As shown in Figure 6, prazosin (0.1 and 0.3 mg kg^{-1}), but not desmethylimipramine (0.1 and 1 mg kg^{-1}) significantly inhibited the effects of methoxamine. Figure 7 shows that the same doses of prazosin and desmethylimipramine significantly inhibited the effect of tyramine. Rightward parallel shifts in the linear portion of the log dose-response curves are summarised in Table 1. For either dose of prazosin, there was no significant difference in the degree of shift of the log dose-response curve to methoxamine compared with that to methoxyphenamine. Similarly, for either dose of desmethylimipramine, there was no significant difference in the degree of shift of the log dose-response curve to tyramine compared to that of methoxyphenamine.

Discussion

In the present study, methoxyphenamine (0.01–30 mg kg^{-1}, i.v.), adrenaline (0.01–3 μg kg^{-1}, i.v.) and pseudoephedrine (0.03–3 mg kg^{-1}, i.v.) reduced the nasal basal resistance in anaesthetized rats. Neither methoxyphenamine nor pseudoephedrine were as potent or as efficacious as adrenaline in this test, but they produced longer-lasting decongestant effects. Adrenaline at a dose as low as 0.01 μg kg^{-1} reduced nasal basal resistance, as has been previously shown by Bentley & Jackson (1970).

The present results also showed that nasal congestion induced by 0.2% histamine solution, passed for 15 s into the ventilated nasal passages of the rat, was reduced by methoxyphenamine, adrenaline, pseudoepedrine, methoxamine or tyramine. Pseudoephedrine was approximately 4–5 times more potent than methoxyphenamine in this test. Pseudoephedrine is a stereoisomer of ephedrine possessing both direct and indirect sympathomimetic activity. Empey *et al.* (1980) showed that an oral dose of 60 mg could inhibit histamine-induced nasal congestion in normal human subjects. Histamine is an important mediator in the symptoms of allergic rhinitis; exposure of the nasal mucosa to histamine can cause irritation, sneezing, vascular engorgement and congestion, oedema of the mucosa and production of excessive nasal secretions (Bentley & Jackson, 1970; Misawa, 1988). Although Brooks *et al.* (1984) found that methoxyphenamine was not effective in patients suffering from ragweed hay fever, combinations of methylprednisolone and methoxyphenamine have been shown to have decongestant effects in patients with allergic rhinitis (Grater, 1960); the effects of the steroid were enhanced in the presence of methoxyphenamine.

The effects of methoxyphenamine on histamine-induced nasal congestion were reduced by desmethylimipramine and prazosin, suggesting an indirect action of methoxyphenamine by its release of neuronal noradrenaline which then acts on α_1-adrenoceptors. The decongestant effect of methoxamine was sensitive to blockade by prazosin but unaffected in the presence of desmethylimipramine. In contrast, the response to tyramine was inhibited by prazosin and desmethylimipramine. The inhibitory effect of prazosin suggests that methoxyphenamine, tyramine and methoxamine all act at least in part via activation of α_1-adrenoceptors in the nasal vasculature of the rat.

The nasal decongestant activity of sympathomimetic agents is thought to be mediated through an action, either directly or indirectly, on α-adrenoceptors on local blood vessels in the nasal mucosa with consequent reduction in oedema. Vessels controlling blood flow to and vascular volume of the nasal mucosa are richly innervated by adrenergic nerve fibres (Dahlström & Fuxe, 1965). Adrenaline or noradrenaline either administered into the circulation or applied directly to the nasal mucosa cause pronounced nasal vasoconstriction (Malcomson, 1959; Jackson, 1970). These vasoconstrictor responses are mediated by α-adrenoceptors and are reduced after administration of α-adrenoceptor antagonists such as phentolamine (Eccles & MacLean, 1977). Drugs such as cocaine and desmethylimipramine which interfere with the neuronal uptake of noradrenaline into nerve endings therefore potentiate the nasal vasoconstriction induced by sympathetic

Table 1 Degree of shift of the linear portion of the log dose-decongestant response curves to agonists in the presence of antagonists

		Degree of shift (and 95% confidence limits)		
Antagonist	*Dose* (mg kg^{-1})	*Methoxyphenamine* ($n = 5$)	*Methoxamine* ($n = 4$)	*Tyramine* ($n = 4$–6)
Prazosin	0.1	2.3 (1.8; 2.98)	2.7 (1.85; 4.03)	1.98 (1.58; 2.52)
	0.3	7.48 (5.39; 11.15)	6.5 (4.25; 10.08)	5.33 (3.77; 8.33)
Desmethylimipramine	0.1	1.75 (5.39; 2.66)	1.01 (0.84; 1.22)	2.42 (1.91; 3.07)
	1	2.96 (1.46; 8.41)	1.17 (0.96; 1.42)	5.72 (4.41; 7.51)

Values are given as the mean with the 95% confidence limits shown in parentheses. The number of animals in the group is given by *n*.

nerve stimulation (Eccles & MacLean, 1977) but reduce the effects of indirectly acting sympathomimetic amines.

In summary, the present study provides evidence that methoxyphenamine has nasal decongestant properties in the rat. Its sympathomimetic action in reducing histamine-induced nasal congestion is probably mediated, at least in part indirectly, with involvement of α_1-adrenoceptors.

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References

BENTLEY, A.J. & JACKSON, R.T. (1970). Changes in the patency of the upper nasal passages induced by histamine and antihistamine. *Laryngoscope*, **80**, 1859–1870.

BOWMAN, W.C. & RAND, M.J. (1980). Chapter 24: The respiratory system and drugs affecting respiratory function. In *Textbook of Pharmacology*. ed. Bowman, W.C. & Rand, M.J., 2nd edn, pp. 24, 26. Oxford: Blackwell Scientific Publications.

BROOKS, C.D., HEISSLER, C.T. & BOHAN, D.F. (1984). Comparison of methoxyphenamine and phenylpropanolamine with placebo for decongestion in patients suffering from Ragweed Hay Fever. In *Upjohn Technical Report* (Code No. 7213/84/012, September 11, 1984), pp. 1–4. Kalamazoo: Upjohn.

CURRY, J.J., FUCHS, J.E. & LEARD, S.E. (1949). Clinical and experimental studies with orthoxine in the treatment of bronchial asthma. *J. Allergy*, **20**, 104–110.

DAHLSTRÖM, A. & FUXE, K. (1965). The adrenergic innervation of the nasal mucosa of certain mammals. *Acta Otolaryngol.*, **58**, 65–72.

DIEM, K. & LEUTNER, C. (1970). *Documenta Geigy Scientific Tables*, 7th edn, pp. 173–178. Basle: J.R. Geigy.

ECCLES, R. & MacLEAN, A.G. (1977). Relaxation of smooth muscle following contraction elicited by sympathetic nerve stimulation *in vivo*. *Br. J. Pharmacol.*, **61**, 551–558.

EMPEY, D.W., YOUNG, G.A., LETLEY, E., JOHN, G.C., SMITH, P., McDONNELL, K.A., BAGG, L.R. & HUGHES, D.T.D. (1980). Dose-response study of the nasal decongestant and cardiovascular effects of pseudoephedrine. *Br. J. Clin. Pharmacol.*, **9**, 351–358.

GRATER, W.C. (1960). Effectiveness of methylprednisolone with methoxyphenamine in respiratory allergy. *Ann. Allergy*, **18**, 615–619.

JACKSON, R.T. (1970). Pharmacologic responses of the nasal mucosa. *Ann. Otol. Rhinol. Laryngol.*, **79**, 461–467.

KONZETT, H. & RÖSSLER, R. (1940) Versuchsanordnung zu Untersuchungen an der Bronchialmuskulatur. *Naunyn Schmiedebergs Arch. Exp. Path. Pharmakol.*, **195**, 71–74.

MALCOMSON, K.G. (1959). The vasomotor activities of the nasal mucous membrane. *J. Laryngol. Otol.*, **73**, 73–98.

MISAWA, M. (1988). A new rhinitis model using chemical mediators in rats. *Jpn. J. Pharmacol.*, **48**, 15–22.

SALEM, H. & CLEMENTE, E. (1972). A new experimental method for evaluating drugs in the nasal cavity. *Acta Otolaryngol.*, **96**, 524–529.

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The effects of ryanodine and caffeine on Ca-activated current in guinea-pig ventricular myocytes

[1]Edward White & [2]Derek A. Terrar

University Department of Pharmacology, South Parks Road, Oxford, OX1 3QT

1 Action potentials from guinea-pig single ventricular myocytes were interrupted by application of a 300 ms voltage clamp to −40 mV in order to evoke the Ca-activated tail current which is thought to be carried by Na:Ca exchange. Stimulation frequency was 1 Hz and temperature 36°C.

2 The actions of ryanodine (1 μM and 10 μM) and caffeine (1 mM and 10 mM) on Ca-activated tail currents were investigated.

3 Exposure to 10 mM caffeine and ryanodine reduced tail currents associated with very abbreviated (12 ms duration) action potentials and greatly reduced the difference between first and steady-state tail currents at this action potential duration. These observations were interpreted in terms of suppression of Ca release from the sarcoplasmic reticulum (SR) stores.

4 Tail current decay during the voltage clamp is thought to reflect the fall in $[Ca]_i$ which accompanies muscle relaxation. Current decay is dependent on Ca extrusion via Na:Ca exchange and on Ca accumulation by the SR stores. Time constants of tail current decay were seen to decrease with increasing action potential duration. This relationship was not affected by 1 mM caffeine or 1 μM ryanodine. Ryanodine at 10 μM and 10 mM caffeine abolished this relationship and increased the time constants of current decay. An increase in the time constant of tail current decay was thought to reflect a reduction in the rate of Ca accumulation by the sarcoplasmic reticulum.

5 The actions of caffeine and ryanodine on the Ca-activated tail currents are consistent with a dose-dependent leakage of Ca from the SR Ca stores. The Ca-activated tail current appears to be a useful tool in the study of Ca homeostasis.

Introduction

A Ca-activated 'tail' current has been demonstrated by several groups in single myocytes from amphibian hearts (Hume & Uehara, 1986; Cambell *et al.*, 1988) and in mammalian hearts following either voltage clamp depolarizations (Fedida *et al.*, 1987; Mitchell *et al.*, 1987; Giles & Shimoni, 1989) or interruption of action potentials (Egan *et al.*, 1989; Terrar & White, 1989). There is general agreement that the major component of the tail current is carried by electrogenic Na:Ca exchange operating in the Ca extrusion mode. However, the presence of a Ca-activated non-specific cation conductance (Ehara *et al.*, 1988) in some situations cannot be discounted. Properties of Na:Ca exchange in single guinea-pig ventricular cells have been described by Kimura *et al.* (1987), Barcenas-Ruiz *et al.* (1987) and Beuckelmann & Wier (1989). In the heart Na:Ca exchange is thought to be a major mechanism in Ca homoeostasis (Mullins, 1979; Chapman, 1983; Noble, 1986).

The time course of the decay of the Ca-activated current is thought to reflect the fall in cytosolic Ca which accompanies relaxation of cardiac muscle. The fall in cytosolic Ca is believed to result from the actions of Na:Ca exchange (which extrudes Ca from the cell) and the sarcoplasmic reticulum (SR) adenosine-5'-triphosphatase (ATP-ase, which loads Ca into the SR for re-release); see for e.g. Hilgemann & Noble (1987). It is predicted that the tail current will be sensitive to actions which influence these mechanisms. In support of this hypothesis, it has been shown that the non-specific Na:Ca exchange inhibitor dichlorobenzamil can slow the decay of tail current (Lipp & Pott, 1988). Agents active upon SR function also influence tail current decay, e.g. adrenaline speeds the decay of the tail current (Fedida *et al.*, 1987). Caffeine slows the tail current decay (Giles & Shimoni, 1989; Terrar & White, 1989) but the effects of ryanodine are less clear (compare Fedida *et al.*, 1987; Giles & Shimoni, 1989; White & Terrar, 1990). In contrast the cardiac glycoside strophanthidin (which is not thought to exert a direct influence on SR function) can increase the magnitude of the tail current without influencing the decay (White & Terrar, 1990). Clearly the Ca-activated tail current should be useful in studying processes which modulate the systolic intracellular Ca transient of cardiac tissue.

Ryanodine and caffeine are important pharmacological tools in the study of Ca regulation in the heart (for ryanodine see Sutko & Willerson, 1980; Sutko *et al.*, 1985; Meissner, 1986; Rousseau *et al.*, 1987: for caffeine, Blinks *et al.*, 1972; Chapman & Loety, 1976; Hess & Weir, 1984; Rousseau & Meissner, 1989). To date there is no quantitative comparative study of the effects of ryanodine and caffeine on the Ca-activated tail current in single myocytes. The major aim of this work was to make such a study. A second aim was to explore the possibility that the study of Ca-activated current in the presence of these drugs might shed further light on the roles of Na:Ca exchange and SR function in Ca homeostasis.

Methods

Experiments were performed on single cardiac ventricular myocytes isolated from guinea-pig. Guinea-pigs were stunned then killed by cervical dislocation. Hearts were excised and single cells isolated as previously described by Powell *et al.* (1980) and Mitchell *et al.* (1984a). After isolation cells were superfused with a oxygenated solution at 36°C containing (mM): NaCl 118.5, $NaHCO_3$ 14.5, KCl 4.2, $K_2H_2PO_4$ 1.2, $MgSO_4$ 1.2, glucose 11.1 and $CaCl_2$ 2.0.

Ca-activated tail currents were evoked by use of the technique of interrupted action potentials. This procedure was used to produce an envelope of tail currents. Currents were analysed with the VCAN software package provided by Dr. J. Dempster. Both these techniques have been described in detail previously (Terrar & White, 1989; White & Terrar, 1990).

[1] Present address: Lab. d'Electrophysiologie et de Pharmacologie Cellulaires, Université de Tours, Parc de Grandmont, F-37200, Tours, France.

[2] Author for correspondence.

Briefly, action potentials were evoked at a frequency of 1 Hz and were interrupted at various times after the upstroke by application of a 300 ms voltage clamp to −40 mV. The pattern of stimulation was always eight full action potentials followed by eight interrupted action potentials. The onset of the voltage clamp evoked a Ca-activated tail current which decayed during the voltage clamp pulse. By fitting a single exponential to the decay of the current (using the VCAN package) the magnitude of the current at the point of interruption of the action potential together with the time constant of the decaying current could be estimated. This technique allowed the separation of Ca-activated tail current from the rapid current transient (thought to consist of capacity currents and current carried by rapidly de-activating Ca channels) which is also evoked by onset of the voltage clamp. In some control cells the first tail current associated with an interrupted action potential duration of 12 ms was seen to rise for a few ms before falling. In these cases a single exponential described the falling section of the current. Current amplitude was extrapolated by eye (White & Terrar, 1990). The rise in tail current following repolarization is thought to reflect continued release of Ca from the SR and has also been described by Egan *et al.* (1989) and Giles & Shimoni (1989).

Throughout this work a tail current 'transient' refers to the relationship between current amplitude and action potential duration (e.g. Figures 1 and 2). 'Decay' of a tail current refers to the fall in current during a single voltage clamp pulse at −40 mV (e.g. Figures 5 and 6).

Cells were exposed to caffeine (1 mM or 10 mM) or ryanodine (1 μM or 10 μM). These drug concentrations were chosen as they represent those most commonly used to achieve an effect on SR function (see references in the final paragraph of Introduction). Exposure was achieved by inclusion of the agent in the superfusing solution and was therefore continuous unless otherwise indicated. It was assumed that under these conditions the effects of both caffeine and ryanodine had reached a steady state before the records presented in the results were collected. It should be stressed in particular that the responses to caffeine were distinct from the 'caffeine contractures' evoked upon initial exposure to this agent (e.g. Chapman & Tunstall, 1983).

It was a constant concern that the experiments should be performed at as close to physiological conditions as the experimental design would allow. For this reason the action potential was used to generate the calcium transient in favour of more controllable voltage clamp steps. As the processes under study are know to be temperature sensitive (see, for example, Egan *et al.*, 1989; Giles & Shimoni, 1989), it was felt important that these studies be carried out at 36°C. Statistical tests were made by either paired or unpaired *t* tests as appropriate.

Results

Tail current transients

Action potentials were stimulated at a frequency of 1 Hz. After eight normal action potentials, eight action potentials were interrupted by a 300 ms voltage clamp pulse to −40 mV. This procedure evoked the Ca-activated tail current which is thought to be carried by Na:Ca exchange. Varying the time of action potential interruption allowed the generation of an envelope of tail currents. Such an envelope is shown in Figure 1. It can be seen that in this cell, current amplitude (following the initial rapid current transient) decreased with increasing action potential duration. The tail current decayed to a steady state level within the 300 ms period for which the voltage clamp was applied.

Amplitude of the first evoked tail current for each action potential duration gives a tail current transient which is thought to reflect qualitatively the cytosolic Ca transient (Egan *et al.*, 1989; Terrar & White, 1989). Figure 2 (c and d,

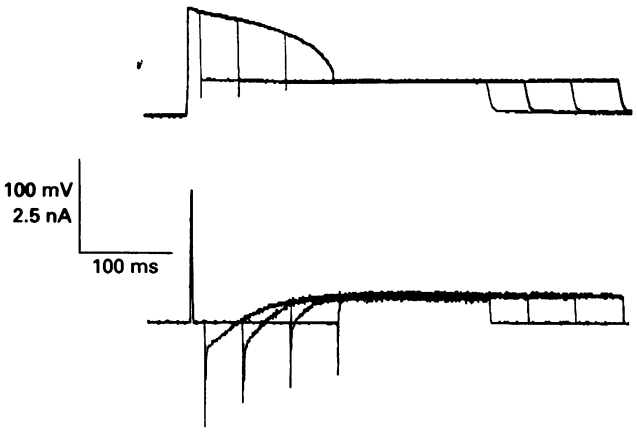

Figure 1 Membrane potential (upper traces) and membrane current (lower traces) in guinea-pig ventricular myocytes. Envelope of Ca-activated tail current, constructed by superimposing records for the first tail currents evoked by interrupting action potentials after 12, 52, 102 and 152 ms with a 300 ms voltage clamp to −40 mV. In all figures zero current is indicated by the level of current before and after the voltage clamp. Stimulation frequency 1 Hz, 36°C.

dotted lines) describes mean tail current transients from 18 cells. Several characteristic features about the time course of this transient should be noted: (1) even very short (12 ms) action potentials can evoke a large tail current; (2) the transient peaks approximately 50 ms into the action potential; (3) there is a rapid fall in the transient to a low level before the end of action potential repolarization. An explanation of our hypothesis of events which determine the shape of this 'control' transient is crucial to the interpretation of the results. It is predicted that the ability of short (12 ms) action potentials to trigger tail currents of almost equal amplitude to those found at the peak of the tail current transient is due to a substantial release of Ca from intracellular SR stores. The fall in the transient after the peak is due to a combination of Ca removal from the cytoplasm by extrusion from the cell by Na:Ca exchange and Ca re-uptake into SR stores. If these assumptions are correct then the tail current transients and the time course of tail currents during the voltage clamp should be affected by ryanodine and caffeine as both are thought to influence SR function.

Figure 2c gives transients for cells exposed to 1 or 10 mM caffeine (6 cells in each case). The transient in cells exposed to 1 mM caffeine was similar to control except that the fall in the transient appeared to be slowed (cf. points after 152 ms). In the presence of 10 mM caffeine the transient was altered in a manner described by Terrar & White (1989) under slightly different conditions (2.5 mM Ca, 0.3 Hz). Currents associated with 12 ms action potentials were reduced (in both absolute amplitude and relative to peak current) and the fall in the transient was greatly delayed. It should also be noted that while exposure to 10 mM caffeine resulted in action potentials with a characteristic delayed phase of repolarization (see Terrar & White, 1989) this was not observed in cells exposed to 1 mM caffeine. If it is assumed that exposure to 10 mM caffeine results in an absence of normal SR function, then the ability of cells to generate Ca-activated tail currents of comparable magnitude to control cells may be associated with the large increase in Ca influx via Ca channels associated with exposure to 10 mM caffeine (Terrar & White, 1989).

Figure 2d shows the transients from cells exposed to 1 μM (10 cells) or 10 μM ryanodine (6 cells). The transients reveal a major depression of current associated with the shortest action potentials and a reduction of peak current, although current appeared to remain close to peak levels for longer than control before falling. The early depression of current is consistent with the removal of a stores component of Ca release which normally allows rapid development of the tail current transient (presumably reflecting a rapid rise of cytosolic Ca). Tail currents in the presence of 1 μM ryanodine were

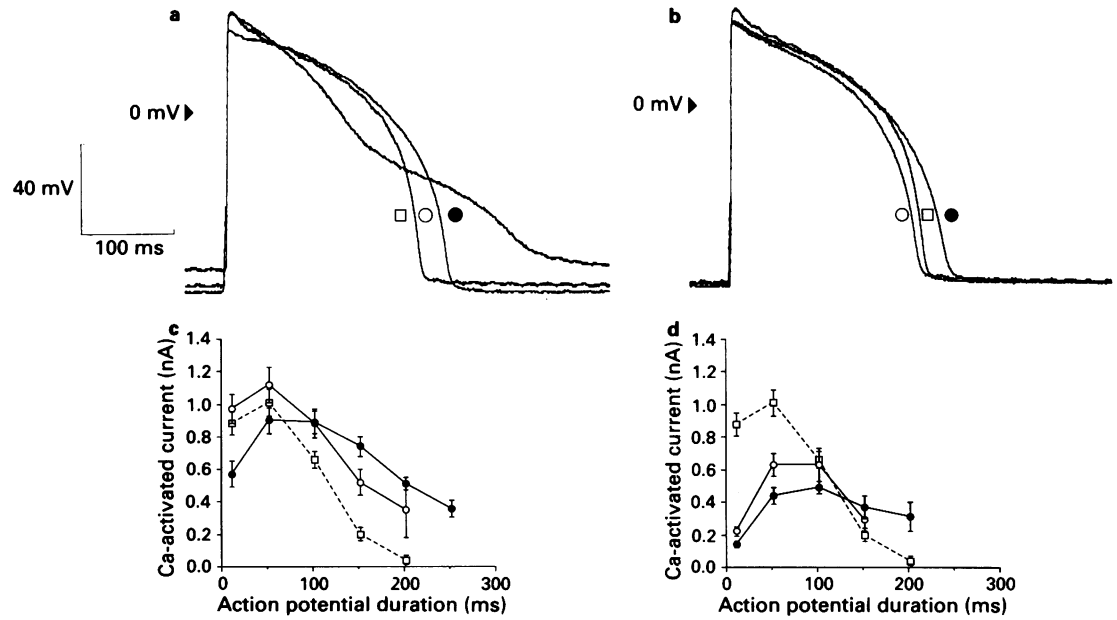

Figure 2 Representative action potentials in guinea-pig ventricular myocytes. (a) Control (□), in the presence of 1 mM caffeine (○) and 10 mM caffeine (●). (b) Control (□), in the presence of 1 μM ryanodine (○) and 10 μM ryanodine (●). Mean Ca-activated tail current transients for control cells are represented by (□---□) in (c) and (d) (n = 18 cells) and for cells exposed to: (c) 1 mM caffeine (○, n = 6 cells); 10 mM caffeine (●, n = 6 cells); (d) 1 μM ryanodine (○, n = 10 cells); 10 μM ryanodine (●, n = 6 cells). Note depression of tail currents at 12 ms in cells exposed to ryanodine and 10 mM caffeine. Mean tail currents at 202 ms were calculated from those cells which had not repolarized beyond −40 mV after this time (control, n = 7; 1 mM caffeine, n = 3; 10 mM caffeine, n = 6; 1 μM ryanodine, n = 2 only and hence not included; 10 μM ryanodine, n = 4). In the presence of 1 mM caffeine tail currents were significantly larger than control at 102 ms ($P < 0.05$), 152 ms ($P < 0.01$) and 202 ms ($P < 0.05$). Tail currents in 10 mM caffeine were smaller than control at 12 ms ($P < 0.001$), larger at 102 ms ($P < 0.05$), 152 ms and 202 ms ($P < 0.001$). In the presence of 1 μM ryanodine tail currents were smaller than control at 12 ms ($P < 0.001$) and 52 ms ($P < 0.01$). In 10 μM ryanodine, currents were smaller at 12 ms and 52 ms ($P < 0.001$) and larger at 152 ms ($P < 0.05$) and 202 ms ($P < 0.01$), P calculated by unpaired t tests. Exposure to 10 mM caffeine and 10 μM ryanodine prolonged the transient. In (c) and (d) vertical lines indicate s.e.

not significantly different from those in the presence of 10 μM ryanodine up to 152 ms.

First/steady state currents

It was consistently observed that in control cells the level of tail current associated with the first action potential of 12 ms duration could not be sustained by the following similarly abbreviated action potentials ($P < 0.001$) (Figure 3a). It may be that the first shortened action potential can trigger Ca release from stores (filled by the preceding full action potentials). As a consequence of reduced Ca entry through Ca channels and increased Ca-extrusion via Na:Ca exchange (which both result from the early repolarization), this high level of stores filling may not be maintained during the subsequent shortened action potentials and hence a reduction in current occurs. Figure 3b shows the differences between first and steady state current remains despite exposure to 1 mM caffeine ($P < 0.001$). This supports the hypothesis that in these experiments controlled release of Ca persists in cells treated with 1 mM caffeine. In contrast the response is abolished or reduced in cells exposed to either 10 mM caffeine (Figure 3c), 1 μM ryanodine (Figure 3d) or 10 μM ryanodine (Figure 3e) ($P > 0.05$ in all 3 treatments). Figure 4 gives mean first and steady state currents. Responses in the control and 1 mM caffeine cells are clearly different from those during other treatments.

Although the magnitude of currents in the presence of ryanodine is much smaller than control there was a reduction of current at the steady state in some cells (5 out of 10 cells exposed to 1 μM ryanodine and 5 out of 6 of those exposed to 10 μM). One explanation is that this observation reflects a small ryanodine insensitive component of stores release. In some cells no difference in first and steady state current was observed but in others a difference was seen despite prolonged (over 1 h) exposure to ryanodine. It was also observed that despite prolonged exposure a difference in first and steady state current was often seen when cells were first stimulated. This difference disappeared or reduced with further stimulation and was thought to be related to the use-dependent nature of ryanodine action (Mitchell *et al.*, 1984b; Malecot & Katzung, 1987). For this reason cells were stimulated for at least 1 min before collection of tail current records.

From these results it seems that the modulation of a large first steady-state response in very abbreviated action potentials may be a useful tool in the study of Ca release by the SR.

Decay of tail currents

After the onset of the voltage clamp pulse the Ca-activated current decayed at a steady state. It has been shown that the decay of the current speeds up as the length of the associated action potential (Terrar & White, 1989) or voltage clamp depolarization (Giles & Shimoni, 1989) increases. This effect is demonstrated by the records in Figure 1. Time constants of current decay decreased with increasing action potential duration as follows: 36, 27, 18, 6 ms. This observation has been explained in terms of increasing activity of the Ca uptake procedure by Giles & Shimoni (1989). Figure 5 shows tail currents recorded after action potentials of 52 ms for control and cells exposed to caffeine and ryanodine. The greatest divergence from control is seen in the cell exposed to 10 mM caffeine (Figure 5c). Mean relationships between current decay and action potential duration in the presence of caffeine are shown in Figure 6a. The decrease in time constant with increasing action potential duration was maintained in cells exposed to 1 mM caffeine but not in the presence of the higher dose of the drug. Exposure to 10 mM caffeine greatly increased time constants of currents associated with action potentials of over 12 ms duration. Figure 6b gives values for cells exposed to ryanodine. Time constants appeared little affected by 1 μM ryanodine. In the presence of 10 μM ryanodine time constants were qualitatively similar to those in the presence of 10 mM caffeine though faster. The very large standard error of current decay associated with 12 ms action potentials in the presence of 10 μM ryanodine is probably due to the difficulty in fitting

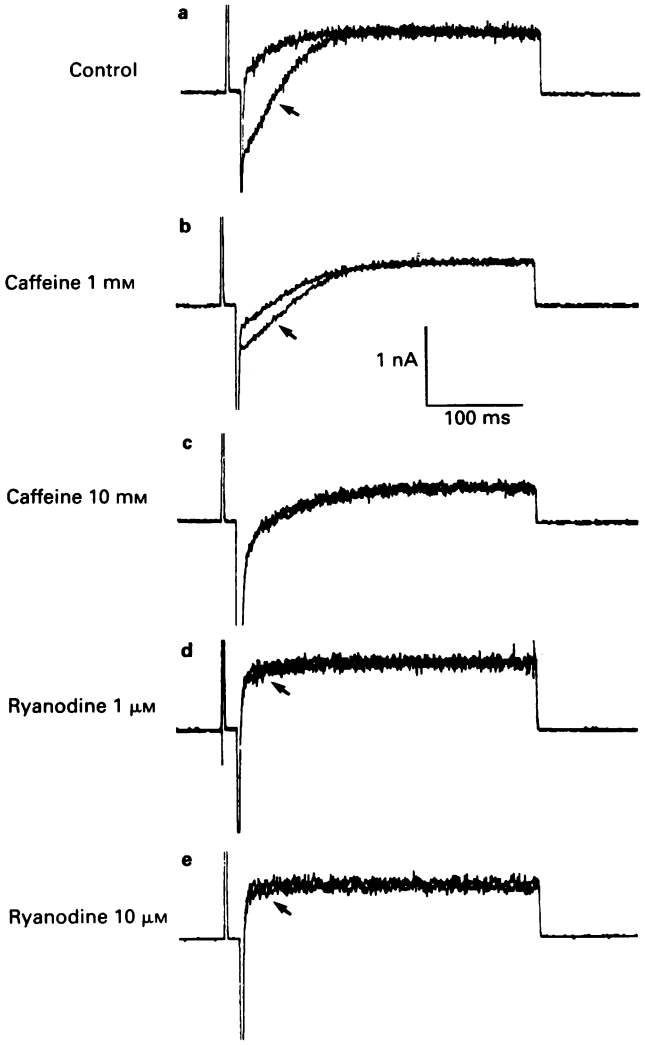

Figure 3 First (arrows where distinguishable) and steady state tail currents associated with action potentials interrupted after 12 ms for: (a) control; (b) 1 mM caffeine; (c) 10 mM caffeine; (d) 1 μM ryanodine; (e) 10 μM ryanodine. Note responses in (a) and (b) are clearly different from others. Stimulation frequency 1 Hz, 36°C.

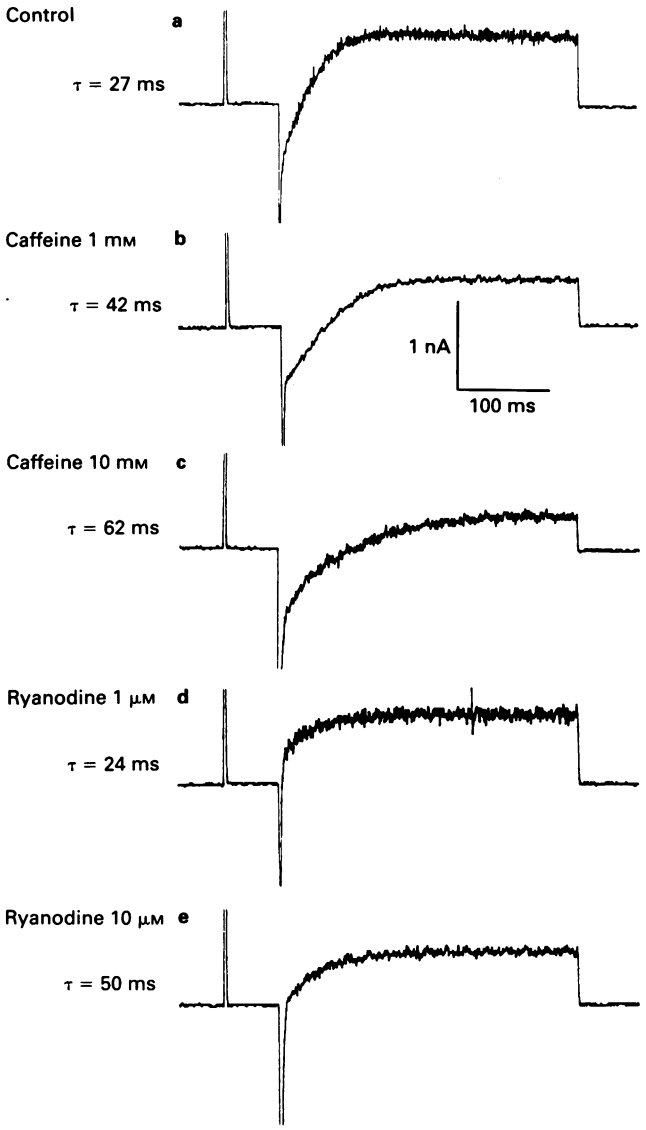

Figure 5 Ca-activated tail currents associated with action potentials interrupted after 52 ms. Time constants of current decay (τ) are given. (a) Control; (b) 1 mM caffeine; (c) 10 mM caffeine; (d) 1 μM ryanodine and (e) 10 μM ryanodine. Stimulation frequency 1 Hz, 36°C.

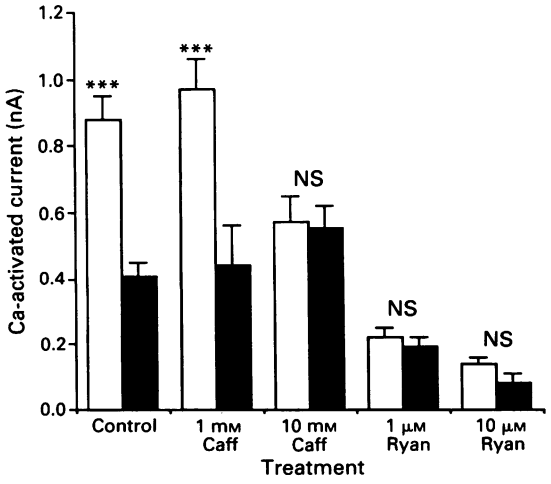

Figure 4 Mean first (open columns) and steady state (solid columns) Ca-activated current associated with action potentials interrupted after 12 ms for control (n = 18 cells); 1 mM caffeine (Caff, n = 6 cells); 10 mM caffeine (n = 6 cells); 1 μM ryanodine (Ryan, n = 10 cells) and 10 μM ryanodine (n = 6 cells). *** $P < 0.001$, NS = not significant, paired t tests. Bars show s.e.mean.

an exponential to such small currents, given the signal to noise ratio of these records.

One further point should also be noted: it was often observed that in control cells the time constant of the first tail current associated with action potentials of 12 ms was greater than that of the steady state ($P < 0.05$); for example, time constants of currents in Figure 3a were 36 and 30 ms for first and steady state respectively. The first abbreviated action potential is thought to be associated with a large release of SR Ca but reduced Ca entry. It would be predicted that this situation leads to a net extrusion of Ca from the cell by Na:Ca exchange until (during subsequent abbreviated action potentials) a steady state is established (at which point Ca entry must equal Ca extrusion, Hilgemann & Noble, 1987). It is possible that the increased amount of Ca extruded during the first abbreviated action potential results in a slower time constant of current decay as compared with the steady state.

In order to test the above observations more fully we also recorded tail currents in some cells which were subsequently exposed to either 1 μM ryanodine or 10 mM caffeine (3 cells for each treatment). In all cases the observed changes in action potential shape, tail current transients, first steady-state and time constant of current decay were consistent with the above observations. This was also true in the case of 3 cells which

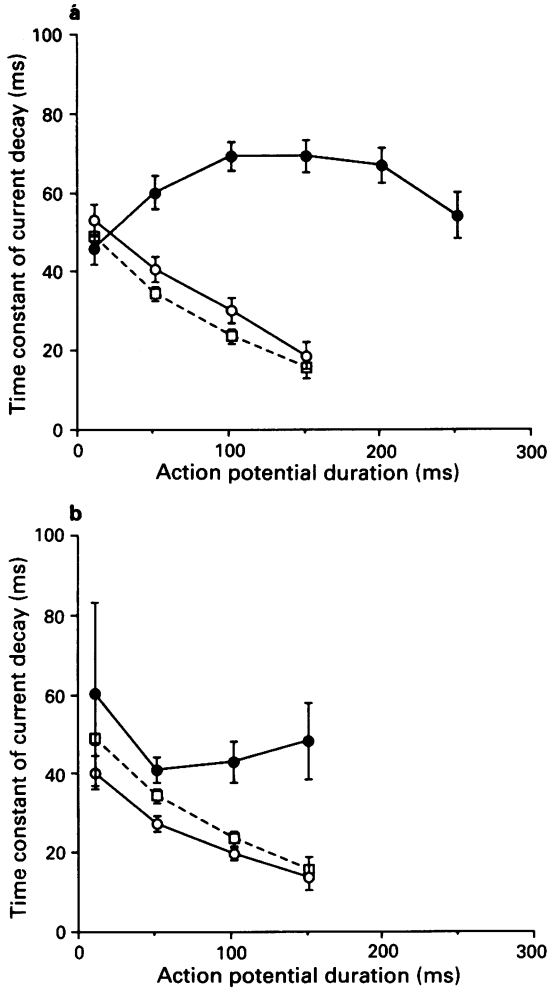

Figure 6 Mean time constants of Ca-activated current decay. Control cells (□ --- □) in (a) and (b) (n = 18 cells). (a) Cells exposed to 1 mM caffeine (○, n = 6 cells); 10 mM caffeine (●, n = 6 cells). (b) Cells exposed to 1 μM ryanodine (○, n = 10 cells); 10 μM ryanodine (●, n = 6 cells). In the presence of 1 mM caffeine time constants were not significantly different from control except at 12 ms ($P < 0.05$). In 10 mM caffeine time constants were greater than control ($P < 0.001$) except at 12 ms. In the presence of 1 μM ryanodine time constants were not different from control except at 52 ms ($P < 0.05$). Time constants were greater than control in the presence of 10 μM ryanodine at 102 ms ($P < 0.001$) and 152 ms ($P < 0.01$), P calculated by unpaired t tests. Note decrease in decay times with increasing action potential duration in control cells and those exposed to lower doses of caffeine and ryanodine. This relationship was disturbed by 10 mM caffeine and 10 μM ryanodine. Vertical lines show s.e.

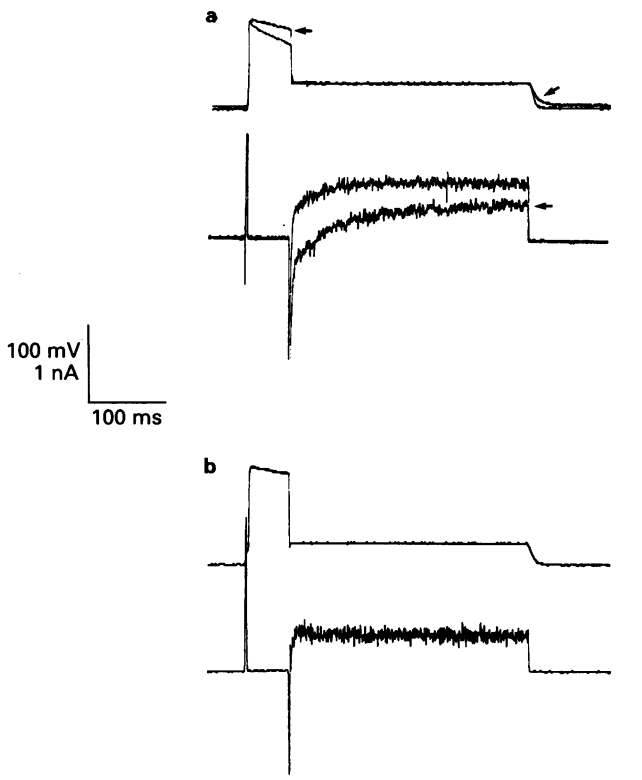

Figure 7 Membrane potential (upper traces) and membrane current (lower traces) for action potentials interrupted after 52 ms. (a) Superimposed records for a cell exposed to 1 μM ryanodine and then additionally to 10 mM caffeine (arrows) for 2 min. Note inward shift in holding current. (b) Cell continuously exposed to 10 mM caffeine and impaled with a microelectrode containing 100 mM BAPTA. Note absence of Ca-activated tail current and lack of significant levels of time-dependent current. Stimulation frequency 1 Hz, 36°C.

were first exposed to 1 μM ryanodine and then 10 μM caffeine. The effects of caffeine were reversible in the absence of ryanodine.

Caffeine-induced current changes

One further consistent observation was that exposure to 10 mM caffeine caused an inward shift in the holding current at the end of the voltage clamp pulse to −40 mV (Figure 7a) (in the presence or absence of 1 μM ryanodine) which reversed on washing. Clearly if this caffeine-induced current change contained any significant time-dependent component the estimates of current magnitude and current decay would be compromised. Cells were impaled with electrodes containing 100 mM BAPTA (1,2-*bis*(2-aminophenoxy)ethane N,N,N',N'-tetraacetic acid which chelates $[Ca]_i$ thus abolishing Ca-activated tail currents and revealing residual currents) and exposed to 10 mM caffeine. It can be seen from Figure 7b that there is no significant time-dependent current present. A rigorous investigation of this caffeine-induced shift in current was beyond the scope of the present study. However, 1 s voltage clamp ramps (in the presence of 10 μM nifedipine to block Ca channels and 100 mM BAPTA containing microelectrodes) from either −80 to 0 mV or −40 to +40 mV revealed a consistent inward shift in current peaking around −40 mV and consistent with a reduction in the inward rectification of the steady-state current-voltage curve. One possible hypothesis to account for these initial observations is that 10 mM caffeine may be blocking the inward rectifying potassium current I_{K1}. This remains open to a further study.

Discussion

Although the actions of ryanodine and caffeine are thought to influence the SR Ca stores the response of the Ca-activated current to these agents differed. In the present study, Ca-activated tail currents were used to investigate mechanisms controlling Ca homeostasis. This method was chosen on the basis of previous observations which support the idea that this is a valid method for measuring changes in cytosolic Ca with minimal disturbance of the intracellular conditions (Egan *et al.*, 1989; Terrar & White, 1989). The method is more direct than the use of contraction (which in these experiments might be influenced by caffeine-induced changes in myofilament sensitivity for Ca) and avoids any possible influence of Ca indicators such as fura-2 on the Ca transient (e.g. Noble & Powell, 1990). Furthermore, under appropriate conditions the tail current may directly measure one important mechanism of

Ca homeostasis, i.e. Na:Ca exchange. The use of the Ca-activated Na:Ca exchange current as a qualitative indicator of changes in $[Ca]_i$ is paralleled by similar use of a Ca-activated Cl current (e.g. in mouse pancreatic acinar cells, Wakim *et al.*, 1989).

Possible contamination of the tail currents with deactivating Ca and potassium currents had been discussed elsewhere (Fedida *et al.*, 1987; Mitchell *et al.*, 1987; Egan *et al.*, 1989; Giles & Shimoni, 1989; Terrar & White, 1989; White & Terrar, 1990) and errors from these currents are not thought to be significant under the conditions of our experiments.

We believe that the contribution of Ca release from the SR to cytosolic Ca transients may be monitored by investigating changes in the amplitude and ratio of steady-state/first Ca-activated tail currents following very abbreviated action potentials (Figures 3 and 4). By clamping to more negative potentials than used in this study it should be possible to bias the system towards greater Ca extrusion (Eisner & Lederer, 1985; Noble, 1986) leading to reduced loading of the stores and to an enhancement in this ratio.

In the following discussion we have assumed throughout that the alterations in the tail current caused by ryanodine and caffeine were a result of actions of these drugs on the SR. Experiments with caffeine need to be interpreted with caution because of its well reported actions at several sites. However, we are unaware of any report suggesting that caffeine (or ryanodine) directly affects the Na:Ca exchange. It should be noted that any inhibition of the Na:Ca exchange would also be expected to delay tail current decay (see Lipp & Pott, 1989). We have also ignored any contribution to Ca extrusion by the sarcolemmal Ca-ATP-ase, assuming this is to be a low level background mechanism (Sipido & Wier, 1989).

Tail currents in the presence of 1 mM caffeine were similar to those of control cells. Cells exposed to 10 mM caffeine appeared to lose the ability to release Ca from the SR, as indicated by a lack of difference in first and steady state currents associated with the same shortened action potentials (Figures 2 and 4). A similar conclusion is drawn for the action of ryanodine, based this time on the marked reduction by ryanodine of the tail currents associated with 12 ms action potentials. This observation is in keeping with the accepted action of ryanodine (e.g. Sutko *et al.*, 1985). Fedida *et al.* (1987) and Giles & Shimoni (1989) demonstrated that 1 μM ryanodine did not reduce tail currents following 50 ms depolarizations at room temperature. The duration of depolarization appears to be critical. It can be seen from Figure 2d that reduction of tail current amplitude by ryanodine decreased with duration of depolarization (time of interruption of action potential). It should also be noted that both the triggering (via Ca entry through sarcolemmal Ca channels) and release of Ca from the SR (via SR Ca channels) are known to be sensitive to temperature. For this reason the effects of ryanodine may be more readily observed at 36°C. Changes in the tail current transients caused by ryanodine and 10 mM caffeine are consistent with a slowing of the time to peak of the $[Ca]_i$ transient (measured with aequorin) observed in the presence of caffeine (Hess & Wier, 1984) and ryanodine (Wier *et al.*, 1985).

Consistent with the data of Giles & Shimoni (1989) and Terrar & White (1989) caffeine (here at a dose of 10 mM) delayed the decay of tail currents and abolished the inverse relationship between duration of depolarization and speed of decay (Figure 6). It was observed here, however, that currents associated with 12 ms action potentials were faster to decay than those associated with longer action potentials. This may be some function of intracellular Ca buffers or intracellular diffusion of Ca. One explanation of the overall slowing of tail current decay on exposure to 10 mM caffeine is a reduction in uptake of Ca into the SR. However, caffeine has been shown to inhibit phoshodiesterase action (Butcher & Sutherland, 1962) and one consequence of this could be the stimulation of the SR Ca-ATP-ase via increased phosphorylation of phospholamban (Rapundalo *et al.*, 1989); this would be expected to speed the decay in contrast to the observed slowing. An alternative possibility is that caffeine induced a leak of Ca from the SR on such a scale that Ca could not be accumulated by the SR and thus the fall in $[Ca]_i$ and tail current was delayed. A leak could be caused by maintained opening of the SR Ca release channel by caffeine. Caffeine has been shown to activate the Ca release channel of cardiac SR membranes in the single channel studies of Ehrlich & Watras (1988) and of Rousseau & Meissner (1989).

Ryanodine 1 μM did not slow the decay of the tail current. It may be that 1 μM ryanodine did not reduce Ca uptake into the SR. Clearly however if 1 μM ryanodine suppressed the release of Ca from the SR but did not reduce uptake, Ca must have been lost at some time during the cardiac cycle. Ryanodine at 1 μM may also induce a leak of Ca from the SR which is insufficient observably to affect Ca uptake (over the time course of the Ca-activated tail current) but sufficient to deplete stores during diastole. A greater leak at 10 μM would explain the increase in current decay times. Again evidence from single channel studies may support this theory since Rousseau *et al.* (1987) demonstrated that ryanodine can promote a subconducting state of the SR Ca release channel with a probability of opening close to unity. Bers & Bridges (1989) observed that, unlike 10 mM caffeine, 0.5 μM ryanodine did not delay the rate of relaxation in rabbit ventricle and explained this in terms of initial accumulation of Ca by the SR followed by Ca leakage back into the cytpoplasm. A model for cardiac SR incorporating a leak has been described by Hilgemann *et al.* (1989).

Our observations indicate that the actions of both caffeine and ryanodine on the Ca-activated tail current can be explained in terms of a leak of Ca from the SR, but that experimental responses will depend on the choice of agent, dosage and experimental conditions (such as depolarization duration). The Ca-activated tail current appears to be a useful tool in the study of Ca homeostasis.

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References

BARCENAS-RUIZ, L., BEUCKELMANN, D.J. & WIER, G.W. (1987). Sodium-calcium exchange in heart: membrane currents and changes in $[Ca]_i$. *Science*, **238**, 1720–1722.

BERS, D.M. & BRIDGE, J.H.B. (1989). Relaxation of rabbit ventricular muscle by Na-Ca exchange and sarcoplasmic reticulum calcium pump. *Circ. Res.*, **65**, 334–342.

BEUCKELMANN, D.J. & WIER, W.G. (1989). Sodium-calcium exchange in guinea-pig cardiac cells: exchange current and changes in intracellular Ca. *J. Physiol.*, **414**, 499–520.

BLINKS, B.R., OLSON, C.B. & JEWELL, B.R. & BRAVENY, P. (1972). Influence of caffeine and other methylxanthines on mechanical properties of isolated mammalian heart muscle. *Circ. Res.*, **30**, 367–392.

BUTCHER, R.W. & SUTHERLAND, E.W. (1962). Adenosine 3',5' phosphate in biological materials. *J. Biol. Chem.*, **237**, 1244–1250.

CAMBELL, D.L., GILES, W.R., ROBINSON, K. & SHIBATA, E.F. (1988). Studies of the sodium-calcium exchanger in bull-frog atrial myocytes. *J. Physiol.*, **403**, 317–340.

CHAPMAN, R.A. (1983). Control of cardiac contractility at the cellular level. *Am. J. Physiol.*, **245**, H535–H553.

CHAPMAN, R.A. & LEOTY, C. (1976). The time-dependent and dose-dependent effects of caffeine on the contraction of the ferret heart. *J. Physiol.*, **256**, 287–314.

CHAPMAN, R.A. & TUNSTALL, J. (1983). The control and strength of the caffeine contracture in frog atrial trabeculae: an activity of the sodium-calcium exchange. *Q.J. Exp. Physiol.*, **68**, 381–393.

EISNER, D.A. & LEDERER, W.J. (1985). Na-Ca exchange: Stoichiometry and electrogenicity. *Am. J. Physiol.*, **248**, C189–C202.

EGAN, T.M., NOBLE, D., NOBLE, S.J., POWELL, T., SPINDLER, A.J. & TWIST, V.W. (1989). Sodium-calcium exchange during the action potential in guinea-pig ventricular cells. *J. Physiol.*, **411**, 639–661.

EHARA, T., NOMA, A. & ONO, K. (1988). Calcium-activated non-selective cation channel in ventricular cells isolated from adult guinea-pig hearts. *J. Physiol.*, **403**, 117–133.

EHRLICH, B.E. & WATRAS, J. (1988). Inositol 1,4,5-trisphosphate activates a channel from smooth muscle sarcoplasmic reticulum. *Nature*, **336**, 583–586.

FEDIDA, D., NOBLE, D., SHIMONI, Y. & SPINDLER, A.J. (1987). Inward current related to contraction in guinea-pig ventricular myocytes. *J. Physiol.*, **385**, 565–589.

GILES, W. & SHIMONI, Y. (1989). Slow inward tail currents in rabbit cardiac cells. *J. Physiol.*, **417**, 447–463.

HESS, P. & WIER, W.G. (1984). Excitation-contraction coupling in cardiac Purkinje fibres. Effects of caffeine on intracellular [Ca^{2+}] transient, membrane currents and contraction. *J. Gen. Physiol.*, **83**, 417–433.

HILGEMANN, D.W. & NOBLE, D. (1987). Excitation-contraction coupling and extracellular calcium transients in rabbit atrium: reconstruction of basic cellular mechanisms. *Proc. R. Soc. Lond.*, **B230**, 163–205.

HILGEMANN, D.W., ROOS, K.P. & BRADY, A.J. (1989). Slowly relaxing caffeine responses in rat ventricle: relationships of ryanodine and caffeine actions. *Am. J. Physiol.*, **256**, H1100–H1109.

HUME, J.R. & UEHARA, A. (1986). 'Creep currents' in single frog atrial cells may be generated by electrogenic Na-Ca exchange. *J. Gen. Physiol.*, **87**, 857–884.

KIMURA, J., MIYAME, S. & NOMA, A. (1987). Identification of sodium-calcium exchange current in single ventricular cells of guinea-pig. *J. Physiol.*, **389**, 199–222.

LIPP, P. & POTT, L. (1988). Voltage dependence of sodium-calcium exchange current in guinea-pig atrial myocytes determined by means of an inhibitor. *J. Physiol.*, **403**, 355–366.

MALECOT, C.O. & KATZUNG, B.G. (1987). Use-dependence of ryanodine effect on post-rest contraction in ferret cardiac muscle. *Circ. Res.*, **60**, 560–567.

MEISSNER, G. (1986). Ryanodine activation and inhibition of the Ca release channel of sarcoplasmic reticulum. *Biol. Chem.*, **261**, 6300–6306.

MITCHELL, M.R., POWELL, T., TERRAR, D.A. & TWIST, V.W. (1984a). The effects of ryanodine, EGTA and low sodium on action potentials in rat and guinea-pig ventricular muscle. *Br. J. Pharmacol.*, **81**, 543–550.

MITCHELL, M.R., POWELL, T., TERRAR, D.A. & TWIST, V.W. (1984b). Ryanodine prolongs Ca-current while suppressing contraction in rat ventricular muscle cells. *Br. J. Pharmacol.*, **81**, 13–15.

MITCHELL, M.R., POWELL, T., TERRAR, D.A. & TWIST, V.W. (1987). Calcium-activated inward current and contraction in rat and guinea-pig ventricular myocytes. *J. Physiol.*, **391**, 545–560.

MULLINS, L.J. (1979). The generation of electric currents in cardiac fibers by Na-Ca exchange. *Am. J. Physiol.*, **239**, C103–C110.

NOBLE, D. (1986). Sodium-calcium exchange and its role in generating electric current. In *Cardiac Muscle: The Regulation of Excitation and Contraction*, ed. Nathan, R.D., pp. 171–200. New York: Academic Press.

NOBLE, D. & POWELL, T. (1990). The attenuation and slowing of calcium signals in cardiac muscle by fluorescent indicators. *J. Physiol.*, **425**, 54P.

POWELL, T., TERRAR, D.A. & TWIST, V.W. (1980). Electrical properties of individual cells isolated from adult rat ventricular myocardium. *J. Physiol.*, **302**, 131–153.

RAPUNDALO, S.T., SOLARO, R.J. & KRANIAS, E.G. (1989). Inotropic responses to isoproterenol and phosphodiesterase inhibitors in intact guinea-pig hearts: comparison of cyclic AMP levels and phosphorylation of sarcoplasmic reticulum and myofibrillar proteins. *Circ. Res.*, **64**, 104–111.

ROUSSEAU, E. & MEISSNER, G. (1989). Single cardiac sarcoplasmic reticulum Ca release channel: activation by caffeine. *Am. J. Physiol.*, **256**, H328–H333.

ROUSSEAU, E., SMITH, J.S. & MEISSNER, G. (1987). Ryanodine modifies conductance and gating behavior of single Ca release channel. *Am. J. Physiol.*, **253**, C364–C368.

SIPIDO, K.R. & WIER, W.G. (1989). $[Ca]_i$ transients in isolated guinea-pig ventricular myocytes in the absence of sodium-calcium (Na-Ca) exchange and sarcoplasmic reticulum function. *J. Physiol.*, **418**, 170P.

SUTKO, J.L., ITO, K. & KENYON, J.L. (1985). Ryanodine: a modifier of sarcoplasmic reticular calcium release in striated muscle. *Fed. Proc.*, **44**, 2984–2988.

SUTKO, L.J. & WILLERSON, J.T. (1980). Ryanodine alteration of the contractile state of rat ventricular tissues. Comparison with dog, cat and rabbit ventricular tissues. *Circ. Res.*, **46**, 332–343.

TERRAR, D.A. & WHITE, E. (1989). Changes in cytosolic calcium monitored by inward currents during action potentials in guinea-pig ventricular cells. *Proc. R. Soc. Lond.*, **B238**, 171–188.

WAKIM, M., POTTER, B.V.L. & PETERSEN, O.H. (1989). Pulsatile intracellular calcium release does not depend on fluctuations in inositol trisphosphate. *Nature*, **339**, 317–320.

WHITE, E. & TERRAR, D.A. (1990). The action of strophanthidin on Ca-activated current and contraction in single guinea-pig ventricular myocytes. *Exp. Physiol.*, **75**, 559–572.

WIER, W.G., YUE, D.T. & MARBAN, E. (1985). Effects of ryanodine on intracellular Ca transients in mammalian cardiac muscle. *Fed. Proc.*, **44**, 2989–2993.

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Influence of N-ethylmaleimide on action potential and force of contraction of guinea-pig papillary muscles

Corona von Poehl, H. Iven & [1]H. Brasch

Department of Pharmacology, Medical University of Lübeck, Ratzeburger Allee 160, D-2400 Lübeck, F.R.G.

1 Standard microelectrode techniques were used to investigate the influence of N-ethylmaleimide on the action potential, slow response action potential and force of contraction of guinea-pig papillary muscles.

2 N-ethylmaleimide, 3×10^{-5} to 10^{-4} mol l^{-1}, concentration-dependently increased the force of contraction. The positive inotropic effect developed quickly and, with the largest drug concentration, was followed by a progressive decline of the contractile force. The action potential duration was progressively shortened by N-ethylmaleimide.

3 The effects of N-ethylmaleimide were not prevented in the presence of tetrodotoxin 3×10^{-8} mol l^{-1} and propranolol 4×10^{-6} mol l^{-1} or by a reduction of the Na^+-concentration to 70 mmol l^{-1}.

4 Verapamil, 10^{-6} mol l^{-1}, reduced the positive inotropic, but not the action potential shortening effect of N-ethylmaleimide.

5 In K^+-depolarized muscles in the presence of propranolol and tetrodotoxin, N-ethylmaleimide 10^{-4} mol l^{-1} increased the maximum depolarization velocity and the duration of the slow response action potential. The latter effect was transient and was followed by a progressive reduction of the action potential duration.

6 The most likely explanation for the action potential shortening effect of N-ethylmaleimide seems to be an increase of an outward potassium current while the transient inotropic effect of the drug may be caused, at least in part, by an increase of the slow inward calcium current.

Introduction

N-ethylmaleimide (NEM) increases the force of contraction of cat papillary muscles (Bennett *et al.*, 1958), guinea-pig papillary muscles (Fricke, 1978) and guinea-pig atria (From, 1970; Ueno, 1971; Yamamoto *et al.*, 1973; Temma *et al.*, 1978). A release of endogenous noradrenaline (Yamamoto *et al.*, 1973) and an inhibition of Na^+-K^+-ATPase (Skou & Hilberg, 1965; Fujita *et al.*, 1968; Tobin & Akera, 1975) have been implicated in the positive inotropic drug effect, but other explanations cannot be ruled out. During prolonged exposure to NEM, an increase of the resting tension, a decline of the contractile force and the appearance of arrhythmias are often observed (From, 1970; Yamamoto *et al.*, 1973). The latter effect suggests that NEM might alter the membrane currents and the time course of the action potential. Indeed, Toda & Konishi (1969) observed a slowing of diastolic depolarization and a progressive shortening of the action potential duration of sinoatrial pacemaker cells by NEM. Braun & Sperelakis (1988), on the other hand, found the action potential of guinea-pig atrium unchanged after a 25 min exposure to NEM, whereas no information is available as to the effect of the drug on ventricular action potentials. We therefore decided to study the effect of NEM on action potential and force of contraction of guinea-pig papillary muscles with standard microelectrode techniques. The results revealed multiple sites of drug action, and the influence of NEM on the calcium current in K^+-depolarized muscles suggests a new interpretation of the transient inotropic effect of this drug.

Methods

Experimental procedure

Male guinea-pigs (250–500 g) were killed by a blow on the head. The heart was rapidly excised and papillary muscles, from the right ventricle, were prepared in cooled, oxygenated Krebs-Henseleit solution. Muscles with a diameter of less than 1 mm were then mounted horizontally in a 20 ml organ bath filled with Krebs-Henseleit solution of the following composition (mmol l^{-1}): NaCl 117.6, KCl 5.8, $CaCl_2$ 2.5, $NaHCO_3$ 25, NaH_2PO_4 1.2, $MgSO_4$ 1.2 and glucose 5.5. The solution was kept at 32 ± 0.2°C and was gassed with a mixture of 95% O_2 and 5% CO_2. The muscles were stimulated via pointed platinum electrodes at a rate of 1 Hz with square pulses of 1 ms duration and 1.5 times threshold voltage. The force of contraction was registered isometrically by a K 30 force-displacement transducer (Hugo Sachs, Hugstetten, F.R.G.). Action potentials were recorded with standard glass microelectrodes which were filled with 3 mol l^{-1} KCl and had a tip resistance of 5–10 MΩ. The maximum depolarization velocity $\dot{V}_{max}$ was obtained by passive signal differentiation. The RC-circuit with a time constant of 2×10^{-4} s had been tested and found to be linear over the desired range in a previous study (Iven & Brasch, 1977). The action potential, $\dot{V}_{max}$ and force of contraction were displayed on a Tektronix 502 A dual-beam oscilloscope (Tektronix Inc., Beaverton, OR, U.S.A.) and photographed with a Grass C4 Camera (Grass Medical Instruments, Quincy, MA, U.S.A.) for subsequent analysis. Drugs were added to the organ bath after an equilibration period of at least 90 min. The effect of N-ethylmaleimide (NEM) was observed for 60 min. To test the reversibility of the drug effect, the organ bath was then perfused with drug-free Krebs-Henseleit solution for 20 min (4 ml min^{-1}) and the remaining drug effect was evaluated after a further 30 min recovery period. An experiment was included in the study only if the microelectrode remained in the same cell during the whole time course of the experiment.

In experiments with low sodium Krebs-Henseleit solution, 73.8 mol l^{-1} NaCl was replaced by 147.6 mmol l^{-1} saccharose. To induce Ca^{2+}-mediated slow-response action potentials, the papillary muscles were depolarized by increasing the KCl concentration to 26 mmol l^{-1}. The muscles were then stimulated at a rate of 0.1 Hz with square pulses of 3 ms duration and 1.5 times threshold voltage. In these experiments, the Krebs Henseleit solution contained no $MgSO_4$ but 0.2 mmol l^{-1} $BaCl_2$ to reduce the outward potassium current (Ehara & Inazawa, 1980). To avoid indirect drug effects caused by a release of endogenous catecholamines the effects of NEM and Bay K 8644 were tested in the presence of propranolol and tetrodotoxin. Propranolol 4×10^{-6} mol l^{-1} and tetrodotoxin 3×10^{-6} mol l^{-1} were added to the organ bath 1 h before the

[1] Author for correspondence.

application of NEM or Bay K 8644 to allow sufficient time for establishing steady-state concentrations in the tissues.

Drugs

The following drugs were used in the study: N-ethylmaleimide (Sigma, Deizenhofen, F.R.G.); tetrodotoxin (Sankyo Co., Tokyo, Japan); propranolol hydrochloride (Rhein-Pharma, Heidelberg, F.R.G.); verapamil hydrochloride (Knoll, Ludwigshafen, F.R.G.) and ouabain (Sigma, Deizenhofen, F.R.G.). Racemic Bay K 8644 (1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluorophenyl) pyridine-5-carboxylic acid) was a gift from the Bayer AG (Leverkusen, F.R.G.). Bay K 8644 was dissolved in DMSO and 20 μl of the solution was added to the 20 ml organ bath. The experiments with Bay K 8644 were carried out under light from a sodium vapour lamp. All other drugs were dissolved in twice distilled water, and stock solutions were prepared daily.

Statistics

Arithmetic means and their standard errors (s.e.) were calculated for the pre-drug values of all parameters. Drug effects were expressed as changes from the pre-drug values for each individual experiment. Mean changes were then calculated and assessed for statistical significance by Student's t test for paired data. $P \leq 0.05$ for the two-tailed test was fixed as criterion for the acceptance of statistical significance throughout.

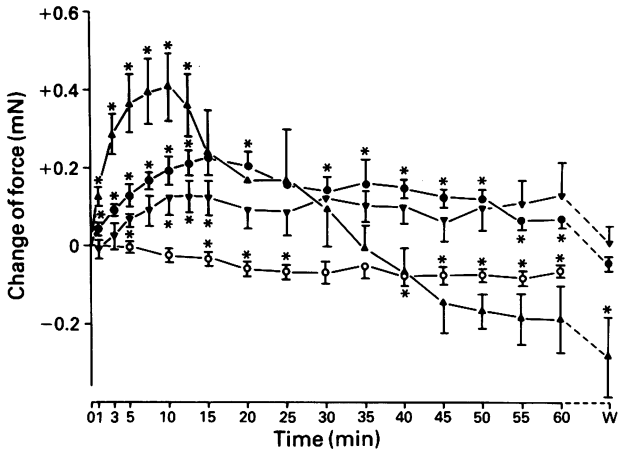

Figure 1 Effect of N-ethylmaleimide (NEM) on the contractile force of guinea-pig papillary muscles. Abscissa scale: time after drug addition (min). W = effect remaining after washout of drug. Ordinate scale: change of the force of contraction (mN). (○) Control experiments, no drug; (▼) NEM 3×10^{-5} mol l^{-1}; (●) NEM 5.6×10^{-5} mol l^{-1}; (▲) NEM 10^{-4} mol l^{-1}. The symbols indicate mean changes and vertical lines show s.e.mean (n = 6). Asterisks indicate changes that are statistically significant (t test for paired data, $P \leq 0.05$ two tailed). Pre-drug values of the contractile force were 0.46 ± 0.13 mN in control experiments and 0.05 ± 0.15, 0.34 ± 0.08 and 0.41 ± 0.12 mN in the experiments with the three concentrations of NEM.

Results

Effects of N-ethylmaleimide

Action potential parameters remained stable in control experiments, but a slow decline of the contractile force was seen during the 60 min observation period (Table 1, Figures 1 and 2). NEM concentration-dependently increased the force of contraction. The positive inotropic effect developed quickly and reached a maximum 10 min after drug addition (Figure 1). With 3×10^{-5} and 5.6×10^{-5} mol l^{-1} NEM, the contractile force remained elevated until the end of the 1 h period of drug exposure. But with 10^{-4} mol l^{-1} NEM the force of contraction declined quickly after the maximum was reached (Figures 1 and 3) and a negative inotropic effect of the drug was obvious towards the end of the observation period, which could not be reversed by washout (Figure 1). During the 50 min after the end of drug exposure (20 min of perfusion

Table 1 Effect of N-ethylmaleimide (NEM) on action potential parameters and force of contraction of guinea-pig papillary muscles

Drug		*MRP* (mV)	*APA* (mV)	$\dot{V}_{max}$ (V s^{-1})	APD_{20} (ms)	APD_{80} (ms)	*Fc* (mN)
None	C	−79.8 ± 1.1	116.7 ± 2.1	92.3 ± 10.1	112.7 ± 8.5	187.8 ± 7.8	0.46 ± 0.13
	Δ 10 min	−0.3 ± 0.8	+0.7 ± 0.5	−0.5 ± 2.2	−0.3 ± 1.0	+1.7 ± 1.8	−0.03 ± 0.02
	Δ 1 h	−0.5 ± 1.5	+0.5 ± 1.4	+1.8 ± 2.5	+4.7 ± 2.2	+0.8 ± 2.7	−0.06 ± 0.02*
NEM 30 μmol l^{-1}	C	−78.5 ± 1.0	115.2 ± 4.4	92.3 ± 5.9	124.2 ± 11.1	210.5 ± 13.2	0.50 ± 0.15
	Δ 10 min	+0.7 ± 0.7	+0.8 ± 0.9	+1.7 ± 1.7	+4.7 ± 1.3*	+1.7 ± 3.9	+0.12 ± 0.04*
	Δ 1 h	+0.7 ± 0.9	−1.5 ± 2.6	+6.5 ± 3.5	−9.8 ± 8.9	−16.8 ± 12.7	+0.12 ± 0.08
NEM 56 μmol l^{-1}	C	−79.3 ± 1.3	114.3 ± 2.8	85.0 ± 7.9	111.2 ± 9.6	201.0 ± 8.7	0.34 ± 0.08
	Δ 10 min	−2.2 ± 0.8*	+2.8 ± 2.2	+1.7 ± 3.2	+4.7 ± 2.7	−3.0 ± 2.6	+0.19 ± 0.04*
	Δ 1 h	−2.3 ± 1.1	−5.2 ± 1.5*	−4.7 ± 4.6	−53.5 ± 9.3*	−70.7 ± 5.1*	+0.07 ± 0.02*
NEM 100 μmol l^{-1}	C	−79.7 ± 1.4	117.5 ± 2.4	88.0 ± 7.9	112.5 ± 3.3	200.8 ± 4.6	0.41 ± 0.12
	Δ 10 min	+0.8 ± 1.2	−1.8 ± 0.8	+0.3 ± 2.3	+2.8 ± 4.5	−7.7 ± 3.5	+0.41 ± 0.10*
	Δ 1 h	−1.8 ± 1.4	−17.7 ± 6.7*	−16.2 ± 5.1*	−70.0 ± 7.7*	−106.0 ± 11.4*	−0.19 ± 0.09
NEM 100 μmol l^{-1} + TTX 30 nmol l^{-1} + Prop 4 μmol l^{-1}	C	−79.8 ± 0.9	111.5 ± 1.4	84.3 ± 3.1	119.2 ± 5.7	205.7 ± 4.7	0.38 ± 0.10
	Δ 10 min	−2.2 ± 0.5*	+1.2 ± 0.6	−0.3 ± 3.0	−0.8 ± 3.1	−6.5 ± 3.3	+0.54 ± 0.11*
	Δ 1 h	−2.8 ± 1.1*	−20.7 ± 2.9*	−16.8 ± 6.4*	−82.0 ± 4.9*	−119.5 ± 8.2*	−0.20 ± 0.09
NEM 100 μmol l^{-1} + Ver 1 μmol l^{-1}	C	−78.5 ± 0.9	107.8 ± 1.3	135.2 ± 8.0	109.7 ± 4.4	200.2 ± 6.8	0.05 ± 0.01
	Δ 10 min	−2.3 ± 0.6*	+0.7 ± 0.7	+2.3 ± 1.7	+8.3 ± 3.0*	+6.0 ± 3.8	+0.11 ± 0.03*
	Δ 1 h	−2.5 ± 1.4	−20.8 ± 10.7	−35.7 ± 20.2	−64.8 ± 9.8*	−98.0 ± 14.6*	+0.07 ± 0.05
NEM 100 μmol l^{-1} Na^+ 70 mmol l^{-1}	C	−79.3 ± 0.7	107.7 ± 0.9	68.3 ± 11.3	81.7 ± 8.3	155.0 ± 12.6	0.35 ± 0.10
	Δ 10 min	+0.3 ± 0.9	+2.7 ± 2.2	+1.7 ± 4.4	+10.0 ± 0.5*	+11.7 ± 1.7*	+0.37 ± 0.04*
	Δ 1 h	+1.0 ± 1.1	−11.0 ± 1.0*	+0.0 ± 5.8	−48.3 ± 8.3*	−85.0 ± 15.3*	−0.27 ± 0.08

Pre-drug values (C) and their mean changes 10 min (Δ 10 min) and 1 h (Δ 1 h) after addition of NEM are given ($\overline{\Delta x}$ ± s.e.mean, n = 6). Asterisks indicate changes that are statistically significant (Student's t test for paired data, $P \leq 0.05$, two-tailed). Abbreviations: MRP = membrane resting potential; APA = action potential amplitude; $\dot{V}_{max}$ = maximum depolarization velocity; APD_{20}, APD_{80} = action potential duration at 20% and 80% of full repolarization; Fc = force of contraction; TTX = tetrodotoxin 3×10^{-8} mol l^{-1}; Prop = propranolol 4×10^{-6} mol l^{-1}; Ver = verapamil 10^{-6} mol l^{-1}: a = 3 experiments only.

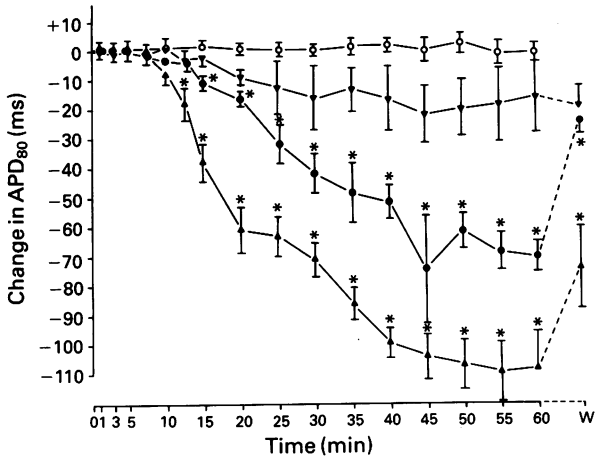

Figure 2 Effect of N-ethylmaleimide (NEM) on the action potential duration at 80% of full repolarization (APD_{80}). Abscissa scale: time after drug addition (min). W = effect remaining after washout of drug. Ordinate scale: change in action potential duration (ms). (○) Control experiments, no drug; (▼) NEM 3×10^{-5} mol l^{-1}; (●) NEM 5.6×10^{-5} mol l^{-1}; (▲) NEM 10^{-4} mol l^{-1}. The symbols indicate mean changes and vertical lines show s.e.mean ($n = 6$). Asterisks mark changes that are statistically significant (t test for paired data, $P \leqslant 0.05$ two-tailed). Pre-drug values of the AP duration were 187.8 ± 7.8 ms in control experiments and 210 ± 13.2, 201.0 ± 8.7 and 200.8 ± 4.6 ms in the experiments with the three concentrations of NEM.

with drug-free solution and 30 further min of recovery without perfusion; see Methods) the force of contraction decreased even further. It is not clear whether this was due to a persistent effect of NEM or merely a consequence of the long duration of these experiments. In many muscles a transient prolongation of the duration of the action potential in the plateau range was observed after the addition of NEM (APD_{20}; Table 1). The overall effect, however, was slight and statistically significant only with the lowest concentration. With 3×10^{-5} mol l^{-1} NEM, the only further change was a minor reduction of the total action potential duration towards the end of the observation period. However, with 5.6×10^{-5} and 10^{-4} mol l^{-1} NEM a progressive shortening of the action potential duration developed (Table 1, Figure 2). The action potential amplitude and the maximum depolarization velocity were likewise reduced. All these effects were only incompletely reversible after washout.

Modification of N-ethylmaleimide effects by other drugs

The addition of propranolol (4×10^{-6} mol l^{-1}, 40 min before NEM) and tetrodotoxin (3×10^{-8} mol l^{-1}, 20 min before NEM) had no effect on the action potential or the force of contraction. After pretreatment with both drugs the control values immediately before addition of NEM, which are shown in Table 1, were similar to the control values in the untreated groups of muscles. In the presence of propranolol and tetrodotoxin NEM 10^{-4} mol l^{-1} still exerted a transient positive inotropic effect which was followed by a rapid decline of the contractile force (Table 1, Figure 3). The time course was similar to that in the experiments with NEM alone. Propranolol and tetrodotoxin did not prevent the decrease of the action potential duration by NEM (Table 1, Figure 3).

Verapamil alone 10^{-6} mol l^{-1}, given 1 h before the addition of NEM, greatly reduced the force of contraction of the muscles, but had little influence on the action potential characteristics (see control values in Table 1). In the presence of verapamil, 10^{-4} mol l^{-1} NEM still exerted a positive inotropic effect (Figure 3). However, the force increased more slowly than with NEM alone, reached a maximum only 30 min after drug addition and remained elevated until the end of the experiments. The maximum increase was much smaller than with NEM alone (Table 1). In contrast, the shortening of the action potential duration by NEM was not attenuated by verapamil (Table 1, Figure 3).

In low sodium Krebs-Henseleit solution, the positive inotropic effect of 10^{-4} mol l^{-1} NEM was not reduced (Table 1). A rapid increase was again followed by a negative inotropic effect of NEM. The progressive shortening of the action potential duration was also still observed.

Effects of ouabain

Ouabain, 10^{-6} mol l^{-1}, had a prominent positive inotropic effect (Figure 4). The effect developed more slowly than with NEM. The force of contraction increased continuously during 1 h of drug exposure. The magnitude of the ouabain effect was

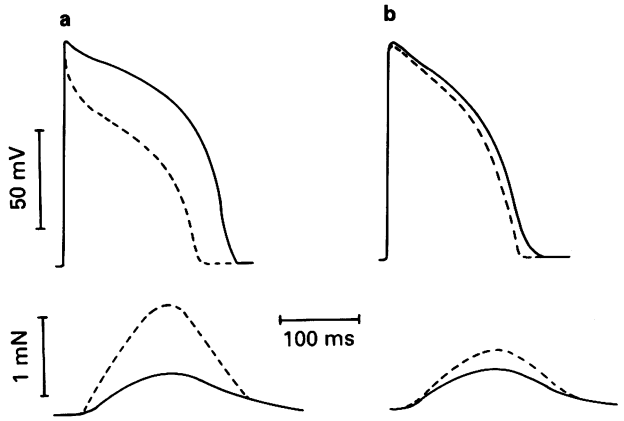

Figure 4 Typical examples of the effect of ouabain 10^{-6} mol l^{-1} on action potential (upper traces) and force of contraction (lower traces) of papillary muscles. (a) Effect of ouabain at normal (143.8 mmol l^{-1}) sodium concentration. (b) Effect of ouabain at reduced (70 mmol l^{-1}) sodium concentration. Solid line: control, before drug addition; broken line, 1 h after addition of ouabain.

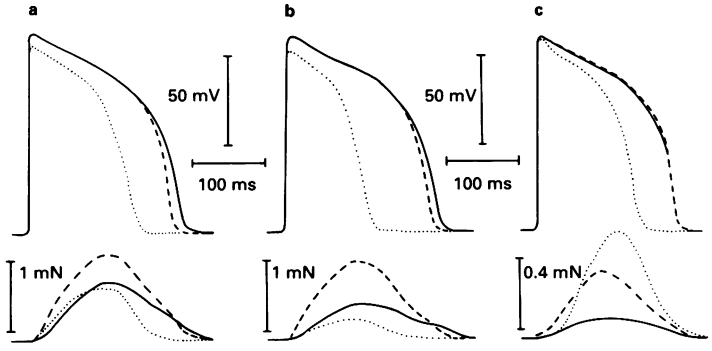

Figure 3 Typical examples for the effect of 10^{-4} mol l^{-1} N-ethylmaleimide (NEM) on action potential (upper traces) and force of contraction (lower traces) of papillary muscles. (a) Effect of NEM alone. (b) Effect of NEM in the presence of propranolol 4×10^{-6} mol l^{-1} and tetrodotoxin 3×10^{-8} mol l^{-1}. (c) Effect of NEM in the presence of verapamil 10^{-6} mol l^{-1}. (——) Control, before addition of NEM; (– – –) 10 min after addition of NEM; (.....) 30 min after addition of NEM.

greatly influenced by the sodium concentration (Figure 4). While a 145 ± 8% increase of the force was seen in normal Krebs-Henseleit solution, a mere 37 ± 10% increase was obtained in sodium-depleted solution (3 experiments each). The decreases of the action potential duration at 80% of full repolarization and of the action potential amplitude (Figure 4), which were prominent in normal solution (−25 ± 5% and −13 ± 3%, respectively) became marginal when the sodium concentration was reduced (−6 ± 3% and −1 ± 0.5%, respectively).

Effect of N-ethylmaleimide on slow response action potentials

NEM, 10^{-4} mol l^{-1}, increased the maximum depolarization velocity and the duration of slow response action potentials. $\dot{V}_{max}$ was nearly doubled (from 11.3 ± 1.1 to 21.3 ± 1.9 V s^{-1}; $P \leq 0.05$) after 25 min of NEM exposure and remained elevated for the rest of the observation period (Figure 5). The increase of the action potential duration (pre-drug value 224.3 ± 12.54 ms), on the other hand, was transient. The maximum prolongation after 15 min of NEM exposure was followed by a progressive shortening (Figure 5). After 40 min the mean reduction was 21.7 ± 12.54 ms and during the next 20 min three of the six preparations became inexcitable. The amplitude of the action potential (pre-drug value 77.8 ± 1.3 mV) was increased and decreased with a similar time course, but the changes were small (about 2 mV) and not statistically significant. The membrane resting potential (−42.8 ± 0.6 mV) was not affected by NEM.

In three preliminary experiments 5×10^{-7} mol l^{-1} of the Ca^{2+}-channel activator Bay K 8644 increased $\dot{V}_{max}$ of slow response action potentials from 14.3 ± 2.0 V s^{-1} to 21.7 ± 4.0 V s^{-1} ($P \leq 0.05$) and increased the action potential duration by 41.7 ± 6.0 ms (from 273.3 ± 34.7 ms to 315.0 ± 30.4 ms; $P \leq 0.05$). The effect of Bay K 8644 remained constant for 1 h and subsequent addition of 10^{-4} mol l^{-1} NEM in these muscles caused no further increase of $\dot{V}_{max}$ and only a marginal (+15 ± 2.9 ms) additional increase of the action potential duration. However, a progressive shortening of the action potential duration was again evident when the exposure to NEM lasted longer than 20 min.

Ouabain, in concentrations up to 10^{-5} mol l^{-1}, had no detectable effect on slow response action potentials (preliminary results, data not shown).

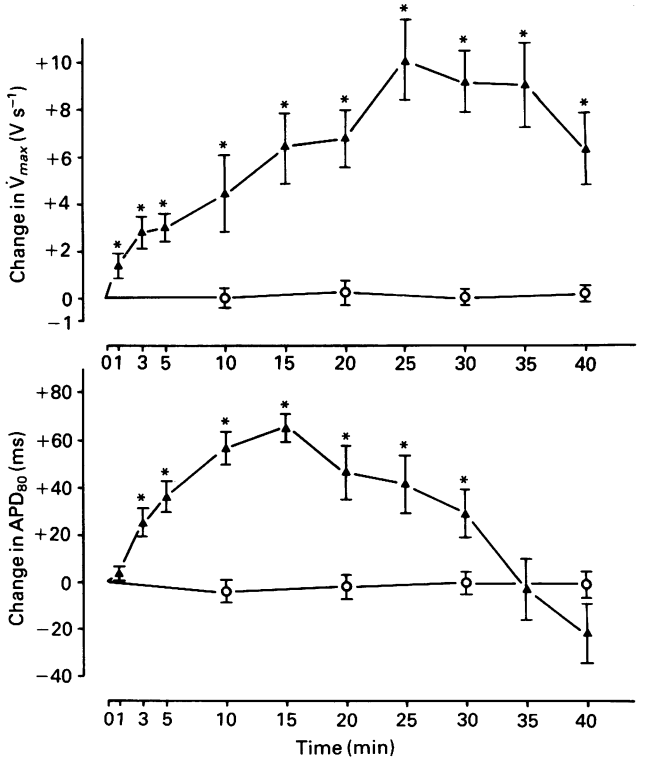

Figure 5 Effect of N-ethylmaleimide 10^{-4} mol l^{-1} (NEM) on the depolarization velocity $\dot{V}_{max}$ (a) and the duration of slow response action potentials at 80% of full repolarization (APD_{80}, b) in K^+-depolarized papillary muscles. Abscissae: time after drug addition (min). Ordinates: change in $\dot{V}_{max}$ (V s^{-1}) or duration (ms) of the action potential. (○) Control experiments, no drug; (▲) NEM 10^{-4} mol l^{-1}. The symbols indicate mean changes and vertical lines show s.e.mean ($n = 6$). Asterisks mark changes that are statistically significant (t test for paired data, $P \leq 0.05$ two-tailed). Pre-drug values for $\dot{V}_{max}$ and AP duration were 11.0 ± 1.0 V s^{-1} and 231.3 ± 14.43 ms in control experiments and 11.3 ± 1.1 V s^{-1} and 224.3 ± 12.54 ms in experiments with NEM.

Discussion

At normal K^+-concentration, NEM caused a transient positive inotropic effect and a progressive decrease of the action potential duration. As judged from its effect on $\dot{V}_{max}$, the influence of NEM on the fast inward Na^+-current is small. The NEM-induced increase of the slow inward Ca^{2+}-current (see below) would prolong rather than shorten the action potential duration. But an increase of the outward K^+-current would be a plausible explanation for the accelerated repolarization. Such an increase would also explain the negative chronotropic effect of NEM in sinoatrial pacemaker cells and the associated slowing of the spontaneous diastolic depolarization (Toda & Konishi, 1969). That the duration of guinea-pig atrial action potentials is not affected by NEM (Braun & Sperelakis, 1988) does not necessarily contradict our hypothesis. Similar diverse effects on the repolarization of atrial and ventricular tissues have been observed, for instance, with Na-salicylate (Cohen *et al.*, 1979a,b; Brasch, 1983) and have been explained by a different sensitivity of the main repolarizing current towards this drug. Data from voltage-clamp or patch-clamp experiments with NEM are needed to differentiate its effects on the various types of K^+-currents.

In our experiments, as in the study of From (1970), the positive inotropic effect of NEM was not affected by propranolol or tetrodotoxin. Thus we cannot confirm previous findings which suggest that the increase of force is due exclusively (Yamamoto *et al.*, 1973), or at least in part (Temma *et al.*, 1978), to a release of endogenous noradrenaline. Furthermore, we have shown recently that NEM does not increase the transmitter release from [^{3}H]-noradrenaline loaded muscles when the myocardium is stimulated with pulses of low voltage, whereas the drug potentiates the noradrenaline efflux when the sympathetic nerve endings are depolarized by field stimulation with supramaximal voltage (Brasch, 1989). The latter effect is caused by an interruption of the α_2-adrenoceptor-mediated autoinhibition. The positive inotropic effect of NEM developed much faster than the effect of ouabain, an observation already made by Fricke (1978), and was not attenuated by a reduction of the extracellular sodium concentration. It is unlikely, therefore, that NEM increased the contractile force by an inhibition of Na^+, K^+-ATPase in our experiments, though such an inhibition has been demonstrated with purified enzyme preparations (Skou & Hilberg, 1965; Fujita *et al.*, 1968; Tobin & Akera, 1975).

In K^+-depolarized muscles, NEM increased $\dot{V}_{max}$ and action potential duration. Similar results were obtained with the Ca^{2+}-channel activator Bay K 8644. Both drugs seem to act on the same membrane currents, as the effect of NEM was attenuated by pretreatment with Bay K 8644. In the presence of K^+-current inactivators ($BaCl_2$ in our experiments), an increase of $\dot{V}_{max}$ and duration of slow response action potentials is thought to be a reliable indicator of an increased slow inward Ca^{2+}-current (Schüttler & Boldt, 1989) and, as the inotropic effect of NEM was greatly reduced by pretreatment with verapamil, we suggest that it is mainly caused by the NEM-induced increase of the Ca^{2+}-current. The transient nature of the positive inotropic effect can be explained by the

shortening of the action potential duration which was observed in normal and K^+-depolarized muscles. This is probably caused by an increased K^+-conductance (see above) and will shorten the time during which the Ca^+-current can flow, while the peak inward Ca^{2+}-current is not reduced, as judged from the persistent increase of $\dot{V}_{max}$ in slow response action potentials (Figure 5). An alternative explanation for the decline of force would be that NEM, like the alkaloid ryanodine, inhibits calcium uptake by the sarcoplasmic reticulum (Kirsten & Kuperman, 1970). Thus prolonged exposure to NEM could lead to a gradual depletion of the intracellular calcium stores.

Yamamoto *et al.* (1973) found that the effects of NEM in guinea-pig atria were prevented in the presence of cysteine. They concluded that NEM acts by alkylating SH-groups on proteins. Pertussis toxin-sensitive G-proteins are the most sensitive targets for the alkylating effects of NEM (Jakobs *et al.*, 1982). Such G-proteins are involved in the regulation of the conductivity of cardiac K^+- and Ca^{2+}-channels (see Robishaw & Foster, 1989 for review). An alteration of their function could therefore explain the effects of NEM. This explanation is speculative, however, as the concentrations of NEM used in our experiments were too large to exclude alkylation of other functionally important proteins (Jakobs *et al.*, 1982). Thus, the exact mechanism(s) of action of NEM remain to be resolved.

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References

BENNETT, D.R., ANDERSEN, K.S., ANDERSEN, M.V. Jr., ROBERTSON, D.N. & CHENOWETH, M.B. (1958). Structure-activity analysis of the positive inotropic action of conjugated carbonyl compounds on the cat papillary muscle. *J. Pharmacol Exp. Ther.*, **122**, 489–498.

BRASCH, H. (1983). Lack of direct antiarrhythmic electrophysiological effects of salicylate on isolated guinea-pig myocardium. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **323**, 343–349.

BRASCH, H. (1989). Pulse duration and α_2-adrenoceptors modify noradrenaline release from field-stimulated atria. *Eur. J. Pharmacol.*, **171**, 49–57.

BRAUN, A.P. & SPERELAKIS, N. (1988). Attenuation by N-ethylmaleimide treatment of the cholinergically induced shortening of action potential duration in guinea-pig right atrium. *Biochem. Pharmacol.*, **37**, 4577–4581.

COHEN, I., NOBLE, D., OHBA, M. & OJEDA, C. (1979a). Action of salicylate ions on the electrical properties of sheep cardiac Purkinje fibres. *J. Physiol.*, **297**, 163–185.

COHEN, I., NOBLE, D., OHBA, M. & OJEDA, C. (1979b). The interaction of ouabain and salicylate on sheep cardiac muscle. *J. Physiol.*, **197**, 187–205.

EHARA, T. & INAZAWA, M. (1980). Calcium-dependent slow action potentials in potassium-depolarized guinea-pig ventricular myocardium enhanced by barium ions. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **315**, 47–54.

FRICKE, U. (1978). Myocardial activity of inhibitors of the Na^+- K^+-ATPase. Differences in the mode of action and subcellular distribution pattern of N-ethylmaleimide and ouabain. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **303**, 197–204.

FROM, A.H.L. (1970). N-ethylmaleimide (NEM) induced inotropism. *Clin. Res.*, **18**, 306.

FUJITA, M., NAGANO, K., MIZUNO, N., TASHIMA, Y., NAKAO, T. & NAKAO, M. (1968). Comparison of some minor activities accompanying a preparation of sodium-plus-potassium ion stimulated adenosine triphosphatase from the pig brain. *Biochem. J.*, **106**, 113–121.

IVEN, H. & BRASCH, H. (1977). Effects of the local anesthetics bufacain and lidocaine on transmembrane action potentials, refractory period, and reactivation of the sodium system in guinea pig heart muscle. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **297**, 153–161.

JAKOBS, K.H., LASCH, P., MINUTH, M., AKTORIES, K. & SCHULZ, G. (1982). Uncoupling of alpha-adrenoceptor-mediated inhibition of human platelet adenylate cyclase by N-ethylmaleimide. *J. Biol. Chem.*, **257**, 2819.

KIRSTEN, E.B. & KUPERMAN, A.S. (1970). Effects of sulphydryl inhibitors on frog sartorius muscle: N-ethylmaleimide. *Br. J. Pharmacol.*, **40**, 827–835.

ROBISHAW, J.D. & FOSTER, K.A. (1989). Role of G proteins in the regulation of the cardiovascular system. *Ann. Rev. Physiol.*, **51**, 229–244.

SCHÜTTLER, K. & BOLDT, W. (1989). Ca-mediated action potentials in ventricular myocardium. Variation by various stimulation patterns and by K-channel blockers. *J. Mol. Cell. Cardiol.*, **21**, 901–909.

SKOU, J.C. & HILBERG, C. (1965). The effect of sulphydryl blocking reagents and of urea on the ($Na^+ + K^+$)-activated enzyme system. *Biochim. Biophys. Acta*, **110**, 359–369.

TEMMA, K., AKERA, T., KU, D.D. & BRODY, T.M. (1978). Sodium pump inhibition by sulfhydryl inhibitors and myocardial contractility. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **302**, 63–71.

TOBIN, T. & AKERA, T. (1975). Showdomycin, a nucleotide-site-directed inhibitor of (Na^+-K^+)-ATPase. *Biochim. Biophys. Acta*, **389**, 126–136.

TODA, N. & KONISHI, N. (1989). Effects of a sulfhydryl reagent, N-ethylmaleimide, on electrical and mechanical activities of isolated atria. *Eur. J. Pharmacol.*, **7**, 5–13.

UENO, A. (1971). Adrenolytic property of NEM in the guinea pig atria *in vitro*. *Jpn. J. Pharmacol.*, **21**, 837–839.

YAMAMOTO, H., KITANO, T., NISHINO, H. & MURANO, T. (1973). Studies on pharmacodynamic action of N-ethylmaleimide (NEM) I.: Influence of NEM on synaptic transmission of sympathetic nerves in guinea-pigs. *Jpn. J. Pharmacol.*, **23**, 151–160.

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Inhibitory effect of streptozotocin-induced diabetes on non-cholinergic motor transmission in rat detrusor and its prevention by sorbinil

G.N. Luheshi & [1]M.A. Zar

The Department of Pharmacological Sciences, The Medical School, Framlington Place, Newcastle Upon Tyne NE2 4HH

1 Non-cholinergic motor transmission in the urinary bladder of streptozotocin (STZ)-diabetic rats was studied by recording contractile activity of strips of detrusor *in vitro*.

2 The neurogenic contractile responses to electrical field stimulation (EFS) of atropine-treated detrusor strips were decreased in 4, 8 and 12 week STZ-diabetic rats. The decrease was most marked in 12 week diabetic rats and least in 4 week ones.

3 Concentration-response curves showed no change in sensitivity of the detrusor to acetylcholine (ACh) in diabetic rats. The maximum tension generated by ACh was similar in diabetic and non-diabetic animals.

4 The contractile responses to EFS at frequencies $\geqslant 1$ Hz were not maintained during stimulation. The 'fade' was significantly greater in detrusor strips of diabetic rats.

5 The contractile response of detrusor to EFS was significantly greater in 12 week diabetic rats treated with the aldose reductase inhibitor sorbinil, than in untreated 12 week diabetic rats. The sensitivity to ACh was similar in the two groups.

6 It is concluded that the reduction of the neurogenic non-cholinergic responses of detrusor to EFS in STZ-diabetic rats is probably caused by a reduction in the release of the non-cholinergic motor transmitter. The results are discussed in relation to bladder dysfunction in human diabetes mellitus.

Introduction

The incidence of bladder dysfunction in diabetic patients is reported to be as high as 87% (Faerman *et al.*, 1971). There is a widely held view that the bladder dysfunction originates as a result of diabetic autonomic neuropathy (Buck *et al.*, 1974; Ellenberg, 1980). Although several studies on different aspects of bladder dysfunction have been reported in the streptozotocin (STZ)-induced diabetic rat, an animal model of human diabetes (Lincoln *et al.*, 1984; Longhurst & Belis, 1986; Moss *et al.*, 1987; Kolta *et al.*, 1985; Santicioli *et al.*, 1987), the effect of diabetes on the non-cholinergic motor transmission to the detrusor has remained unclear. The results from some of the studies lead to divergent conclusions regarding the effects of diabetes on the non-cholinergic motor transmission to the detrusor. Thus the findings of Moss *et al.* (1987) have indicated no change in the overall response to non-cholinergic motor nerve stimulation, even though postjunctional sensitivity to a stable purinoceptor agonist ranged from high in 8 week diabetic animals to low in 16 week diabetic animals. Using non-atropinised strip preparations from STZ-diabetic rats, Longhurst & Belis (1986) have reported a drastic reduction in neurogenic responses without any significant alteration in adenosine 5'-triphosphate (ATP) sensitivity. Lincoln *et al.* (1984) found no impairment in the ability of the detrusor muscle to respond to cholinergic nerve stimulation. The results of the latter two investigations, taken together, imply that the reduction in the motor transmission, observed by Longhurst & Belis (1986), was ascribable to the non-cholinergic component of the transmission.

In the present study, isolated strips of rat detrusor have been contracted by acetylcholine (myogenic) and by electrical field stimulation (neurogenic) the latter being performed in the presence of atropine. These myogenic and non-cholinergic neurogenic contractions have been compared in non-diabetic controls and STZ-diabetic rats, in order to determine the effect of STZ-diabetes on the non-cholinergic motor transmission and detrusor contractility.

The accumulation of excess sorbitol within peripheral nerves as a direct consequence of diabetes has been advanced as one of the principal causes of neuropathy (Green *et al.*, 1985) and has been described in human (Mayhew *et al.*, 1983) and experimental (Clements, 1979; Mayer & Tomlinson 1983) diabetes. In the present investigation we have therefore also examined the effect of inhibition of sorbitol formation by an aldose reductase inhibitor, sorbinil, on the damaging effect of STZ-induced diabetes upon non-cholinergic motor transmission in this organ.

Methods

Animals

All experiments were performed on young adult male Wistar rats, 8 weeks old, weighing 180–200 g at the start of the experiment. The animals were randomly allocated to different experimental groups each consisting of 6 animals. The animals were individually weighed and the weight recorded.

Induction of diabetes

The rats to be made diabetic were fasted overnight and their blood glucose levels estimated. Diabetes was induced by administration of a single intraperitoneal injection of streptozotocin (STZ), 75 mg kg^{-1}, to each rat. Three days after the STZ treatment, the animals were fasted for 2 h before obtaining another blood sample from each animal in order to estimate the blood glucose level. This was to ensure that diabetes had been induced. Animals with blood glucose levels $\geqslant 300$ mg 100 ml^{-1} were deemed to be diabetic and therefore suitable for the study. The blood glucose levels of the diabetic rats were thereafter monitored at regular intervals. Age- and weight-matched controls of every diabetic group were treated

[1] Author for correspondence.

in an identical fashion except that saline was injected instead of STZ.

Experimental organisation

The diabetic animals were divided into four groups of 6 rats each. The first three groups were kept for 4, 8 and 12 weeks respectively after the induction of diabetes. The fourth group of 6 diabetic animals was maintained for 12 weeks while receiving treatment with sorbinil (see below). Each of the aforementioned groups except the last one, had a corresponding group of age- and weight-matched controls which were treated in an identical fashion. The group of diabetic animals maintained for 12 weeks served as control for the sorbinil-treated group.

Sorbinil-treatment

Treatment with the aldose reductase inhibitor, sorbinil (Pfizer research, Pfizer corporation, Groton, Connecticut, U.S.A.) was begun on the day of injection of STZ. The sorbinil was administered by gavage in a freshly made daily dose of 20 mg kg^{-1} suspended in 0.1 M NaOH titrated down to pH 7 with HCl. This treatment was maintained for 12 weeks.

Maintenance of animals

Each group of 6 animals was caged separately and kept under precisely the same conditions as the other groups. All groups were kept in a temperature controlled room (22 ± 2°C), artificially lit from 06 h 00 min–18 h 00 min each day. The animals were fed with standard laboratory diet and provided with water *ad libitum*. After the initial weights had been noted, each rat was routinely weighted once a week and the weight recorded up to the end of the study.

Estimation of blood glucose levels

Periodic routine estimation was carried out by taking a drop of blood from the tail vein of the rat and estimating its glucose level with BM-Test-Glycemie strips (Boehringer-Manheim).

The final estimate of blood glucose was carried out after the animal had been killed. Blood samples and glucose standards (50 μl) were collected into ice cold 0.016% uranyl acetate (1 ml). The mixture was agitated, centrifuged at 800 *g* and the supernatant assayed for glucose spectrophotometrically by the standard glucose oxidase method using the GOD-PAP assay kit (Boehringer-Ingelheim, London).

Experimental procedures

At the end of the allocated period for maintenance (4, 8 or 12 weeks) the animals were killed by concussion and decapitation. Final blood samples were collected for estimation of their glucose contents.

(a) *Preparation of the detrusor strip* The strip was prepared according to the procedure of Ambache & Zar (1970), as subsequently modified by Zar *et al.* (1990). After the animal had been killed the lower abdomen was opened and the bladder exposed. The urine present in the bladder was withdrawn into a 5 ml syringe and its volume recorded. The bladder was then held at its apex slightly stretched and the investing layers of serosal coat, connective tissue and accompanying blood vessels were cut away as close as possible from the outer surface of the bladder wall. The bladder was excised by a cut above the trigone and rapidly weighed. The bladder was then placed in a Petri dish and washed with several changes of Krebs-Henseleit solution. The bladder was opened by two lateral incisions and then unfolded to give a rectangular sheet of tissue. The unfolded tissue was laid on Krebs-soaked tissue paper and strips of bladder 1–1.5 cm long and 0.2 cm wide were cut with a pair of fine scissors. The strip preparation of the detrusor was suspended in a 1 ml jacketed organ bath between built-in vertical platinum electrodes, at a resting tension of 0.5 g. The resting tension was kept constant at 0.5 g throughout the experiment by appropriate adjustments whenever needed. The preparation was maintained at 37°C in Krebs-Henseleit solution gassed in the reservoir and in the organ bath with 95% O_2 plus 5% CO_2 mixture. Indomethacin 10 μM was present in the Krebs solution throughout the duration of the experiment in order to minimize the spontaneous activity of the isolated preparation (Zar *et al.*, 1990). For recording the tension of the detrusor muscle, the preparation was connected to an isometric transducer and a potentiometric recorder. An equilibration period of 30 min was allowed before the start of further experimentation (see below); during this period the preparation was repeatedly washed with fresh Krebs solution.

(b) *Experiments on isolated detrusor strips* In each experiment, isolated detrusor strips were used to obtain contractile responses to two types of stimuli: electrical field stimulation and acetylcholine. The two types of stimuli were not delivered to the same strip. One, or if necessary, more strips were reserved for each type of stimulus.

(i) *Electrical field stimulation* The strips reserved for electrical field stimulation (EFS) were treated with atropine 3 μM. EFS was started after 30 min exposure to atropine and during its continued presence for the rest of the experiment. The parameters of EFS were as follows for trains of pulses: pulse duration = 0.1 ms; train duration = 10 s; frequency = 1, 2, 4, 6, 8 and 10 Hz; interval between two trains = 60 s; voltage = supramaximal for evoking maximal contraction at each frequency. The chosen concentration of atropine (3 μM) was based upon observations in several experiments that the responses to EFS were maximally depressed by atropine 0.5 μM and atropine in higher concentrations was no more effective than atropine 0.5 μM. It was therefore considered prudent to employ atropine in a concentration (3 μM) which would be supramaximal for blocking the cholinergic component of the EFS-evoked response but yet be sufficiently low not to exert any non-specific depressent effect on the EFS-evoked response. The choice of parameters of EFS was based upon the satisfaction of two criteria: first, reproducibility of the response at each frequency of stimulation and second, the ability of tetrodotoxin, 0.5 μM to abolish the response. Fulfilment of both criteria was deemed to imply that the EFS evoked responses were neurogenic and supramaximal.

(ii) *Construction of dose-response curves to acetylcholine* The object of the experiments was to assess the ability of the detrusor to generate tension in response to a spasmogen. The spasmogen chosen for this purpose was ACh which was used in the bath concentration range of 10^{-8}–10^{-3} M, in a non-cumulative manner. Exposure to a dose of ACh was maintained until the maximal response to that concentration of ACh was obtained. The preparation was then repeatedly washed with several changes of Krebs solution. The preparation was allowed to relax fully before exposing it to the next higher concentration of ACh.

Statistics

At the end of each experiment, the bladder strip was detached from the recording set up, blotted dry and weighed. In order to render the results comparable from one strip to another and from strips of different animals, the tension generated by the strip was then used to calculate the tension/100 mg bladder tissue. All values are expressed as mean ± s.e.mean and the statistical significances were calculated by Student's *t* test.

Materials

The drugs used and their sources were: acetylcholine chloride, atropine sulphate, indomethacin, streptozotocin (Sigma), tet-

Table 1 Body weight (g) and blood glucose concentration (mg 100 ml^{-1}) in streptozotocin-diabetic and control rats

	Duration of diabetes (weeks)						
	4		8		12		
	Control	*Diabetic*	*Control*	*Diabetic*	*Control*	*Diabetic*	*Diabetic + sorbinil*
Weight (g)							
Initial	220 ± 5	219 ± 3	203 ± 5	201 ± 3	210 ± 5	203 ± 4	199 ± 5
Final	340 ± 8	195 ± 7***	419 ± 12	162 ± 8***	480 ± 11	172 ± 8***	165 ± 11
Blood glucose (mg 100 ml)$^{-1}$	63 ± 7	410 ± 29***	58 ± 2	446 ± 17***	70 ± 3	468 ± 37***	520 ± 45

All values are means ± s.e.mean ($n = 6$ in each group). The values in diabetic animals have been compared with their corresponding controls and the level of significant difference is indicated by *** $P < 0.001$. There was no significant difference between the values from 12 week diabetic animals and 12 week sorbinil-treated diabetic animals.

rodotoxin (Sankyo) and sorbinil (Pfizer). Solutions of drugs were made fresh on the day of their use with the exception of tetrodotoxin the stock solution of which was stored at −20°C.

Preparation of drug solutions

Streptozotocin: this was dissolved immediately prior to its injection in 50 mM sodium citrate buffer and titrated to pH 4.5 with HCl. The drug was kept on ice at all times before its use.

Sorbinil: this drug was dissolved in freshly prepared 0.1 M NaOH on the day of treatment. The resulting alkaline solution was titrated down to a neutral pH with HCl.

Indomethacin: absolute ethanol was used to dissolve this drug to make a solution of 10^{-2} M.

All other drugs were dissolved in distilled water.

Composition of Krebs-Henseleit solution

The composition (in mM) was: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11.

Results

Body-weight and blood glucose level

Diabetic animals in all groups (4, 8 and 12 weeks) with or without sorbinil-treatment did not gain weight over the course of the investigation. By contrast, non-diabetic controls acquired significant weight gains during this period. The % gain in mean body weight of non-diabetic animals was 55, 106 and 128 after 4, 8 and 12 weeks respectively (Table 1). As was to be expected, the blood glucose levels in all diabetic animals were high and treatment with sorbinil had no significant effects on either the blood glucose levels or on loss of body weight (Table 1).

Bladder weight and residual urine

The weight of the whole urinary bladder increased only very modestly with increasing age in control (non-diabetic) rats (mean weights in mg after 4, 8 and 12 weeks = 63, 65 and 75 respectively). In sharp contrast to the control animals, the weight of urinary bladder rose sharply in diabetic animals and there appeared to be a direct relationship between the duration of diabetes and the weight of the bladder (mean weights of bladder in mg 4, 8 and 12 weeks following the induction of diabetes = 287, 710 and 820 respectively). Sorbinil-treatment of diabetic rats did not affect the weight-gain of bladder and no significant difference in the weights of the urinary bladder was seen in 12 week diabetic animals treated or untreated with sorbinil (Table 2).

The volume of residual urine (VRU) i.e. the volume of urine present in the bladder after the death of the animal was considerably higher in diabetic animals compared to their controls (Table 2). The VRU increased with increasing age both in controls and diabetic animals. Mean VRU in sorbinil-treated diabetic rats was 2.8 ml compared to 3.8 ml in the corresponding sorbinil-untreated diabetic controls, a difference that was statistically significant at the 5% level (Table 2).

Contractile responses to electrical field stimulation (EFS)

The experiments were conducted in the presence of atropine (3 μM) in order to eliminate the cholinergic contribution to the transmission and limit the motor transmission to its non-cholinergic component alone. EFS, at every frequency used (1, 2, 4, 6, 8 and 10 Hz), evoked contractions of the detrusor in all groups of animals. Three phases in the contractile response to EFS were distinguishable.

Phase 1: during the first 2 s of the onset of stimulation, the detrusor strip contracted sharply and the attainment of the peak tension signalled the end of phase 1.

Phase 2 was the plateau phase, following the termination of phase 1. Its duration was inversely proportional to the frequency of stimulus burst. At the lowest frequency of 1 Hz it was very prolonged but at the highest frequency (10 Hz), it was extremely brief (Figure 1).

Phase 3 was the phase of relaxation during which the strip lost tension despite continuing stimulation. This phase was virtually non-existent at 1 Hz but pronounced at higher frequencies, specially at 10 Hz (Figure 1).

Table 2 Weight of bladder and volume of residual urine in streptozotocin-diabetic and control rats

	Duration of diabetes (weeks)						
	4		8		12		
	Control	*Diabetic*	*Control*	*Diabetic*	*Control*	*Diabetic*	*Diabetic + sorbinil*
Bladder weight (mg)	58 ± 6	287 ± 35***	65 ± 5	710 ± 33***	75 ± 4	820 ± 29***	790 ± 19
Residual urine (ml)	0.2 ± 0.05	1.1 ± 0.3***	0.3 ± 0.05	2.2 ± 0.2***	0.3 ± 0.04	3.8 ± 0.16***	2.8 ± 0.12

All values are means ± s.e.mean ($n = 6$ in each group). The values in diabetic animals have been compared with their corresponding controls and the level of significant difference is indicated by *** $P < 0.001$. There was no significant difference between the values from 12 week diabetic animals and 12 week sorbinil-treated diabetic animals. Volume of residual urine represents the volume of urine present in the bladder immediately after exsanguination of the animal.

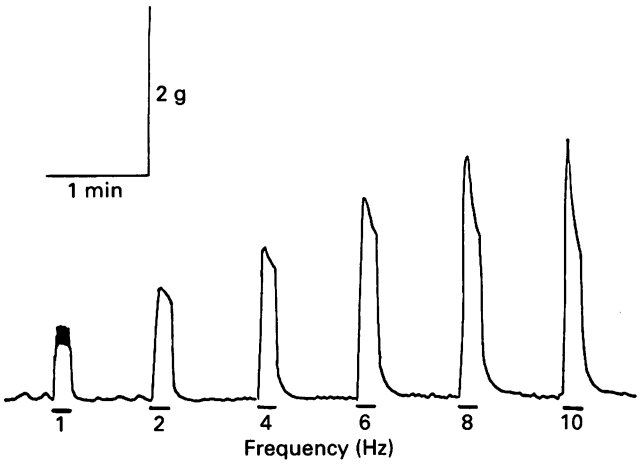

Figure 1 Rat isolated detrusor: a typical experiment showing contractile responses to electrical field stimulation in the presence of atropine 3 μM. The frequency of pulses within each train is given by the subscripts (1–10 Hz) and the duration of each stimulus burst (10 s) is indicated by a horizontal bar. Note that the peak tension was maintained only at 1 Hz and the 'non-sustainability' of the peak tension increased with increasing stimulation-frequency.

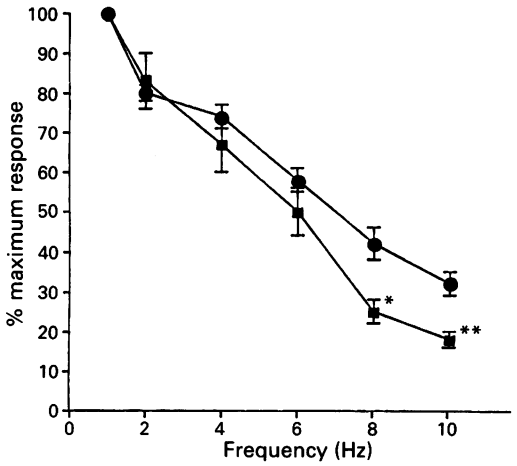

Figure 2 Comparison of the degree of 'non-sustainability' of the peak tension in response to electrical field stimulation at 1–10 Hz between detrusors from 12 week streptozotocin-diabetic (■) and 12 week non-diabetic controls (●). Each value is the mean of $n = 6$; vertical bars show s.e.mean. In each experiment the value was calculated by expressing the tension of the strip, during the delivery of the last pulse in the train, as % of the maximum tension to the same train. Note that the degree of 'non-sustainability' of the peak tension increases with increasing frequency in both diabetic and non-diabetic controls but is greater in diabetic detrusor. * $P \leq 0.05$, ** $P \leq 0.01$ indicate a significant difference from the non-diabetic control value.

Figure 1 is a record of part of a typical experiment on a detrusor strip from a non-diabetic control. The preparation responded to each stimulus burst by a contraction and the peak tension was reached approximately within the first 2 s of the 10 s bursts. The tension was not maintained at any frequency of stimulation except 1 Hz and the strip partially relaxed during the remaining period of EFS. The degree of relaxation after reaching the peak tension was directly reciprocal to the frequency of pulses within the stimulus burst, being least at 2 Hz and highest at 10 Hz (Figures 1 and 2). The responses to EFS were qualitatively and in general similar in strips from diabetic and non-diabetic animals. But there were significant quantitative differences in the responses to EFS between diabetic and non-diabetic detrusor strips. Diabetic detrusor developed a significantly lower peak tension in response to EFS compared to the non-diabetic controls. Peak tensions developed by 4, 8 and 12 week diabetic rat strips and their respective controls are presented in Figure 3 as % of the maximum tension. At every frequency of stimulation, the peak tension generated by diabetic detrusor was lower than the corresponding control value and the difference was statistically significant at every frequency used. The adverse effect on the generation of peak tension in response to EFS appeared to be related to the duration of diabetes. It was most pronounced in 12 week diabetic rats and least in 4 week diabetic ones. It has already been shown that the peak tension in response to EFS at all frequencies except 1 Hz was not sustained and the preparation started to relax during maintained stimulation (Figure 1). The degree of non-sustainability of peak tension was compared in 12 week diabetic rats and their non-diabetic controls (Figure 2). The final tension values (tension of the strip when the last pulse of the stimulus was delivered) are plotted in Figure 2 as a percentage of the peak tension and therefore serve as inverse indices of the degree of relaxation. These values are significantly lower in diabetic rats compared to the controls at 8 and 10 Hz, thus indicating an acceleration of the non-sustainability of the peak tension in diabetic animals.

Sorbinil-treated diabetic rats

The tension generated in response to EFS by detrusor strips from sorbinil-treated diabetic rats was markedly greater in comparison to that generated by the untreated diabetic ones. This was particularly noticeable at 4, 6, 8 and 10 Hz. Figure 4 allows a direct comparison to be made of the tension generated by the two groups of diabetic animals in response to EFS, at different frequencies. The peak tension developed by sorbinil-treated diabetic rats was greater at every frequency of stimulation, being 144%, 142%, 167%, 187%, 190%, 191% of the control tension, at 1, 2, 4, 6, 8 and 10 Hz respectively.

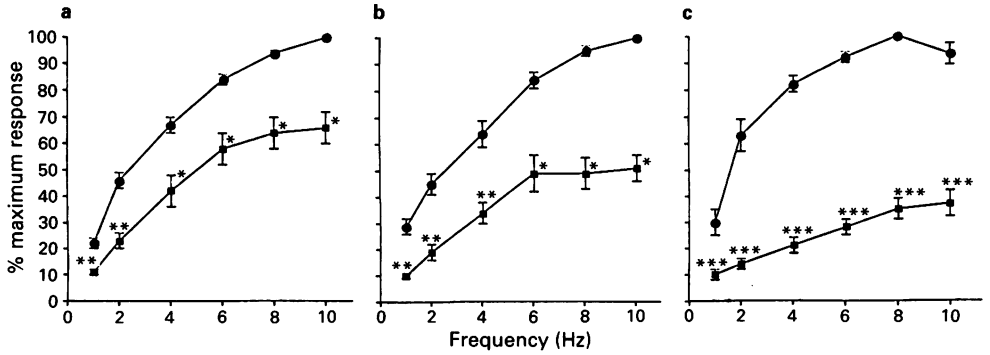

Figure 3 Non-cholinergic contractile responses of detrusor from streptozotocin (STZ)-diabetic (■) and non-diabetic control (●) rats to electrical field stimulation (EFS), 4 (a), 8 (b) and 12 (c) weeks following the induction of STZ-diabetes. All values are means (s.e.mean shown by vertical bars) and have been plotted as % of the maximum responses in their responsive group, (a), (b) or (c). Atropine (3 μM) was present throughout the experiment. Note that the degree of reduction of the EFS-evoked response increases with the increase in the duration of diabetes. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

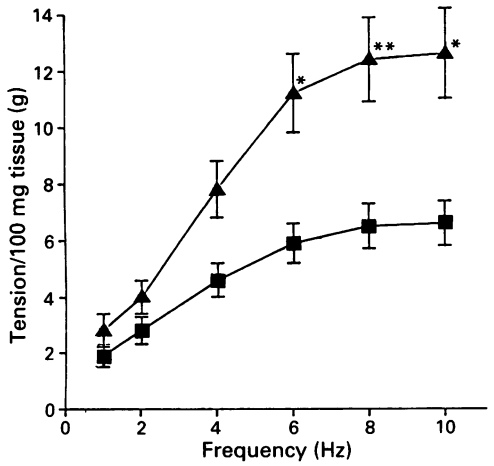

Figure 4 Contractile responses of detrusor from sorbinil-treated (▲) and nontreated (■) 12 week diabetic rats to electrical field stimulation. Atropine, (3 μM) was present throughout the experiment. All values are means (s.e.mean shown by vertical bars) ($n = 6$) of the tension (g) developed by 100 mg bladder tissue (further details are given in the methods). Note the greater tension developed by the detrusor from sorbinil-treated rats. * $P \leqslant 0.05$; ** $P \leqslant 0.01$.

Contractile responses to acetylcholine

ACh in concentrations ranging from 10^{-7} M to 10^{-3} M caused contractions of the detrusor. Dose-response curves to ACh in 12 week diabetic, non-diabetic and sorbinil-treated diabetic animals were very similar (Figure 5). Threshold concentration of ACh for evoking a contractile response in all 3 groups was 10^{-7} M and 50% of the maximal response was obtained with ACh concentrations ranging between 10^{-5} M and 3×10^{-5} M. Compared to normal, the peak tension achieved was somewhat greater in the two diabetic groups (Figure 5) but the differences were not significant.

Discussion

It is now widely recognised that the motor transmission in the rat urinary bladder, in common with other mammalian species, contains a large non-cholinergic component (Ambache & Zar, 1970; Taira, 1972). Impairment of this component of the motor transmission in streptozotocin-diabetic animals and the partial prevention of the impairment by an aldose reductase inhibitor, sorbinil, were the most striking

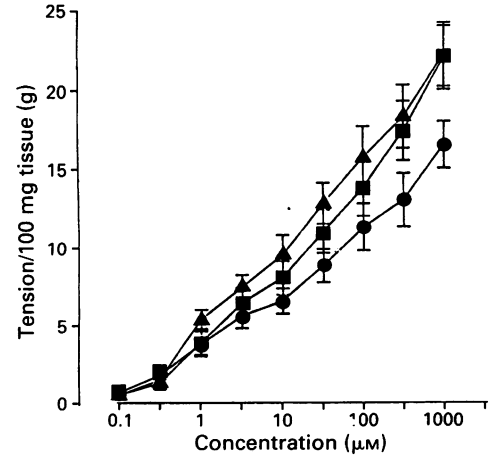

Figure 5 Concentration-response curves to acetylcholine (ACh) in detrusor strips from 12 week sorbinil-treated diabetic (▲), untreated diabetic (■) and non-diabetic control (●) rats. All values are means ($n = 6$) and represent the tension developed by 100 mg of bladder tissue. There was no significant difference between the three curves at any concentration of ACh.

findings of the present investigation. There are sound reasons to believe that the observed impairment of the non-cholinergic motor transmission in the diabetic bladder might have a prejunctional mechanism, as opposed to a postjunctional disturbed state of detrusor contractility. Our results indicate that the sensitivity of the detrusor to the spasmogenic action of ACh, a physiological spasmogen in this tissue, was not diminished in diabetic animals (ED_{50} for ACh was 4.02×10^{-5} M in controls and 3.51×10^{-5} M in diabetics). Nor can the impaired response to EFS in diabetic animals be attributed to a reduction in the tension generating capacity of the detrusor. The peak tension generated by the detrusor in response to ACh was greater in the diabetic animals. The impression that the STZ-induced diabetes does not adversely affect the contractility of the detrusor is also consistent with the observations of other workers in this field; Lincoln *et al.* (1984), Kolta *et al.* (1985) and Moss *et al.* (1987) also found no impairment in the ability of the detrusor from the STZ-diabetic rats to respond to ACh. A large body of evidence has accumulated over the years, in support of the original proposal by Burnstock and his colleagues that ATP might be the non-cholinergic motor transmitter in the mammalian urinary bladder (Burnstock *et al.*, 1972; 1978; Downie & Dean, 1977; Moss *et al.*, 1985). It is of considerable importance to recall that Longhurst & Belis (1986) found no statistically significant difference in the contractile responses of bladder strips to ATP between control and STZ-diabetic rats. If ATP is indeed the non-cholinergic transmitter, it would seem reasonable to conclude that the impairment of the non-cholinergic transmission observed in this investigation was prejunctional in origin and reflects a diminished release of the non-cholinergic (purinergic) transmitter in diabetic animals. A close examination of the time course of the response to EFS also appears to provide further corroborative evidence for this conclusion. The atropine-resistant response of the detrusor to 10 s bursts of EFS consisted of 3 phases: phase 1 was a sharp rise in tension lasting about 2 s; phase 2 was the plateau phase during which the tension remained stable. Its duration was dependent on the stimulation-frequency and was most prolonged at 1 Hz and least at 10 Hz. Phase 3 followed the plateau phase and was characterized by a gradual decline in tension despite continuing stimulation. EFS at 1 Hz produced a response in which phase 3 was missing while phase 2 was very prolonged. With increasing frequency the duration of phase 2 declined while phase 3 became more prominent. Thus the peak tension could be maintained at 100% throughout the duration of stimulation at 1 Hz whereas the tension towards the end of the stimulation period at 10 Hz was less than 35% of the peak value (Figure 2). Since the duration of the stimulus period remained constant at 10 s for both 1 Hz and 10 Hz stimulation schedules, the main difference between the two schedules lay in the total number of pulses delivered i.e. 10 at 1 Hz and 100 at 10 Hz. Although in theory it is possible that the failure to sustain the tension at the higher frequencies of EFS was in part, or wholly attributable to a decline in postjunctional sensitivity to the non-cholinergic transmitter, the complete absence of the occurrence of the fade at 1 Hz, indicates the improbability of this explanation being correct. It therefore seems attractive to suggest that the fade at the higher frequencies was caused by the high demand on releasable transmitter stores leading to a prejunctional failure to maintain an undiminished non-cholinergic transmitter release. Given this scenario, it is highly relevant to find that the failure to maintain tension at higher frequencies was significantly greater in diabetic animals, thus supporting the notion that a prejunctional mechanism was involved in the transmission failure following STZ-induced diabetes (Figure 2). This conclusion is in agreement with that of Santicioli *et al.* (1987) who also noted the reduced responses to field stimulation in STZ pretreated rats and ascribed this reduction, at least in part, to the ongoing diabetic neuropathy. They also made the important observation that unlike the diabetic animals, the bladder strips of sucrose-fed animals, having normal blood glucose

levels but a similar increase in urine production responded to EFS with normal or above normal contractions. Their results clearly show that prejunctional failure of transmission in diabetic animals is not a consequence of a saccharide (glucose)-led polyuria since another saccharide (sucrose)-led polyuria of comparable magnitude exerted no such effect.

Two further aspects of the transmission failure in diabetic rat detrusor merit some comments. First the reduced response to EFS was seen even at the lowest frequency of stimulation. Given that the failure is prejunctional, this finding implies that the transmitter output in response to even very short trains (10 pulses, 1 Hz) is reduced in diabetic animals. Secondly the degree of transmission failure bears a direct relationship to the duration of diabetes. Responses to field stimulation were most reduced in 12 week diabetic rats and least but still substantially in 4 week ones, suggesting that the process has a rapid onset and is progressive. Our findings of a reduced non-cholinergic neurogenic response in STZ-diabetic rats seem at first glance to run counter to those of Moss *et al.* (1987) who concluded that the atropine-resistant neurogenic response was increased in 8 week diabetic animals and was not significantly reduced in 16 week diabetic animals. Although no obvious reason for this discrepancy is evident, it may perhaps be attributable to the different experimental methodologies in the two investigations. Unlike the present investigation which was conducted on isolated detrusor strips, Moss *et al.* (1987) used *in vitro* whole bladder preparations. Another possible explanation may lie in the development of hypersensitivity of the postjunctional ATP receptors, noted by Moss *et al.*; ATP being the prime candidate for the role of the non-cholinergic transmitter in this tissue, the postjunctional hypersensitivity to ATP, developed as a consequence of diabetes-induced neuronal damage, might have served to mask the prejunctional transmission failure in the investigation of Moss *et al.* (1987).

The biochemical basis of diabetic autonomic neuropathy remains uncertain. There is considerable evidence for the suggestion that enhanced sorbitol concentration within nerves, secondary to diabetes, constitutes the prime factor in the induction of diabetic autonomic neuropathy. It has been known that peripheral nerves contain aldose reductase, the enzyme responsible for the conversion of glucose into sorbitol (Gabbay *et al.*, 1966). The activity of aldose reductase is dependent upon blood glucose concentration and is enhanced by hyperglycaemia (Gabbay, 1973). It has been hypothesised that hyperglycaemia-induced enhanced activity of aldose reductase in diabetes leads to accumulation of sorbitol within nerves which in turn directly or indirectly causes structural damage of the nerves and produces the neuropathy (Green *et al.*, 1985). If indeed excessive sorbitol accumulation within nerves on account of diabetes, is detrimental to nerve function, the inhibition of aldose reductase in diabetic subjects should result in an improvement of neuropathy by effecting a fall in intraneuronal sorbitol concentration. In diabetic rats, the aldose reductase inhibitor, sorbinil has been shown to enhance axonal transport (Tomlinson *et al.*, 1984) and to improve motor nerve conduction velocity (Yue *et al.*, 1982). In human diabetics, sorbinil has been found to improve motor and sensory nerve conduction velocities (Judzewitsch *et al.*, 1983) and to ameliorate painful diabetic neuropathy (Jaspan *et al.*, 1983). In our experiments the tension generated by the detrusor from the sorbinil-treated diabetic animals in response to EFS was significantly greater than the corresponding value from tissues obtained from diabetic rats not treated with sorbinil. Since the response to ACh was not affected by sorbinil treatment, indicating a lack of effect on detrusor contractility, the finding that treatment of diabetic animals with sorbinil partially prevented the failure of non-cholinergic motor transmission, is consistent with a prejunctional (neuronal) basis of the failure and lends support to the sorbitol accumulation hypothesis of diabetic autonomic neuropathy.

An intriguing question arising out of the results of the present investigation concerns the applicability of these findings to human diabetes mellitus. Occurrence of bladder dysfunction in human diabetes is widespread and its incidence high (Faerman *et al.*, 1971). Also, an excellent correlation exists between the incidence of bladder dysfunction and of peripheral neuropathy in diabetic patients indicating a neuronal origin of the dysfunction in man as well (Frimodt-Moller, 1980). The findings of the present investigation relate solely to the non-cholinergic motor transmission in diabetic and non-diabetic rats. Although early reports have cast doubt on the presence of a non-cholinergic component in the motor transmission of the human bladder (Nergardh & Kinn, 1983; Sibley, 1984; Kinder & Mundy, 1985), two more recent studies have provided strong evidence for its presence in human detrusor (Hoyle *et al.*, 1989; Luheshi & Zar, 1990). Hoyle *et al.* (1989) have demonstrated not only the presence of a non-adrenergic, non-cholinergic element of neuromuscular transmission in the human detrusor but also that this component is purinergic. Luheshi & Zar (1990) have additionally given a valid explanation for the negative findings of the earlier workers. In the light of the foregoing observations, it seems reasonable to expect that the bladder dysfunction in human diabetics may in part be attributable to impaired non-cholinergic motor transmission. It would be interesting to know whether administration of an aldose reductase inhibitor to human diabetics exerts a beneficial effect on bladder function as it does in diabetic rats.

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References

AMBACHE, N. & ZAR, M.A. (1970). Non-cholinergic transmission by post-ganglionic motor neurons in the mammalian bladder. *J. Physiol.*, **210**, 761–783.

BUCK, A.C., McRAE, C.U., REED, P.I. & CHISLOM, G.D. (1974). The diabetic bladder. *Proc. R. Soc. Med.*, **67**, 81–83.

BURNSTOCK, G., DUMSDAY, B. & SMYTHE, A. (1972). Atropine resistant excitation of the urinary bladder: the possibility of transmission via nerves releasing a purine nucleotide. *Br. J. Pharmacol.*, **44**, 451–461.

BURNSTOCK, G., COCKS, T., CROWE, R. & KASAKOV, L. (1978). Purinergic innervation of the guinea-pig urinary bladder. *Br. J. Pharmacol.*, **63**, 125–138.

CLEMENTS, R.S. (1979). Diabetic neuropathy: New concepts of its etiology. *Diabetes*, **28**, 604–611.

DOWNIE, J.W. & DEAN, D.M. (1977). The contribution of cholinergic postganglionic neurotransmission to contractions of rabbit detrusor. *J. Pharmacol. Exp. Ther.*, **203**, 417–425.

ELLENBERG, M. (1980). Development of urinary bladder dysfunction in diabetes mellitus. *Ann. Intern. Med.*, **92**, 321–323.

FAERMAN, I., MALER, M., JADZINSKY, M., FOX, D., ALVAREZ, E., ZILVERBAG, J., CIBEIRA, J.B. & COLINAS, R. (1971). A symptomatic neurogenic bladder in juvenile diabetics. *Diabetologia*, **7**, 168–172.

FRIMODT-MOLLER, C. (1980). Diabetic cystopathy. *Ann. Int. Med.*, **92**, 318–321.

GABBAY, K.H. (1973). The sorbitol pathway and the complications of diabetes. *N. Eng. J. Med.*, **288**, 831–836.

GABBAY, K.H., MEVOLA, L.O. & FIELD, R.A. (1966). Sorbitol pathway: Presence in nerve and cord with substrate accumulation in diabetes. *Science*, **151**, 209–210.

GREEN, D.A., LATTIMER, S., ULBRECHT, J. & CARROLL, P. (1985). Glucose-induced alterations in nerve metabolism: current perspective on the pathogenesis of diabetic neuropathy and future directions for research and therapy. *Diabetes Care*, **8**, 290–299.

HOYLE, C.H.V., CHAPPLE, C. & BURNSTOCK, G. (1989). Isolated human bladder: evidence for an adenine dinucleotide acting on P_{2X}-purinoceptors and for purinergic transmission. *Eur. J. Pharmacol.*, **174**, 115–118.

JASPAN, J., MASELLI, R., HEROLD, K. & BARTKINS, C. (1983). The treatment of severely painful diabetic neuropathy with an aldose reductase inhibitor: relief of pain and improved somatic and automatic nerve function. *Lancet*, **ii**, 758–762.

JUDZEWITSCH, R.G., JASPAN, J.B., POLONSKY, K.S., WEINBERG, C.R., HALTER, J.B., HALAR, E., PFEIFER, M.A., VUKADINOVIC, C., BERNSTEIN, L., SCHNEIDER, M., LIANG, K.Y., GABBAY, K.H.,

RUBENSTEIN, A.H. & PORTE, D. Jr. (1983). Aldose reductase inhibition improves nerve conduction velocity in diabetic patients. *N. Engl. J. Med.*, **308**, 119–125.

KINDER, R.B. & MUNDY, A.R. (1985). Atropine blockade of nerve-mediated stimulation of the human detrusor. *Br. J. Urol.*, **57**, 418–421.

KOLTA, M.G., WALLACE, L.J. & GERALD, M.C. (1985). Streptozotocin-induced diabetes affects rat urinary bladder response to autonomic agents. *Diabetes*, **34**, 917–921.

LINCOLN, J., CROCKETT, M., HAVEN, A.J. & BURNSTOCK, G. (1984). Rat bladder in the early stages of streptozotocin-induced diabetes: adrenergic and cholinergic innervation. *Diabetologia*, **26**, 81–87.

LONGHURST, P.A. & BELIS, J.A. (1986). Abnormality of rat bladder contractility in streptozotocin-induced diabetes mellitus. *J. Pharmacol. Exp. Ther.*, **238**, 773–777.

LUHESHI, G.N. & ZAR, M.A. (1990). Presence of non-cholinergic motor transmission in isolated human bladder. *J. Pharm. Pharmacol.*, **42**, 223–224.

MAYER, J.H. & TOMLINSON, D.R. (1983). The influence of aldose reductase inhibition and nerve myoinositol on axonal transport and nerve conduction velocity in rats with experimental diabetes. *J. Physiol.*, **340**, 25–26.

MAYHEW, J.A., GILLON, K.R.W. & HAWTHORNE, J.N. (1983). Free and lipid inositol, sorbitol and sugars obtained post mortem from diabetic and control subjects. *Diabetologia*, **24**, 13–15.

MOSS, H.E. & BURNSTOCK, G. (1985). A comparative study of electrical field stimulation of the guinea-pig, ferret and marmoset urinary bladder. *Eur. J. Pharmacol.*, **114**, 311–316.

MOSS, H.E., LINCOLN, J. & BURNSTOCK, G. (1987). A study of bladder dysfunction during streptozotocin-induced diabetes in the rat using an in vitro whole bladder preparation. *J. Urol.*, **138**, 1279–1284.

NERGARDH, A. & KINN, A-C. (1983). Neurotransmission in activation of the contractile response in the human urinary bladder. *Scand. J. Urol. Nephrol.*, **17**, 153–157.

SANTICIOLI, P., GAMSE, R., MAGGI, C.A. & MELI, A. (1987). Cystometric changes in the early phase of streptozotocin-induced diabetes in rats: evidence for sensory changes not correlated to diabetic neuropathy. *Naunyn-Schmiesdebergs Arch. Pharmacol.*, **335**, 580–587.

SIBLEY, G.N.A. (1984). A comparison of spontaneous and nerve-mediated activity in bladder muscle from man, pig and rabbit. *J. Physiol.*, **354**, 431–443.

TAIRA, N. (1972). The autonomic pharmacology of the bladder. *Ann. Rev. Pharmacol.*, **12**, 197–208.

TOMLINSON, D.R., MORIARTY, R.J. & MAYER, J.H. (1984). Prevention and reversal of defective axonal transport and motor nerve conduction velocity in rats with experimental diabetes by treatment with the aldose reductase inhibitor sorbinil. *Diabetes*, **33**, 470–476.

YUE, D.K., HANWELL, M.A., SATCHELL, P.M. & TURTLE, J.R. (1982). The effect of an aldose reductase inhibitor on motor nerve conduction velocity in diabetic rats. *Diabetes*, **31**, 789–794.

ZAR, M.A., IRAVANI, M.M. & LUHESHI, G.N. (1990). Effect of nifedipine on the contractile responses of the isolated rat bladder. *J. Urol.*, **143**, 835–839.

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Kinins and peritoneal exudates induced by carrageenin and zymosan in rats

[1]Jacques Damas, Victor Bourdon, Gaby Remacle-Volon & *Albert Adam

Physiologie humaine, Université de Liège, 17 Place Delcour, B-4020 Liège, Belgium and *Faculté de Pharmacie, Université de Montreal, Quebec, Canada

1 Kinins were measured by a radioimmunoassay in the inflammatory exudates induced by carrageenin or zymosan in the peritoneal cavity of normal Wistar rats and of kininogen-deficient Brown Norway rats.

2 After administration of carrageenin to normal rats, levels of immunoreactive kinins showed a single peak during the first two hours and then decreased. The presence of kinins preceded and accompanied the exudation of ^{125}I-labelled albumin. Kinins were identified as bradykinin by chromatography.

2 Captopril, an inhibitor of kininase 2, increased the level of kinins and the volume of the exudates after carrageenin treatment. In Brown Norway rats, the volume of the exudates was small and contained little or undetectable amounts of immunoreactive kinins.

4 During zymosan-induced peritonitis, the exudates were devoid of immunoreactive kinins in both species. The volume of the exudates was larger in kininogen-deficient rats than in normal rats.

5 We conclude that in rats, the kinin system is a major factor responsible for the development of the inflammatory reactions induced by carrageenin, but is not involved in the reactions induced by zymosan.

Introduction

Since their first description by Winter *et al.* (1962), the inflammatory reactions induced in rats by carrageenin are considered the conventional model to detect anti-inflammatory drugs. The presence of kinins, endogenous pro-inflammatory peptides, in the fluids withdrawn from carrageenin-induced inflammatory reactions has been demonstrated in a few studies (Willis, 1969; Capasso *et al.*, 1975; Barlas *et al.*, 1985; Kumakura *et al.*, 1988; Katori *et al.*, 1989; Adam *et al.*, 1989), but there is much circumstantial evidence which indicates that kinins could play a major role in the development of these inflammatory reactions (reviewed in Damas & Remacle-Volon, 1981). Several attempts have been made to develop other inflammatory models. Among these attempts, cobra venom factor or zymosan were used to induce complement-dependent inflammatory reactions in the skin (Konno & Tsurufuji, 1983), paws (Gemmel *et al.*, 1979; Leyck *et al.*, 1983; Damas & Remacle-Volon, 1986; Damas *et al.*, 1986), and the pleural (Martins *et al.*, 1989) or abdominal cavities of rats (Wood *et al.*, 1988; Griffiths & Wood, 1989). To compare both models, we examined the peritonitis induced by zymosan and by carrageenin. Though carrageenin-induced pleurisy has been widely described, the peritonitis induced by this polysaccharide has not yet been studied. For this reason, we examined the presence of immunoreactive kinins in the peritoneal exudates induced by carrageenin and zymosan and the involvement of the kinin system in these inflammatory reactions.

Methods

Wistar rats were used as normal controls. The kininogen-deficient Brown Norway rats (BN/May Pfd) were bred by the farm of the Catholic University of Leuven (Heverlee, Belgium). The mean weight of the animals was 240 g. The plasma of our Brown Norway rats is devoid of high and low molecular weight kininogens but contains T-kininogen or alpha-1-cysteine proteinase inhibitor (Hayashi *et al.*, 1984; Damas & Adam, 1985).

The animals were treated with an intraperitoneal injection of either 5 ml lambda carrageenin solution (1% in saline), or 5 ml zymosan suspension (1% in saline) or 5 ml physiological saline. At various times thereafter, they were anaesthetized with ether and exsanguinated. In some instances, blood was taken by cardiac puncture. The abdomen was opened and the fluid withdrawn with a plastic syringe. The 1st ml was immediately mixed with 4 ml cold ethanol. After 30 min, the mixture was centrifuged at 2500 *g* for 15 min. The supernatant was lyophilized and stored at −25°C until assay of the kinins. The other fractions of peritoneal exudates were mixed with 0.1 vol. 3.8% trisodium citrate and used for protein determination and leucocyte counting. Any peritoneal fluid containing blood was discarded.

In other rats, lambda carrageenin (0.1 ml; 1%) was injected in the plantar region of the right hind paw and a control injection of saline was performed in the contralateral paw. At various times thereafter, the animals were anaesthetized with ether and the paws cut at the ankle. The swelling was calculated as a percentage increase in the weight of the carrageenin-injected paw compared to the weight of the control paw.

For studies of plasma exudation in peritoneal cavities or in paws, 1.0 μCi of ^{125}I-labelled human serum albumin mixed with 0.5 ml of saline was injected i.v. into the tail vein of the animals. Fifteen minutes later, the rats were anaesthetized with ether, a blood sample was withdrawn by cardiac puncture and mixed with 0.1 vol. 3.8% trisodium citrate, the peritoneal fluid was harvested or the paws cut. Blood samples were centrifuged at 1500 *g* for 10 min and the plasma was separated. The radioactivity of plasma samples, exudates or paws was counted in a gamma spectrometer (Packard Instrument Co.). The results were calculated for peritoneal exudates as μl of plasma extravasated into the peritoneal cavity during the 15 min period, and for the swelling of the paws by the ratio: radioactivity of carrageenin-treated paw minus radioactivity of saline-treated paw/radioactivity of saline-treated paw.

Kinin levels in exudates were measured by a specific radioimmunoassay (RIA) already described (Adam *et al.*, 1989) with the slight modification that the separation of bound and free labelled peptide was performed with 0.4 ml of incubation buffer $KH_2PO_4/Na_2HPO_4 \cdot 2H_2O$ 200 mM, pH 7.5 containing charcoal (5 g l^{-1}), and dextran sulphate (0.5 g l^{-1}). Samples were assayed in duplicate. The RIA was standardized against bradykinin, but Ile-Ser-bradykinin, kallidin and des-Arg^9-bradykinin showed complete cross-immunoreactivity on an equimolar basis. In some cases, kinins in exudates were also separated by a reversed phase high performance liquid chromatography (h.p.l.c.) prior to RIA (Damas *et al.*, 1989).

[1] Author for correspondence.

After dilution of the exudates with saline and incubation with trypsin (0.5 mg ml^{-1}) for 30 min at 37°C, a total cell count was performed by phase contrast microscopy using a Thoma's plate. Total exudated protein was quantified by the Folin-Ciocaltaeu reagent as described by Lowry *et al.* (1951). The haematocrit was measured by means of capillary tubes centrifuged in a Haemofuge (Heraeus).

Bradykinin and kallidin were purchased from UCB (Brussels, Belgium), T-kinin, des-Arg^9-bradykinin, trypsin, zymosan and lambda carrageenin from Sigma (St Louis, MI, U.S.A.). ^{125}I-labelled serum albumin and bradykinin (8-tyrosine)-triacetate were from New England Nuclear (Boston, MA, U.S.A.). Captopril was a gift from Squibb (Brussels, Belgium).

Results are expressed as the mean ± s.e.mean. The data were evaluated by Student's unpaired *t* test and a *P* value less than 0.05 was regarded as significant.

Results

Carrageenin-induced peritonitis

Physiological saline injected into the peritoneal cavity was slowly reabsorbed (Figure 1). Similarly, in normal Wistar rats, 1 h after the administration of the carrageenin solution, the volume of fluid that we could withdraw from the peritoneal cavity was relatively small, in part because carrageenin stuck to the intestinal wall. Thereafter, an exudate developed (Figure 1), the delayed appearance being corroborated by changes in haematocrit. During the first hour, the haematocrit diminished from 38.4 ± 0.8% ($n = 7$) to 35.6 ± 0.8% ($n = 9$; $P < 0.02$), indicating a plasma dilution. During the following hours, the haematocrit returned to control values: 37.8 ± 0.9% ($n = 7$) at 2 h, 38.0 ± 1.2% ($n = 8$) at 4 h and 39.2 ± 1.6% ($n = 7$) at 6 h. This gradual increase indicates a secondary plasma extravasation. Indeed, after carrageenin injection, extravasation of ^{125}I-labelled albumin was slight at 1 h, but was maximal at 2 h. It then decreased over the next 4 h but was still higher than control values (Figure 1). The protein content of carrageenin-induced exudates was significantly increased after 1 h in Wistar rats and raised during the following hours while the leucocyte content was only increased at 6 h (Table 1).

In Wistar rats, the levels of immunoreactive kinins in carrageenin-exudates were significantly elevated during the first two hours (Table 1). Maximum values were recorded after 1 h. Kinins present in samples withdrawn during this time were identified by h.p.l.c. as bradykinin ($n = 6$).

In order to demonstrate further the involvement of the kinins in this inflammatory reaction, two means were used. First, captopril (20 μg ml^{-1}), a kininase 2 inhibitor (Boura & Svolmanis, 1984), was injected with carrageenin. Captopril significantly increased the volume of the exudate withdrawn at 2 h and the level of immunoreactive kinins (Figure 2).

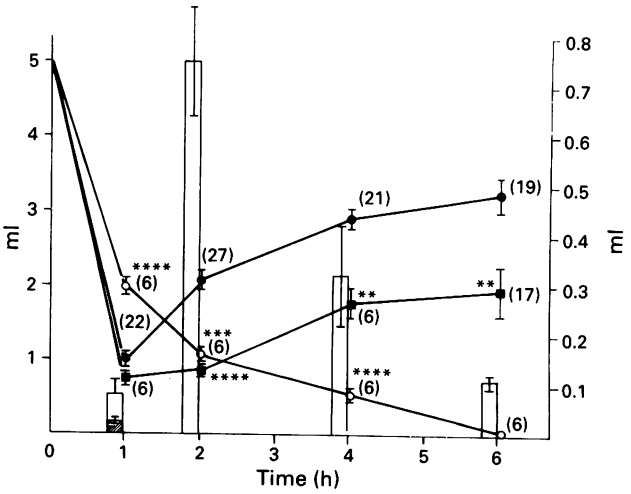

Figure 1 Volume of the peritoneal fluid and exudation of ^{125}I-labelled albumin after injection of carrageenin or saline. Ordinate scales: on the left, volume (ml) of the peritoneal exudates withdrawn from normal Wistar rats (●) or from kininogen-deficient Brown Norway rats (■) or volume of fluid after injection of physiological saline in Wistar rats (○); on the right, albumin exudation (ml of plasma) after carrageenin (open columns) or after physiological saline (hatched column) in Wistar rats. Abscissa scale: time (h) after carrageenin or saline injection. Mean of (*n*) determinations for the volumes and 6 to 10 determinations for the exudation of albumin; s.e.mean shown by vertical bars. The statistical analysis was made by comparing the effects of carrageenin in Wistar rats versus saline or versus carrageenin in Brown Norway rats. ** $P < 0.02$; *** $P < 0.005$; **** $P < 0.001$.

Second, we examined the carrageenin-induced peritonitis in kininogen-deficient Brown Norway rats. In these animals, the development of the exudate was delayed and its volume was reduced compared to Wistar rats (Figure 1). The protein levels were significantly lower than in Wistar rats up to 2 h (Table 1). However, in both strains, the leucocyte content was similarly increased at 6 h. In Brown Norway rats, the kinin levels were lower than the assay limit of detection (0.05 ng). In these rats, captopril (20 μg ml^{-1}) had no influence on the volume of the exudates and their content of kinins.

Carrageenin-induced paw oedema

During carrageenin-induced peritonitis in normal Wistar rats, the maximum increase in vascular permeability was recorded

Table 1 Levels of proteins, leucocytes and kinins in peritoneal exudates induced by carrageenin in Wistar and Brown Norway (BN) rats

	Proteins (mg ml^{-1})		*Leucocytes* (10^3 μl^{-1})		*Kinins* (ng ml^{-1})
Delays	Wistar	BN	Wistar	BN	Wistar
0 h[a]	3.4 ± 0.4 (6)		4.7 ± 0.5 (8)		0.11 ± 0.04 (10)
1 h	8.6 ± 1.1** (12)	5.1 ± 0.4* (6)	2.9 ± 1.3 (6)	2.9 ± 0.3 (6)	1.16 ± 0.35* (11)
2 h	14.2 ± 0.7** (12)	8.1 ± 0.4** (6)	3.7 ± 1.3 (6)	4.6 ± 0.7 (7)	0.67 ± 0.12** (14)
4 h	22.6 ± 0.7** (13)	21.8 ± 2.7 (6)	13.5 ± 4.8 (6)	14.2 ± 2.9 (6)	0.32 ± 0.22 (14)
6 h	30.0 ± 2.1** (14)	27.1 ± 3.7 (7)	24.6 ± 7.7* (6)	33.3 ± 3.9 (7)	0.12 ± 0.04 (10)

[a] Protein, leukocyte and kinin contents of a peritoneal lavage with 5 ml physiological saline. Numbers in parentheses show the number of observations. The statistical analysis was made in Wistar rats as peritonitis versus peritoneal lavage on one hand and on the other hand Brown Norway rats versus Wistar rats. * $P < 0.05$; ** $P < 0.005$.

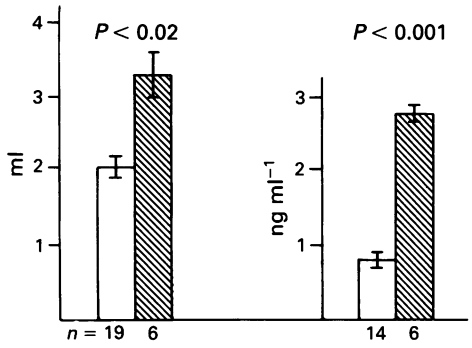

Figure 2 Effect of captopril on the volume of the exudates and their content of immunoreactive kinins after carrageenin injection in normal Wistar rats. Two hours after the intraperitoneal injection of carrageenin alone (open columns) or carrageenin plus captopril ($20\,\mu g\,ml^{-1}$) (hatched columns), the peritoneal exudates were harvested, their volumes (left scale, ml) and their levels of kinins (right scale, $ng\,ml^{-1}$) measured. Mean of n determinations; vertical bars show s.e.mean.

at 2h. Thus, the vascular permeability increase during carrageenin-induced paw oedema in normal Wistar rats was examined to compare the reactivity of the two vascular networks. Results (Figure 3) showed that the changes of vascular permeability in the paw had the same time course as in the peritoneal cavity, with maximum increases being recorded 2h after the injection of carrageenin.

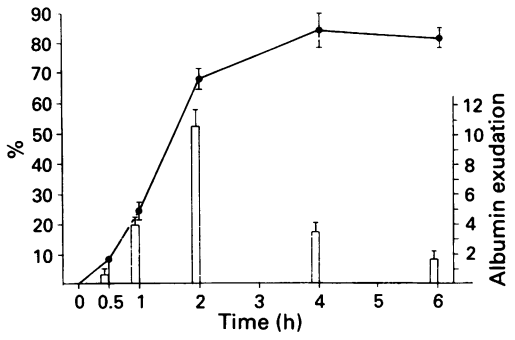

Figure 3 Swelling and exudation of ^{125}I-labelled albumin after injection of carrageenin in the paws of normal Wistar rats. The swelling (left hand scale) was calculated as the percentage increase in weight of the paw (●), the exudation of albumin (right hand scale) was calculated by the ratio: radioactivity of carragenin-treated paw minus radioactivity of saline-treated paw/radioactivity of saline-treated paw (columns). Mean of 6 determinations.

Zymosan-induced peritonitis

In Wistar rats, the volume of the exudates induced by zymosan progressively diminished but taking into account the reabsorption of physiological saline, the amount of the inflammatory fluids increased with time (Figure 4). A significant extravasation of ^{125}I-labelled albumin took place during the whole reaction, maximum values being recorded between 2 and 4h (Figure 4). The protein content of the exudates was increased from the first hour (Table 2). The leucocyte levels were difficult to determine because numerous clumps of agglutinated cells were often seen. Thus in order to perform leucocyte counts, we had to incubate the exudates with trypsin. In these conditions, we observed that the level of leucocytes were significantly raised from 2h. The levels of immunoreactive kinins were only elevated in a few samples (3/10) at 6h (Table 2).

In kininogen-deficient Brown Norway rats, the volume of the exudates induced by zymosan was significantly larger than

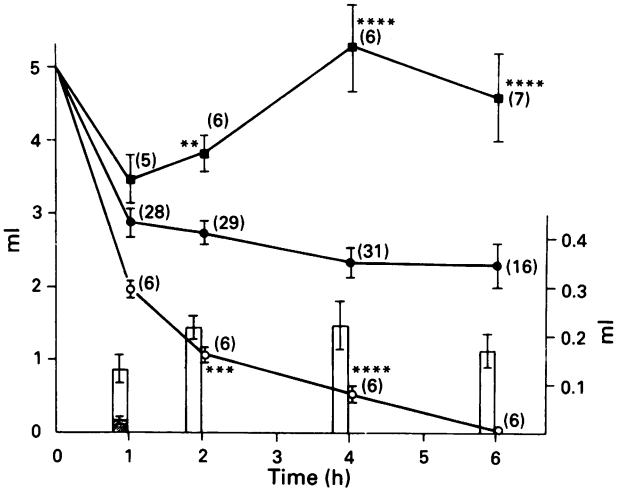

Figure 4 Volume of the peritoneal fluid and exudation of ^{125}I-labelled albumin after injection of zymosan or saline. Ordinate scales: on the left, volume (ml) of the peritoneal exudates withdrawn from normal Wistar rats (●) or from kininogen-deficient Brown Norway rats (■) or volume of fluid after injection of physiological saline in Wistar rats (○); on the right, albumin exudation (ml of plasma) after zymosan (open columns) or after physiological saline (hatched column) in Wistar rats. Abscissa scale: time (h) after zymosan or saline injection. Mean of (n) determinations for the volumes and 6 to 10 determinations for the exudation of albumin; vertical bars show s.e.mean. The statistical analysis was made by comparison of zymosan in Wistar rats versus saline or versus zymosan in Brown Norway rats. ** $P < 0.02$; *** $P < 0.005$; **** $P < 0.001$.

Table 2 Levels of proteins, leucocytes and kinins in peritoneal exudates induced by zymosan in Wistar and Brown Norway (BN) rats

	Proteins ($mg\,ml^{-1}$)		*Leucocytes* ($10^3\,\mu l^{-1}$)		*Kinins* ($ng\,ml^{-1}$)
Delays	Wistar	BN	Wistar	BN	Wistar
0h[a]	3.4 ± 0.4 (6)		4.7 ± 0.5 (8)		0.11 ± 0.04 (10)
1h	21.0 ± 1.7** (17)	17.1 ± 3.3 (6)	5.7 ± 0.6 (8)	10.7 ± 2.6 (6)	0.08 ± 0.06 (6)
2h	24.7 ± 1.4** (21)	19.8 ± 2.3 (6)	16.7 ± 3.3* (10)	29.4 ± 4.3* (7)	0.10 ± 0.05 (7)
4h	33.1 ± 1.6** (20)	25.1 ± 2.9* (6)	20.7 ± 3.7** (10)	49.0 ± 3.4** (6)	0.29 ± 0.16 (8)
6h	36.9 ± 1.5** (15)	28.7 ± 2.3** (6)	23.1 ± 3.2** (6)	68.9 ± 6.1** (6)	0.42 ± 0.19 (10)

[a] Protein, leucocyte and kinin contents of a peritoneal lavage with 5ml physiological saline. Numbers in parentheses show the number of observations. The statistical analysis was made in Wistar rats as peritonitis versus peritoneal lavage on one hand and on the other hand Brown Norway rats versus Wistar rats. * $P < 0.05$; ** $P < 0.005$.

in Wistar rats (Figure 4). The accumulation of leucocytes developed more quickly and reached higher levels than in Wistar rats and than during carrageenin-induced peritonitis (Table 2). The protein contents were smaller than in Wistar rats at 4 and 6 h. This could be explained by the larger volume of the exudates. In these exudates, we did not find any kinins at the various times of observation.

Discussion

Carrageenin-induced peritonitis developed slowly during the first hour in normal rats. The vascular permeability increase was maximum after 2 h. Similar features were observed during the development of paw oedema. The peritonitis thus gives a good general picture of the inflammatory reactions induced by carrageenin in rats. Previous workers (Di Rosa *et al.*, 1971; Garcia Leme *et al.*, 1972; Bolam *et al.*, 1974; Doherty & Robinson 1975; Zanin & Ferreira, 1978) sometimes reported quite different results concerning plasma exudation during carrageenin-induced oedema. However, according to Zanin & Ferreira (1978), the duration of plasma exudation increases with the dose of carrageenin. On the other hand, we injected purified lambda carrageenin, the most effective phlogistic agent of the various types of carrageenin and mixtures of these types were used in other studies (Moore & Trottier, 1974). Thus, these differences may be explained by the use of various doses or of different types of carrageenin (Damas & Remacle-Volon, 1986).

Since the observations of Di Rosa *et al.* (1971), several endogenous pro-inflammatory mediators have been proposed as responsible for the development of the inflammatory reactions induced by carrageenin. Our results indicate that the major part is probably played by kinins. Bradykinin was present in the exudate and prolongation of its half-life by captopril simultaneously increased the volume of the exudates and their content of kinins. The lack of kinin formation in Brown Norway rats prevented the development of the peritonitis, particularly during the two first hours but also during the late phase. Similar results have been obtained with paw oedema and pleurisy induced by this polysaccharide (Damas *et al.*, 1986; Oh-ishi *et al.*, 1987). Indeed, carrageenin activates *in vitro* the contact system of plasma, Hageman factor and plasma prekallikrein, activation which results in the release of bradykinin from high molecular weight kininogen (Schwartz & Kellermeyer, 1969). The time course of a measurable level of kinins during carrageenin-induced peritonitis was similar to that observed during other inflammatory reactions induced by the activators of Hageman factor (Hori *et al.*, 1988; Kumakura *et al.*, 1989; Katori *et al.*, 1989; Damas *et al.*, 1990). The formation of kinins preceded and accompanied the increase of vascular permeability. However, besides the major role played by the kinin system in these reactions, other factors are involved as shown by the small inflammatory responses occurring in kininogen-deficient rats.

After zymosan injection, the peritoneal accumulation of fluid started earlier, the vascular permeability increased more progressively and the accumulation of leucocytes occurred more quickly than after carrageenin. In zymosan-induced peritonitis, the volume of the exudates was also larger in kininogen-deficient rats than in normal rats. This latter observation would indicate first that the reduction of the responses to carrageenin in Brown Norway rats did not depend on a low reactivity of the blood vessels. It suggests also that the kinin system is not involved during zymosan-induced peritonitis. Indeed, the exudate did not contain significant levels of kinins except in a few cases (3/10). The dilution or the extravasation of plasma in inflammatory exudate thus did not result in a significant activation of the kinin system. The formation of kinins during inflammatory reactions would only depend on either the presence of activators of Hageman factor or on the release of tissue kininogenases (Proud & Kaplan, 1988). Inflammatory reactions could thus occur without the active participation of the kinin system. Similar conclusions were obtained from the comparison of zymosan-induced paw oedemas in normal and kininogen-deficient rats (Damas *et al.*, 1986).

Zymosan activates complement factors by the alternative pathway (Schorlemmer *et al.*, 1977). In rabbits, this activation results in inflammatory responses where the leucocytes played a prevailing role (Forrest *et al.*, 1986). In rats, the accumulation and aggregation of leucocytes during peritonitis suggest a similar stimulation of polymorphonuclear cells. Using indirect pharmacological means, Griffiths & Wood (1989) proposed two phases of development of this peritonitis, one of which was dependent on polymorphonuclear leucocytes. Similarly, paw oedema induced by zymosan was reduced by leucopenia (Damas *et al.*, 1986). The other phase could depend on the stimulation of resident peritoneal cells such as mast cells.

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References

ADAM, A., DAMAS, J., CALAY, G., RENARD, Cl., REMACLE-VOLON, G. & BOURDON, V. (1989). Quantification of rat T-kininogen using immunological methods. Application to inflammatory processes. *Biochem. Pharmacol.*, **38**, 1569–1575.

BARLAS, A., SUGIO, K. & GREENBAUM, L.M. (1985). Release of T-kinin and bradykinin in carrageenin induced inflammation in the rat. *FEBS Letters*, **190**, 268–270.

BOLAM, J.P., ELLIOTT, P.N.C., FORD-HUTCHINSON, A.W. & SMITH, M.J.H. (1974). Histamine, 5-hydroxytryptamine, kinins and the anti-inflammatory activity of human plasma fraction in carrageenin-induced paw oedema in the rat. *J. Pharm. Pharmacol.*, **26**, 434–440.

BOURA, A.L.A. & SVOLMANIS, A.P. (1984). Converting enzyme inhibition in the rat by captopril is accompanied by potentiation of carrageenin-induced inflammation. *Br. J. Pharmacol.*, **82**, 3–8.

CAPASSO, F., BALESTRIERI, B., DI ROSA, M., PERSICO, P. & SORRENTINO, L. (1975). Enhancement of carrageenin oedema by 1,10-phenanthroline and evidence for the bradykinin as endogenous mediator. *Agents Actions*, **5**, 359–363.

DAMAS, J. & ADAM, A. (1985). The relationship between kininogens and kallikreins in deficient Brown Norway rat plasma. *Mol. Physiol.*, **8**, 307–316.

DAMAS, J. & REMACLE-VOLON, G. (1981). Rôle des kinines dans l'oedème tégumentaire déclenché par les carragénines chez le rat. *J. Pharmacol. (Paris)*, **13**, 225–239.

DAMAS, J. & REMACLE-VOLON, G. (1986). Mast cell amines and the oedema induced by zymosan and carrageenins in rats. *Eur. J. Pharmacol.*, **121**, 367–376.

DAMAS, J., REMACLE-VOLON, G. & DEFLANDRE, E. (1986). Further studies of the mechanism of counter-irritation by turpentine. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **332**, 196–200.

DAMAS, J., ADAM, A., BOURDON, V. & REMACLE-VOLON, G. (1989). Presence of T-kininogen and kinins in sponge-induced exudates in rats. *Br. J. Pharmacol.*, **97**, 1343–1349.

DAMAS, J., BOURDON, V., REMACLE-VOLON, G. & ADAM, A. (1990). Proteinase inhibitors, kinins and the inflammatory reaction induced by sponge implantation in rats. *Eur. J. Pharmacol.*, **175**, 341–346.

DI ROSA, M., GIROUD, J.P. & WILLOUGHBY, D.A. (1971). Studies on the mediators of the acute inflammatory response induced in rats in different sites by carrageenin and turpentine. *J. Pathol.*, **104**, 15–29.

DOHERTY, N.S. & ROBINSON, B.V. (1975). The inflammatory response to carrageenin. *J. Pharm. Pharmacol.*, **27**, 701–703.

FORREST, M.J., JOSE, P.J. & WILLIAMS, T.J. (1986). Kinetics of the generation and action of chemical mediators in zymosan-induced inflammation of the rabbit peritoneal cavity. *Br. J. Pharmacol.*, **89**, 719–730.

GARCIA LEME, J., HAMAMURA, L., LEITE, M.P. & ROCHA E SILVA, M. (1973). Pharmacological analysis of the acute inflammatory

process induced in the rat's paw by local injection of carrageenin and by heating. *Br. J. Pharmacol.*, **48**, 88–96.

GEMMEL, D.K., COTTNEY, J. & LEWIS, A.J. (1979). Comparative effects of drugs on four paw oedema models in the rat. *Agents Actions*, **9**, 107–116.

GRIFFITHS, R.J. & WOOD, B.E. (1989). Zymosan induces PMN-dependent and independent inflammatory responses in the rat. *Br. J. Pharmacol.*, **96**, 39P.

HAYASHI, I., INO, T., KATO, H., IWANAGA, S., NAKANO, T. & OH-ISHI, S. (1984). Demonstration of the third kininogen in high and low molecular weight kininogens-deficient Brown Norway katholiek rat. *Thrombosis Res.*, **36**, 509–516.

HORI, Y., JYOYAMA, H., YAMADA, K., TAKAGI, M., HIROSE, K. & KATORI, M. (1988). Time course analyses of kinins and other mediators in plasma exudation of rat kaolin-induced pleurisy. *Eur. J. Pharmacol.*, **152**, 235–245.

KATORI, M., MAJIMA, M., HARADA, Y. & UENO, A. (1989). A significant role of plasma kallikrein-kinin system in plasma exudation of rat carrageenin-induced pleurisy. In *Advances in Experimental Medicine and Biology*. ed. Abe, K., Moriya, H. & Fuji, S. vol. 247, pp. 137–144. New York, London: Plenum Press.

KONNO, S. & TSURUFUJI, S. (1983). Induction of zymosan-air-pouch inflammation in rats and its characterization with reference to the effects of anticomplementary and anti-inflammatory agents. *Br. J. Pharmacol.*, **80**, 269–277.

KUMAKURA, S., KAMO, I. & TSURUFUJI, S. (1988). Role of bradykinin in the vascular permeability response induced by carrageenin in rats. *Br. J. Pharmacol.*, **93**, 739–746.

LEYCK, S., ETSCHENBERG, E., HADDING, U. & WINKELMANN, J. (1983). A new model of acute inflammation: cobra venom factor induced paw oedema. *Agents Actions*, **13**, 437–438.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

MARTINS, M.A., e SILVA, P.M.R., CASTRO FARIA NETO, H.C., BOZZA, P.T., DIAZ, P.M.L.F., LIMA, C.M., CORDEIRO, R.S.B. & VARGAFTIG, B.B. (1989). Pharmacological modulation of Paf-induced rat pleurisy and its role in inflammation by zymosan. *Br. J. Pharmacol.*, **96**, 363–371.

MOORE, E. & TROTTER, R.W. (1974). Comparison of various types of carrageenin in promoting pedal edema in the rat. *Res. Commun. Chem. Pathol. Pharmacol.*, **7**, 625–628.

OH-ISHI, S., HAYASHI, I., UTSUNOMIYA, I., HAYASHI, M., YAMAKI, K., YAMASU, A. & NAKANO, T. (1987). Roles of kallikrein-kinin system in acute inflammation: studies on high- and low-molecular weight kininogens-deficient rats (B/N-Katholiek strain). *Agents Actions*, **21**, 384–386.

PROUD, D. & KAPLAN, A.P. (1988). Kinin formation: mechanisms and role in inflammatory disorders. *Ann. Rev. Immunol.*, **6**, 49–83.

SCHORLEMMER, H.U., EDWARDS, J.H., DAVIES, P. & ALLISON, A.C. (1977). Macrophage responses to mouldy hay dust, Micropolyspora faeni and zymosan, activators of complement alternative pathway. *Clin. Exp. Immunol.*, **27**, 198–207.

SCHWARTZ, H.J. & KELLERMEYER, R.W. (1969). Activation of Hageman factor by carrageenin and its possible significance. *Proc. Soc. Exp. Biol. Med.*, **132**, 1021–1024.

WILLIS, A.L. (1969). Release of histamine, kinin and prostaglandin during carrageenin-induced inflammation of the rat. In *Prostaglandins, Peptides and Amines*. ed. Mantegazza, P. & Horton, E.W. pp. 31–38. London: Academic Press.

WINTER, C.A., RISLEY, E.A. & NUSS, G.W. (1962). Carrageenin-induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. *Proc. Soc. Exp. Biol. Med.*, **111**, 544–547.

WOOD, B.E., GRIFFITHS, R.J., LI, S.W., BLACKHAM, A. & ENTWHISTLE, N. (1988). Effects of phenidone, indomethacin and dexamethasone on zymosan induced peritoneal inflammation in the rat. *Br. J. Pharmacol.*, **94**, 416P.

ZANIN, M.T. & FERREIRA, S.H. (1978). Relationship between oedema and plasma exudation in rat paw carrageenin inflammation. *Agents Actions*, **8**, 606–609.

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The effect of capsaicin on voltage-gated calcium currents and calcium signals in cultured dorsal root ganglion cells

D. Bleakman, J.R. Brorson & R.J. Miller
Dept. of Pharmacological and Physiological Sciences, University of Chicago, 947 E. 58th Street, Chicago, Illinois 60637, U.S.A.

1 The effects of capsaicin on voltage-gated Ca^{2+} currents (I_{Ca}), and intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in cultured dorsal root ganglion (DRG) neurones of the rat were examined *in vitro* by use of combined patch clamp-microfluorometric recordings.

2 Under voltage-clamp conditions, capsaicin (0.1–10 μM) caused a concentration-dependent decrease in the magnitude of the I_{Ca}, an elevation in the holding current (I_h) and a concomitant rise in the $[Ca^{2+}]_i$ in most cells examined. Repeated application of capsaicin produced marked desensitization.

3 Some decrease in the I_{Ca} produced by capsaicin was also observed when the rise in $[Ca^{2+}]_i$ was buffered with EGTA or BAPTA and when Ba^{2+} was used as the charge carrier; under these conditions the desensitization previously observed was smaller.

4 The decrement in voltage-gated current was smaller in Ba^{2+} containing solutions than in Ca^{2+} containing solutions suggesting that the capsaicin-induced influx of Ca^{2+} partially mediated the observed decrease in the voltage-gated current. In cells which showed a marked response to capsaicin an outward (positive) current was sometimes observed upon depolarization from −80 to 0 mV. This effect was consistent with an outward movement of cations through the capsaicin conductance pathway which may also account, in part, for the apparent reduction in I_{Ca} by capsaicin.

5 The effects of capsaicin under voltage-clamp conditions were prevented by ruthenium red (1 μM).

6 Under current clamp conditions, capsaicin depolarized and caused a rise in $[Ca^{2+}]_i$ in the majority of DRG cells examined. Both of these effects could be prevented by ruthenium red (500 nM).

7 It is concluded that capsaicin reduces the I_{Ca} of rat DRG neurones primarily by indirect mechanisms.

Introduction

Capsaicin (8-methyl-N-vanillyl-6-nonemamide) is the pungent component of peppers and similar plants which has a variety of effects on sensory neurones (see review by Bevan *et al.*, 1987). Capsaicin has been shown to cause the release and depletion of neuropeptides (Gamse *et al.*, 1981; Fitzgerald, 1983, review), cell excitation and fibre discharge (Foster & Ramage, 1981), and neuronal cell death at high concentrations (Jancso *et al.*, 1985). In rat dorsal root ganglion cells (DRG), capsaicin has been shown to cause cell depolarization (Heyman & Rang, 1985) and increased cell excitability (Baccaglini & Hogan, 1983) as well as an increase in the accumulation of ^{45}Ca (Wood *et al.*, 1988). When using relatively low concentrations of capsaicin (<10 μM), these responses seem to be restricted to the C-type sensory cells responsible for the transmission of nociceptive information (Szolcsanyi, 1983). In this respect, capsaicin is an important tool for investigating the mechanism by which noxious stimuli are detected and sensory information transmitted. The inorganic dye, ruthenium red (RR), which is an inhibitor of mitochondrial and Ca^{2+} transport (Moore, 1971; Swanson *et al.*, 1974), has also recently been shown to antagonize capsaicin-dependent increases in ^{45}Ca uptake (Wood *et al.*, 1988), transmitter release (Maggi *et al.*, 1989), and primary afferent neurone excitation (Amann *et al.*, 1989). In the present study we have examined the effect of capsaicin on both Ca^{2+} (I_{Ca}) currents and associated Ca^{2+} concentration signals ($[Ca^{2+}]_i$) in cultured DRG cells of the rat. In particular we have observed a capsaicin-dependent decrease in I_{Ca} in these cells and have investigated the possible mechanisms underlying this response. Our results help to define some of the properties of the capsaicin-induced conduction pathway in these cells.

Methods

Instrumentation

The methods used for this study have been described previously in detail (Thayer *et al.*, 1988b). Briefly, for excitation of the fluorescent Ca^{2+} probe fura-2 (pentapotassium salt), a collimated beam of light from a 200 W Hg arc lamp was passed through a dual spectrophotometer (Phoenix Instruments) which alternated wavelengths from 340 to 380 nm by means of a spinning chopper (60 Hz). The light source was placed outside a darkened Faraday cage which enclosed the vibration isolation table supporting a microscope. A fused silica lens was positioned to focus light upon a liquid light guide (3 mm × 1 mm, Oriel, Stratford, CT, U.S.A.) and a similar lens, placed at the terminating end of the guide, was positioned to direct light through the epifluorescent illuminator of the microscope. The light guide eliminated problems associated with the vibration from the chopper and electrical noise from the arc lamp. The light was reflected off a dichroic mirror (Nikon, DM 400) and focused through a 70 × oil immersion objective (E. Leitz Inc., Rockleigh, NJ, USA, numerical aperture 1.15). The emission fluorescence was selected for wavelengths with a 480 nm barrier filter and recordings were spatially defined with an adjustable rectangular diaphragm. The fluorescence emission was analyzed with a photomultiplier tube (bialkali) and discriminator (APED II; Thorn EMI Gencom Inc., Plainview, NY, U.S.A.). The discriminator output was converted to pulses which were then integrated by passing the signal through an 8 pole Bessel filter at 500 Hz. The gain on this detection system could be adjusted from 1 to 100 fold by increasing the pulse length. The conversion of light intensity to voltage by this process was confirmed to be linear over the range of the light levels used in these experiments. The signal from the filter was fed into one channel of an analogue to digital convertor (PDP-11/73) computer system (Index Systems, Sunnyvale, CA). The signals from two photodiodes, each placed in a small portion of the light beam directed toward the monochromators, were connected to two additional channels of the analogue to digital convertor.

Sorting the fluorescence output into signals corresponding to excitation at these two wavelengths was performed entirely by software written in BASIC-23 (Cheshire Data, Indec Systems). The photomultiplier output was sorted into signals from 340 and 380 nm excitation by use of the photodiode outputs as timing signals.

Cover slips (25 mm diameter), plated with cells, were mounted in the continuous perfusion chamber (see Thayer et al., 1988a) which was positioned on the opening of the microstage. The solution change in the cell superfusion system approximated a step occurring between 10–20 s. The tubing between the large media reservoirs and the inlet to the chambers delayed the onset of the solution change by an additional 10 s. For experiments which did not involve electrical recordings, the cells were loaded with fura-2 acetoxymethyl ester (fura-2 AM) (5 μM) for 30 min at 37°C and unloaded for 40 min at the same temperature. For the digital imaging experiments, preloaded and washed fura-2 AM loaded cells were imaged according to the methods previously described (Glaum et al., 1990).

Calibration and analysis

Records were corrected for experimentally determined background values and the ratio of 340/380 nm fluorescence calculated off-line. Ratios were converted to free Ca^{2+} by the equation, $Ca^{2+} = K(R - R_{min})/(R_{max} - R)$ in which R is the 340/380 nm fluorescent ratio (Grynkiewicz et al., 1985). The maximum ratio (R_{max}), the minimum ratio (R_{min}) and the constant K, the product of the dissociation product for fura-2 and the ratio of the free and bound forms of the dye at 380 nm, were determined from a fit to a standard curve by use of the above equation with a non-linear least squares analysis computer programme. The standard curve was determined for the fura-2 salt in calibration buffer (in mM: 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) 20, KCl 120, NaCl 5, $MgCl_2$ 1, pH 7.1) containing 10 mM ethylene glycol *bis* (β-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA), $Ks = 3.696 \times 10^6$ M $- 1$ (Fabatio & Fabatio, 1979), with calculated amounts of Ca^{2+} added to give free Ca^{2+} concentrations ranging between approximately 0 to 2000 nM. Experiments performed over long periods of time (> 30 s) were digitally filtered with an algorithm which added 1/2 the value of each data point to 1/4 of the value of each neighbouring point. The data were cycled through this routine 5 times. The $[Ca^{2+}]_i$ traces in patch-clamp experiments were digitally filtered by a single cycle through an 11 point moving average algorithm.

Cell culture

Neurones were cultured from the dorsal root ganglia (DRG) of neonatal rats essentially as described previously (Thayer et al., 1988a). Briefly DRG neurones were dissected from the thoracic and lumbar segments of 1–3 day old Sprague-Dawley rats, incubated for 15 min at 37°C in collagenase/dispase (0.8 and 0.4 u ml^{-1}) and then dissociated into single cells by trituration through a Pasteur pipette. The cells were then plated on laminin-fibronectin-coated coverglasses (25 mm diameter) and incubated in Ham's medium mixture F-12, supplement (GIBCO, Grand Island, NY, U.S.A.) with 5% heat inactivated rat serum, 4% 17-day embryonic extract, 50 ng ml^{-1} nerve growth factor, 44 mM glucose, 2 mM L-glutamine, 1% MEM 100 × vitamins and penicillin/streptomycin (100 u ml^{-1} and 100 mg ml^{-1} respectively) which was replaced every 2–3 days. Cultures were maintained at 37°C in a water-saturated atmosphere with 5% CO_2.

Whole-cell patch clamp

The tight seal whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) was used to record transmembrane I_{Ca} from single cells while simultaneously measuring $[Ca^{2+}]_i$ transients. Cells were mounted in a perfusion chamber and thoroughly rinsed with a buffer composed of (in mM): tetraethylammonium chloride (TEACl) 143, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, glucose 10, pH adjusted to 7.4 with TEAOH. A cell was then approached with a fire-polished pipette containing a solution composed of (in mM): fura-2 pentapotassium salt 0.1, CsCl 135, $MgCl_2$ 1, HEPES 10, diTris phosphocreatinine 14, MgATP 3.6 and creatinine phosphokinase 50 u ml^{-1}; adjusted to pH 7.1 with CsOH. In experiments which involved only current measurements, 100 μM fura-2 pentapotassium salt was replaced by 10 mM EGTA or 20 mM 1,2-*bis*(2-aminophenoxy)ethane N,N,N',N'-tetracetic acid (BAPTA) in the pipette solution. For Ba^{2+} current measurements, 2 mM $CaCl_2$ was replaced with 2 mM $BaCl_2$ in the superfusate. Current clamp experiments were performed with a pipette solution composed of (in mM); fura-2 pentapotassium salt 0.1, KCl 135, $MgCl_2$ 1, HEPES 10, diTris phosphocreatinine 14, MgATP 3.6, and creatinine phosphokinase 50 u ml^{-1}; pH to 7.1 with KOH and an extracellular solution containing NaCl 143, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, glucose 10, pH adjusted to 7.4 with NaOH. Background fluorescence was recorded after formation of a gigaseal but before breaking into the cell, thus accounting for fluorescence contributed by the fura-2 in the pipette. Since the pipette approached the cell from above, the objective was focused below the pipette near the middle of the cell to minimize the pipette fluorescence. Full diffusion of the fura-2 into the cell occurred over a period of about 2 min. Current records were stored on a computer used for the fluorescence data acquisition. All experiments were performed at room temperature. For experiments which examined Ca^{2+} currents alone, the holding current and the voltage gated current were sampled every 7.5 s with 200 ms depolarizations from a holding potential (HP) of −80 mV to a test potential (TP) of 0 mV. In order to illustrate the effects of capsaicin on the holding current, the current versus time data are plotted without leak current subtraction. In voltage clamp experiments where linear leak corrections were performed, an average of 16, 10 mV hyperpolarizing pulses were made from the holding potential. The d.c. component of the averaged leak component was then modelled so as to increase the signal to noise ratio. Digital summation of this leak template after appropriate scaling with the current obtained during depolarizing test pulses provided the leak correction. All experiments were performed at room temperature.

Drugs and chemicals

All reagents were of Analar grade. Fura-2 pentapotassium salt and ester were obtained for Molecular Probes Inc., Eugene, Oregon, U.S.A. Capsaicin was obtained from Sigma Chemical Company, St. Louis Missouri, U.S.A. and dissolved in 70% ethanol to a 10 mM stock solution. Ethanol, at the maximal final concentration of 0.07% had no effects on either I_{Ca} or $[Ca^{2+}]_i$ in these cells. Other agents were obtained from Sigma Chemical Company.

Results

Experiments were performed on a total 188 cells. Of this total, 19 were examined by use of combined fura-2-based microfluorimetry and voltage clamp conditions and 17 with combined fura-2 based microfluorimetry and current clamp conditions; 72 cells were examined under whole cell voltage clamp mode alone and a total of 90 cells were examined with fura-2 based microfluorimetry alone.

Effects of capsaicin on voltage-clamped DRG cells

Depolarizing voltage clamp steps of 80 ms in duration from an HP of −80 mV to a TP of 0 mV were applied to single DRG neurones every 40 s as shown in Figure 1a and b. This interval was sufficient to allow recovery from the rise in $[Ca^{2+}]_i$ which results from Ca^{2+} influx through voltage gated Ca^{2+} channels and lasts approximately 20–30 s (Thayer et al., 1988b). In the cell shown, the peak I_{Ca} was relatively stable at approximately

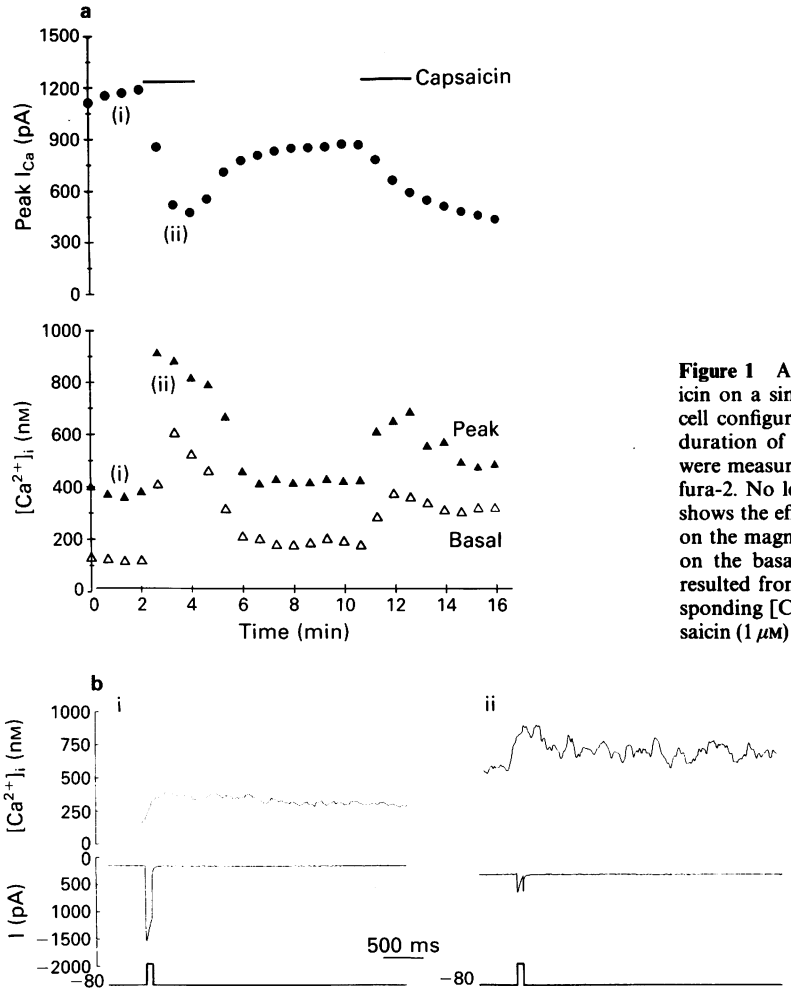


Figure 1 A representative experiment showing the effects of capsaicin on a single DRG cell, voltage-clamped to −80 mV in the whole cell configuration. The cell was depolarized to 0 mV every 40 s for a duration of 80 ms whilst Ca^{2+} currents (I_{Ca}) and $[Ca^{2+}]_i$ transients were measured simultaneously. The pipette solution contained 100 μM fura-2. No leak subtraction has been performed. (a) The upper panel shows the effect of capsaicin (1 μM), as indicated by the horizontal bar, on the magnitude of the peak I_{Ca} (●) and in the lower trace the effect on the basal $[Ca^{2+}]_i$ (△) and the peak rise in $[Ca^{2+}]_i$ (▲) which resulted from the depolarizing step. (b) The I_{Ca} traces and the corresponding $[Ca^{2+}]_i$ transients in the absence (i) and presence (ii) of capsaicin (1 μM) as indicated in (a). The duration of the sweep is 4 s.

1 nA with the basal Ca^{2+} of the cell at 125 nM. The depolarizing pulse resulted in a $[Ca^{2+}]_i$ transient of approximately 300 nM in magnitude. Perfusion of the cell shown in Figure 1 with solutions containing 1 μM capsaicin resulted in the following effects: (A) A reduction in the peak I_{Ca} from approximately 1 nA to 450 pA (Figure 1a, top panel). The mean maximum decrease in the peak I_{Ca} within 2 min of application of 1 μM capsaicin for those cells responding with changes in the three parameters examined simultaneously, namely I_h (see below), I_{Ca} and $[Ca^{2+}]_i$, and showing reversible responses, was a 3.5 ± 0.9 fold decrease in the I_{Ca} from a mean control I_{Ca} of 854 ± 241 pA ($n = 6$ cells responding reversibly; an additional 7 cells responded without recovery, of 19 cells in total examined). (B) The holding current (I_h) necessary to voltage clamp the cell at −80 mV increased from 160 pA to 275 pA. The increase in I_h within 2 min application of 1 μM capsaicin (using the same criteria as (A)) was from a mean value of 114 ± 20 pA to 250 ± 75 pA ($n = 6$ cells). (C) An increase in the basal $[Ca^{2+}]_i$ from 125 nM to 600 nM. The mean increase above basal $[Ca^{2+}]_i$ within 2 min application of 1 μM capsaicin was 4.1 ± 0.7 fold (criteria as (A) and (B), mean basal $[Ca^{2+}]_i = 94 \pm 22$ nM, $n = 6$ cells).

The threshold capsaicin concentration for a response in sensitive cells was between 0.1–0.5 μM. Concentrations of 10 μM or above often produced large irreversible changes in the holding current, I_{Ca} and $[Ca^{2+}]_i$, and normally resulted in a loss of voltage-clamp control. Even taking into consideration the time-dependent rundown of Ca^{2+} channels under voltage-clamp, recovery of the I_{Ca} from capsaicin (1–10 μM) was usually incomplete. A second application of capsaicin (1 μM) after a prolonged washout (7–10 min) and almost complete recovery of the basal Ca^{2+} levels to control values, resulted in a qualitatively similar response. The second response however was much smaller in magnitude and the degree of recovery following a second application was also markedly reduced.

Figure 1b shows individual sweeps at the timepoints indicated in Figure 1a in the absence (i) and presence (ii) of capsaicin. During the depolarizing step an increase in the $[Ca^{2+}]_i$ of approximately 300 nM resulted. It is interesting to note that in the presence of capsaicin (1 μM), this change in $[Ca^{2+}]_i$ was

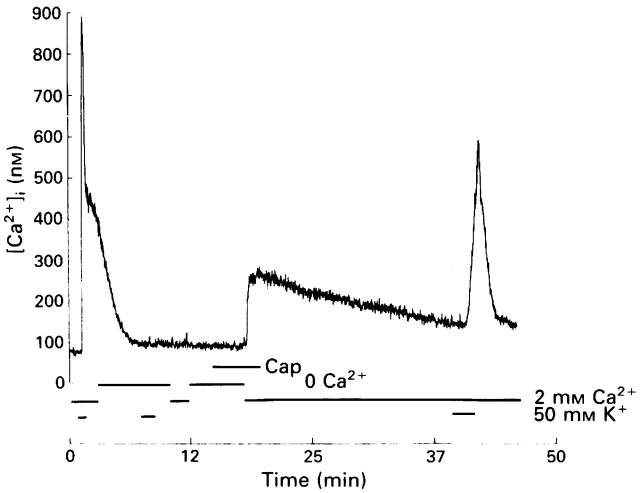


Figure 2 The effect of capsaicin (Cap) on the $[Ca^{2+}]_i$ in a fura-2 AM loaded cell in the presence and absence of extracellular Ca^{2+}. Low Ca^{2+} solutions contained zero added Ca^{2+} and 20 μM BAPTA. The initial depolarizations were made in extracellular solutions containing 50 mM KCl substituted iso-osmotically for NaCl.

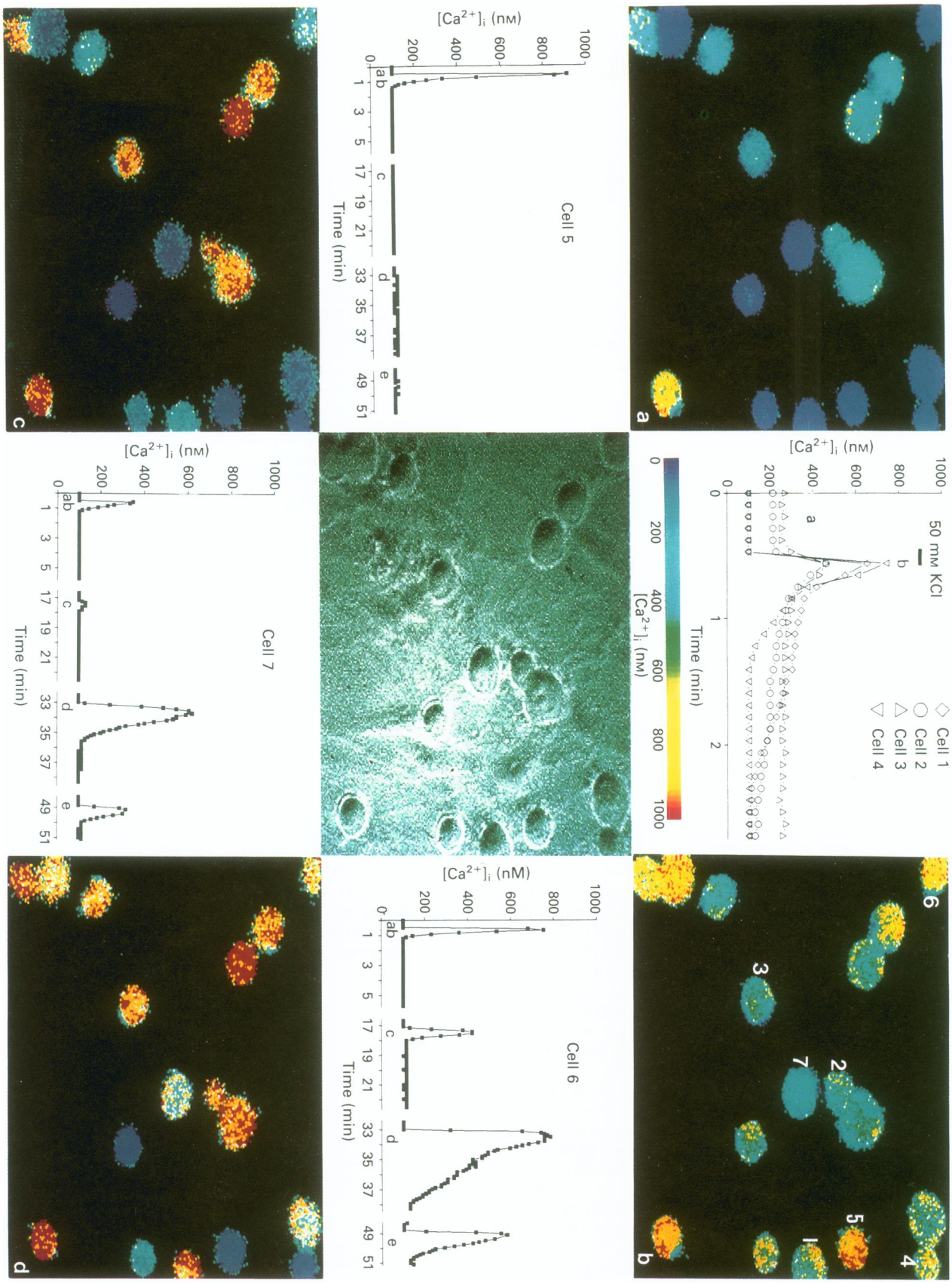


Figure 3 The responses of a representative field of cultured DRG cells to capsaicin as indicated by the digital imaging of a field of 17 DRG cells and the measurement of increases in $[Ca^{2+}]_i$ with fura-2 AM based microfluorimetry. Shown in the centre panel is a phase contrast image of the DRG cells. (a) Unstimulated cells, (b) during exposure to 50 mM KCl (KCl substituted iso-osmotically for NaCl), (c) during exposure to 1 μM capsaicin and (d) during exposure to 10 μM capsaicin. These timepoints in the course of the experiment are indicated on the traces, as is (e) exposure to 100 μM capsaicin. Time courses of responses are shown for selected individual cells labelled 1 to 7 inclusive (see (b)).

All cells in this field responded to 50 mM KCl with an increase in $[Ca^{2+}]_i$ although there was wide variability from cell to cell in both the peak response and the time course of recovery from this challenge (see cells 1–4, top centre). Cell 5 (right trace) failed to respond to capsaicin although it was sensitive to 50 mM KCl. Several patterns of response to capsaicin were evident with (cell 6 left trace, cell 7 bottom trace) which varied in degrees of sensitivity and desensitization to capsaicin exposure.

still preserved although the magnitude of the measured I_{Ca} was markedly reduced.

The observed increase in $[Ca^{2+}]_i$ in the presence of capsaicin or upon depolarization with 50 mM K^+ was absent if extracellular Ca^{2+} was omitted from the external solution. The response recovered when 2 mM Ca^{2+} was returned to the superfusion solutions indicating that the rise in $[Ca^{2+}]_i$ was due to Ca^{2+} influx (Figure 2). In order to estimate unambiguously the percentage of DRG cells in our cultures which were sensitive to capsaicin, digital microfluorometric imaging of fields of fura-2 AM loaded DRG cells in culture was performed and the number of cells responding to 1 and 10 μM capsaicin recorded (Figure 3). From these pseudocolor images and the timecourse of the responses in this example, it is possible to see that of the 17 cells present, all responded to a 50 mM KCl-induced depolarization, although to varying degrees, 8 cells responded to 1 μM capsaicin and 13 to 10 μM capsaicin. There was a wide degree of variability in the nature of the cell responsiveness and the degree of recovery and desensitization to capsaicin (see Figure 3 insets), an observation which correlated with the variability observed in the patch-clamp experiments (see below). Of the 87 cells examined by digital microfluorometric imaging which responded to 50 mM KCl, 29 (33%) responded to 1 μM and 48 (54%) responded to 10 μM capsaicin.

In order to examine the nature of the capsaicin-dependent reductions in I_{Ca} in more detail, experiments were performed in which current measurements were made alone with either Ca^{2+} or Ba^{2+} as the charge carrier, and with 10 mM EGTA or 20 mM BAPTA in the patch pipette as a buffer ($n = 72$ cells). Capsaicin often produced a large increase in the I_h and a large or complete inhibition of the I_{Ca} which reversed on washout of capsaicin from the test chamber; similar but smaller effects were seen in extracellular Ba^{2+}. The threshold capsaicin concentration for both the increase in I_h and the decrease in I_{Ca} or I_{Ba} was 0.1 to 0.5 μM. A dose-response curve in extracellular Ba^{2+} is shown in Figure 4a. Recovery of the voltage gated currents after capsaicin washout was greater in experiments performed with EGTA or BAPTA in the patch pipette than in the experiments without chelators, and was almost complete when the charge carrier was Ba^{2+} instead of Ca^{2+}. In addition, repeated applications of capsaicin to the cells in these experiments showed much less desensitization than in the previously discussed experiments where $[Ca^{2+}]_i$ was measured with fura-2 and consequently the $[Ca^{2+}]_i$ was not buffered (compare Figure 1 with Figures 4 and 6).

To compare the effects of capsaicin in extracellular Ca^{2+} to those in extracellular Ba^{2+} directly in the same cells, experiments were performed in which a trial with one ion was interposed between trials with the other ion, in order to account for the effects of current rundown and desensitization. In the representative experiment shown in Figure 4b, the washout of one ion for the other is marked in the current vs. time record by sharply diminished currents which appear to represent the anomalous mole-fraction effect previously noted by others (Hess & Tsien, 1984; Almers & Mcleskey, 1984). The large response to capsaicin (1 μM) in extracellular Ca^{2+} is preceded and followed by smaller responses in extracellular Ba^{2+}. In such studies, the mean inhibition of I_{Ba} was found to be 39 $\pm$ 6% of the inhibition of I_{Ca} and the change in I_h in Ba^{2+} only 22 $\pm$ 5% of the change in I_h in Ca^{2+} ($n = 6$ cells).

Positive currents were sometimes observed in Ca^{2+} during depolarizations to 0 mV in DRG cells which responded strongly to capsaicin, such as is seen in Figure 4b. Such positive currents were never observed when Ba^{2+} was the charge carrier. To investigate the nature of these positive currents, as well as that of the capsaicin-induced increase in the holding current, current-voltage relationships were measured with 200 μM cadmium extracellularly to block the voltage-gated I_{Ca}. One such measurement is shown in Figure 4c. Prior to application of capsaicin, only a small residual current was seen; when capsaicin (10 μM) was applied, an increased negative holding current at -80 mV was generated which reversed

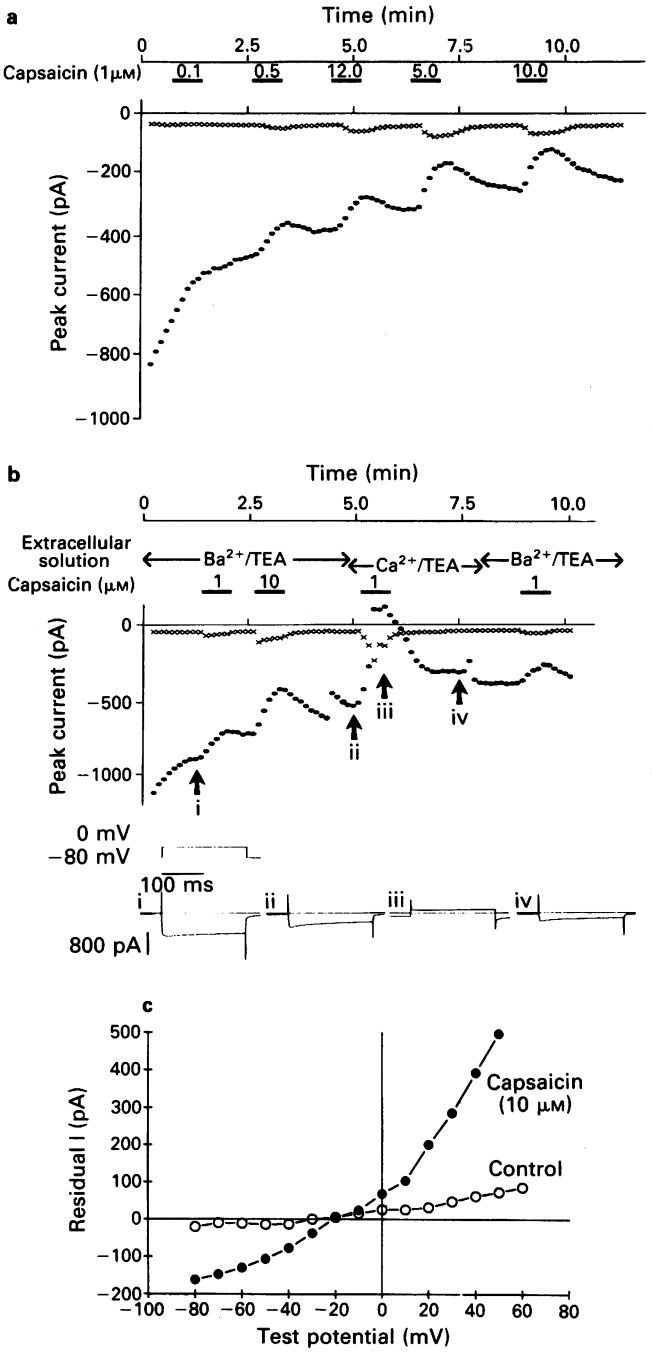


Figure 4 (a) Time course of an experiment on a voltage-clamped DRG cell at a holding potential of -80 mV depolarized to test potentials of 0 mV in Ba^{2+}. The dose-response of the capsaicin-dependent changes in the negative I_{Ba} (●) and holding current I_h (×) in $BaCl_2$. Most cells showed responses at concentrations between 0.1 and 0.5 μM capsaicin. Extracellular solution contained 2 mM Ba^{2+}/TEA and the pipette solution 10 mM EGTA. (No leak subtractions were made in this or the following Figures.). (b) The differential response of a cell to capsaicin with Ba^{2+} compared to Ca^{2+} as the change carrier. A cell was exposed to capsaicin while bathed in an extracellular solution of 2 mM Ba^{2+}/TEA and showed a moderate response to capsaicin as seen with the changing I_h (×) and decrease in magnitude of I_{Ba} (●). The extracellular solution was changed to 2 mM Ca^{2+}/TEA indicated by a transient decline in magnitude of the voltage gated current during mixing of the two solutions which correspond to the anomalous mole-fraction effect. Capsaicin was reapplied with an observed increased response. After the return to solutions containing Ba^{2+} capsaicin was reapplied. The current traces at the time points indicated are shown. Pipette solutions contained 10 mM EGTA. (c) The current-voltage relationship in the absence and presence of capsaicin with cadmium (200 μM) blockade of the voltage-gated I_{Ca}. Capsaicin (10 μM) induced an outwardly rectifying current which was positive at 0 mV and accounted for the increased negative holding current at -80 mV. Pipette solutions contained 10 mM EGTA.

at about −20 mV to a positive current at 0 mV and above, with outward rectification. With 10 mM EGTA as the chelator, the baseline mean slope conductance at −70 mV was 0.29 ± 0.05 nS, which increased to 1.90 ± 0.46 nS in capsaicin (10 μM) ($P < 0.01$, $n = 5$ cells). A similar capsaicin-induced change in conductance (Δg) was seen when substituting 20 mM BAPTA for EGTA in the pipette in an attempt to block fully any Ca^{2+}-activated conductances (Δg = 1.16 ± 0.15 nS, $n = 5$ cells). The magnitude of the capsaicin-induced positive current at 0 mV with cadmium blocking the I_{Ca} was similar to that of the positive currents seen when the I_{Ca} was abolished by capsaicin (compare Figures 4b and c). Also, it was of about the same magnitude as the net decrease in I_{Ba} induced by capsaicin in these cells, suggesting that induction of these positive currents may account for the apparent inhibition of I_{Ba} by capsaicin.

Effects of capsaicin on current-clamped DRG cells

Under current clamp conditions the resting membrane potential of DRG cells ranged between −50 and −60 mV. For the present experiments the membrane potential was routinely adjusted to −60 mV by current injection. Action potentials were elicited by brief current injection, and several action potentials fired in succession produced a stepwise increase in $[Ca^{2+}]_i$ in the cells shown in Figure 5, 5 action potentials fired at an interval of 80 ms (12.5 Hz) produced an increase in $[Ca^{2+}]_i$ from approximately 150 nM to 450 nM. Individual traces of the sweeps indicated in Figure 5a are shown in Figure 5b. Figure 5a illustrates the effect of increasing concentrations of capsaicin on the membrane potential and $[Ca^{2+}]_i$. The threshold concentration was approximately 500 nM. However, a marked depolarization and increase in basal $[Ca^{2+}]_i$ resulted at a capsaicin concentration of 1 μM. The mean increase in $[Ca^{2+}]$ was 4.37 ± 1.25 fold above basal $[Ca^{2+}]$ ($n = 12$ responding cells of 17 cells examined) and the mean decrease in membrane potential was 21.15 ± 5.03 mV ($n = 12$ cells) for 1 μM capsaicin. The depolarization induced was sometimes large enough to produce spontaneous action potential firing, which ceased when the membrane potential reached still more positive values. Recovery of the membrane potential to pre-capsaicin values was normally complete. However, this was not always the case for recovery of the basal $[Ca^{2+}]_i$. Repeated application of 1 μM capsaicin resulted in a second depolarization and increase in basal $[Ca^{2+}]_i$. Cells showed signs of desensitization to repeated application as they did in the voltage-clamp experiments.

Effects of ruthenium red on capsaicin responses in DRG cells

We also examined the effects of ruthenium red (RR) on the response of DRG cells to capsaicin under both voltage-clamp and current-clamp conditions. Figure 6 shows that under voltage-clamp conditions (HP = −80 mV, TP = 0 mV), the repeatable capsaicin-dependent increases in I_h and the decrease in I_{Ca} were blocked after exposure to RR (1 μM). RR alone at a concentration of 1 μM also produced a small inhibi-

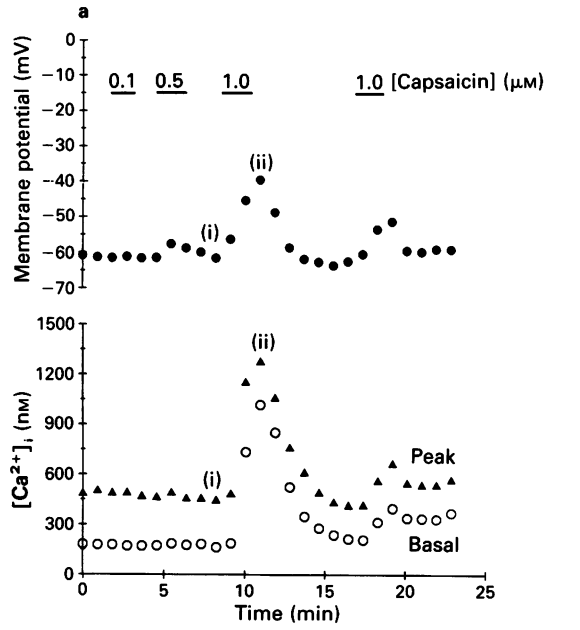
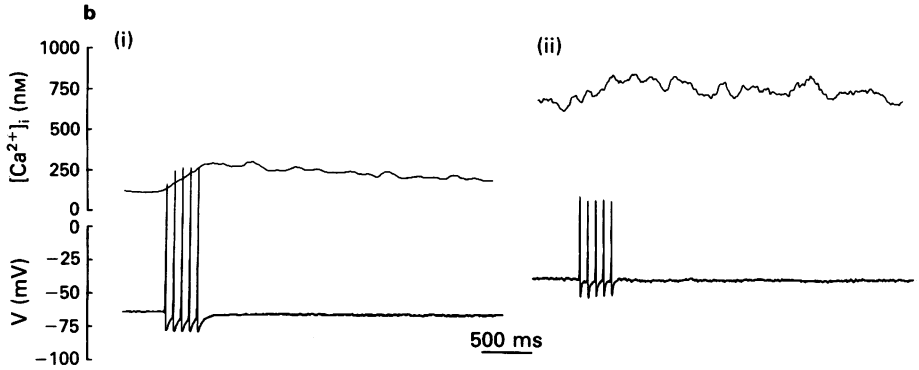


Figure 5 Simultaneous current clamp recordings and $[Ca^{2+}]_i$ in a DRG cell after readjustment of the membrane potential to −60 mV by constant current injection. The top panel shows the time course of membrane potential (●) measured every 40 s. The bottom panel show the basal $[Ca^{2+}]_i$ (○) and the peak value of the $[Ca^{2+}]_i$ transient (△) which results from 5 action potentials elicited by brief current injection (12.5 Hz) every 40 s. As indicated by the horizontal bars, cells were exposed to various concentrations of capsaicin and the parameters described recorded. (b) Individual records of the timepoints indicated in the absence (i) and presence (ii) of capsaicin (1 μM).



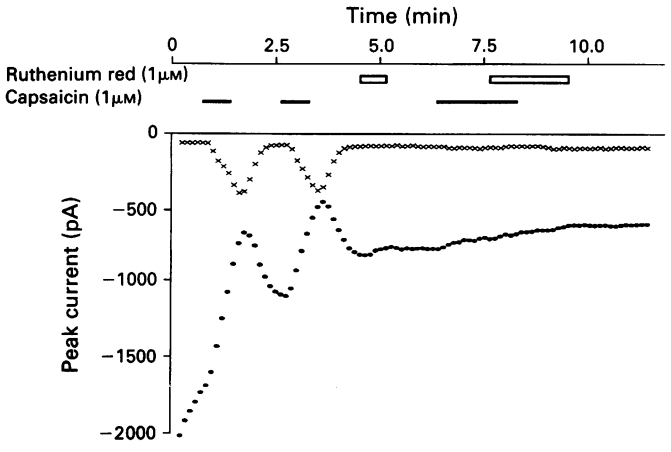


Figure 6 The effect of ruthenium red on the capsaicin-dependent changed in holding current (×) and the I_{Ca} (●). This cell showed a brisk response to 1 μM capsaicin which was repeatable and showed little sign of desensitization. After exposure to ruthenium red (1 μM) as indicated by the horizontal bars, capsaicin was reapplied, without effect. Concurrent exposure to ruthenium red is also indicated. Pipette solution contained 10 mM EGTA.

tion of the I_{Ca} (without affecting the holding current) which may indicate that it had a small blocking effect on voltage-gated Ca^{2+} channels in these cells. The effect of RR was slow to wash out, persisting for several minutes after washout as shown in Figure 6. We found that in cells which lacked any response to 1 μM capsaicin, and lacked any observable change in the holding current on exposure to 10 μM, a very small inhibition of the I_{Ca} could still be observed. These effects were of similar magnitude in both extracellular Ca^{2+} and Ba^{2+} and were unaffected by RR (not shown).

Figure 7 illustrates the effects of RR on capsaicin-dependent changes in membrane potential and basal $[Ca^{2+}]_i$ under current-clamp conditions. In the presence of RR (500 nM), capsaicin (1 μM) produced little or no change in membrane potential or basal $[Ca^{2+}]_i$. After an extensive washout period, the re-application of capsaicin resulted in a depolarization and an increase in the basal $[Ca^{2+}]_i$. However, the onset of the

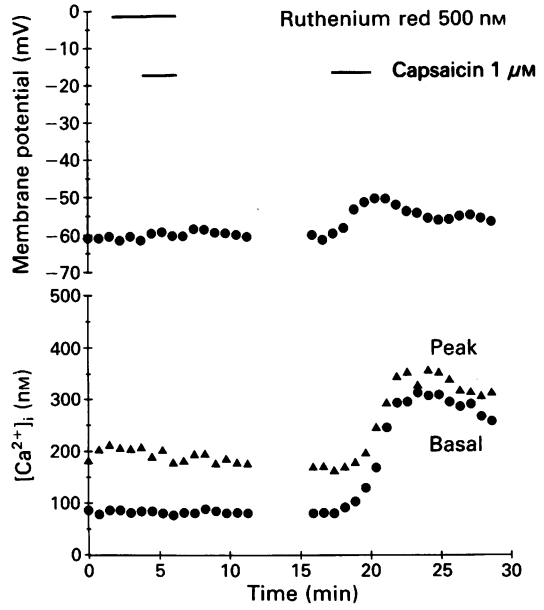


Figure 7 The effect of ruthenium red (500 nM) on the capsaicin-dependent responses in single DRG cell under current clamp conditions showing the membrane potential (●), basal $[Ca^{2+}]_i$. (●), and the peak $[Ca^{2+}]_i$ transient resulting from 5 action potentials elicited by brief current injection (▲).

effects of capsaicin after superfusion with solutions containing RR appeared to be slower, possibly indicating incomplete washout.

Discussion

The present series of experiments have examined the effects of capsaicin on cultured rat sensory neurones. Using the combined patch-clamp-microfluorometric technique for simultaneously measuring the I_{Ca} and $[Ca^{2+}]_i$ we have been able to study the relationship between the capsaicin-dependent increase in $[Ca^{2+}]_i$ and the observed reduction in the I_{Ca}. It has recently been reported that capsaicin inhibits voltage-gated calcium currents in adult rat DRG neurones (Robertson *et al.*, 1989).

Capsaicin-dependent increase in $[Ca^{2+}]_i$

It is clear that the rise in $[Ca^{2+}]_i$ produced by exposing DRG cells to capsaicin results from Ca^{2+} entry from the extracellular solution rather than release of Ca^{2+} from intracellular stores since it was abolished in a Ca^{2+}-'free' solution. For cells which were voltage-clamped at −80 mV, the route of Ca^{2+} entry is likely to be via the established capsaicin-induced conductance which has previously been shown to be permeable to Ca^{2+}, Na^+, guanidinium and Rb^+ ions (Bevan *et al.*, 1987; Wood *et al.*, 1988). Under current-clamp conditions, the rise in $[Ca^{2+}]_i$ could be due to entry via the capsaicin-induced conductance and also through voltage-gated Ca^{2+} channels which open in response to depolarization. Although Wood *et al.* (1988) reported no reduction in capsaicin-dependent Ca^{45} fluxes into cultured DRG cells by dihydropyridine drugs, not all Ca^{2+} channels in rat DRG neurones are dihydropyridine-sensitive (see Miller, 1987) and for the large depolarizations and spontaneous firing observed we would expect some Ca^{2+} entry through voltage-gated channels. However, the currents through the capsaicin-induced conductance, as measured by the change in I_h in voltage-clamp mode, are substantial and would be expected to lead to a large influx of Ca^{2+}.

Capsaicin-dependent changes in holding current and I_{Ca}

The reversible capsaicin-dependent increases in the holding current, I_h, are also likely to be due to the opening of the same capsaicin-induced conductance pathway. Under voltage-clamp conditions, the holding current must precisely match the ionic membrane current in order to maintain the potential constant; thus, an increase in the holding current indicates an increased membrane conductance. In these voltage-clamp experiments, influx of Ca^{2+} is likely to be primarily responsible for the measured changes in holding current since the capsaicin conductance pathway has been reported to be impermeant to Cl^- ions (Wood *et al.*, 1988), Na^+ ions have been replaced with TEA, and the driving force for H^+ ions is relatively small compared to that for Ca^{2+}. It remains possible that a Ca^{2+}-activated channel, not blocked by Cs^+ or TEA, could contribute to the increased holding current. In voltage-clamp experiments performed in extracellular Ba^{2+}, the capsaicin-induced increase in holding current was 22% of that in Ca^{2+}, indicating that the permeability of the capsaicin-induced conductance for Ba^{2+} is approximately one fifth of that of Ca^{2+} (assuming that these are the only contributing ions under these conditions). This may suggest a small pore size or cation binding selectivity for Ca^{2+} ions over Ba^{2+} ions for the channel involved (see Almers & McCleskey, 1984). Under current clamp conditions, both Na^+ and Ca^{2+} can enter the cells and both ions would contribute to the large depolarizations observed.

Since sustained Ca^{2+} entry occurs on exposing DRG cells to capsaicin, one might suppose that the observed inhibition of the I_{Ca} is due to an increase in basal $[Ca^{2+}]_i$ as it has been

shown that many Ca^{2+}-currents are inhibited by raising $[Ca^{2+}]_i$ (see Eckert & Chad, 1984). However, it is also conceivable that an outward current through the observed capsaicin-induced conductance contributes to the apparent inhibition of the I_{Ca}, or that capsaicin directly inhibits I_{Ca}. Thus several types of experiment were performed to resolve these possibilities. Initially, attempts were made to block the calcium-mediated effects of capsaicin by the use of Ca^{2+} chelators in the pipette solution. Buffering of the $[Ca^{2+}]_i$ with 10 mM EGTA reduced, but did not prevent, the capsaicin-induced decrease in the I_{Ca}. More complete buffering with 20 mM BAPTA similarly left residual capsaicin effects (not shown). Thus these experiments suggest that the intracellular chelators may reduce the effects of the capsaicin-induced rise in $[Ca^{2+}]_i$ but inadequately buffer Ca^{2+} sufficiently to prevent effects on channels close to the site of entry.

To eliminate any effects produced by elevated $[Ca^{2+}]_i$ levels, Ba^{2+} was substituted as the extracellular ion. In Ba^{2+} containing solutions, the effects of capsaicin on the holding current and the I_{Ba} were markedly reduced in comparison to the responses in extracellular Ca^{2+} in the same cell. These results strongly favour a major role for Ca^{2+} as the mediator of the capsaicin-induced inhibition of the I_{Ca}. The residual apparent inhibition of the I_{Ba} by capsaicin must take place by a process not involving a raised $[Ca^{2+}]_i$. Two possible explanations arise. The magnitude of the outward currents observed with Ca^{2+} as the extracellular ion were comparable in size to the decreases in the I_{Ba} observed (see Figure 4b). Thus the apparent decrease in the inward I_{Ba} in the presence of capsaicin may be due to a capsaicin-induced outward current partially cancelling the total I_{Ba}. Such an outward current was observed at 0 mV when the capsaicin-dependent changes in the current-voltage relation in the presence of cadmium were measured. This outward current would most likely be Cs^+ flux through the capsaicin-induced conductance, although Cs^+ flux through a Ca^{2+}-activated K^+ channel insensitive to block by TEA cannot be ruled out (Cook, 1988). Alternatively, it may be that Ca^{2+}-mediated processes which lead to the inhibition of the I_{Ca} are also partially activated by Ba^{2+}. Such activation by Ba^{2+} is seen, for example, of the phosphatase calcineurin in the presence of calmodulin but not in its absence (Pullen & Wang, 1984). In either of these possibilities, the inhibition of the I_{Ca} by capsaicin is an indirect effect, mediated by the elevation of the $[Ca^{2+}]_i$. Similar findings describing the effects of capsaicin on the voltage-gated I_{Ca} in adult rat DRG neurones have recently been reported and attributed to increases in $[Ca^{2+}]_i$ (Robertson *et al.*, 1989).

It is interesting to note that the nonapeptide, bradykinin, increases the $[Ca^{2+}]_i$ in approximately 20% of cultured rat DRG cells via Ca^{2+} mobilization (Thayer *et al.*, 1988a; Bleakman *et al.*, 1989) an effect which may contribute to an observed bradykinin-dependent decrease in I_{Ca} (Ewald *et al.*, 1989; Bleakman *et al.*, 1989). However, bradykinin additionally inhibits the I_{Ca} in many cells in which increases in $[Ca^{2+}]_i$ are not observed, hence a different mechanism for the inhibition of I_{Ca} exists for bradykinin from that observed for capsaicin in the present experiments (Ewald *et al.*, 1989).

Desensitization of the capsaicin response

The desensitization to the capsaicin effects in these cells was markedly increased when the cell Ca^{2+} was not buffered. This observation suggests that Ca^{2+} entry may be producing long lasting changes in the capsaicin 'receptor' or associated channel, leading to desensitization.

Inhibition of the effects of capsaicin by ruthenium red

The inhibitory effects of RR on the capsaicin changes in I_h and Ca^{2+} influx which we observed are consistent with the previous reported effects of this inhibitor of transmembrane Ca^{2+} transport (Moore, 1971; Swanson *et al.*, 1974), including its inhibition of capsaicin-dependent increases in Ca^{45} uptake (Wood *et al.*, 1988). It has been reported that primary afferent neurone excitation by capsaicin in the absence of extracellular Ca^{2+} is also prevented by RR (Amann *et al.*, 1989). In our experiments all the observed effects of capsaicin were prevented by RR with the exception of the small inhibition of the I_{Ca} which was found at high capsaicin concentrations and occurred in the absence of changes in I_h. These results suggest that virtually all of the capsaicin effects are likely to be mediated by a common site of action, sensitive to ruthenium red.

From the present series of experiments we can conclude that the primary action of capsaicin is to allow entry of Ca^{2+} into DRG cells via a RR sensitive conductance. This ion movement appears to be responsible for the depolarization observed under current clamp conditions since the depolarization is prevented by RR. Ca^{2+} entry produces the Ca^{2+}-dependent inhibition of the I_{Ca} and the desensitization of the capsaicin response. If the depolarization is large enough, then spontaneous action potential firing can occur, although further depolarization results in a loss of spontaneous activity presumably due to inactivation of Na^+ channels at depolarized potentials. The capsaicin-dependent inactivation of the I_{Ca} and the large depolarization and inactivation of Na^+ channels may in part account for the analgesic effect of capsaicin when applied topically (Carpenter & Lynn, 1981). Thus the inhibitory effects of capsaicin on the I_{Ca} appear to involve three components. The greatest of these is due to the capsaicin-induced increase in $[Ca^{2+}]_i$ and feedback inhibition of the I_{Ca}. Secondly, an outward current through the capsaicin-induced channel at positive potentials may partially mask the voltage-activated inward current. Finally, there may also be a small direct blocking effect.

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References

ALMERS, W. & McCLESKEY, E.W. (1984). Non-selective conductance in Ca^{2+} channels of frog muscle; calcium selectivity in a single-file pore. *J. Physiol.*, **353**, 585–608.

AMANN, R., DONNERER, J. & LEMBECK, F. (1989). Capsaicin-induced stimulation of polymodal nociceptors is antagonized by ruthenium red independently of extracellular calcium. *Neurosci.*, **32**, 255–259.

BACCAGLINI, P.I. & HOGAN, P.G. (1983). Some rat sensory neurones in culture express characteristics of different pain sensory cells. *Proc. Natl. Acad. Sci., U.S.A.*, **80**, 594–598.

BEVAN, S.J., JAMES, I.F., RANG, H.P., WINTER, J. & WOOD, J.N. (1987). The mechanism of action of capsaicin—a sensory neurotoxin. In *Neurotoxins and their Pharmacological Implications*. ed. Jenner, P. New York: Raven Press.

BLEAKMAN, D., THAYER, S.A. & MILLER, R.J. (1989). Bradykinin modulation of calcium currents and $[Ca^{2+}]_i$ in rat dorsal root ganglion cells *in vitro*. *Soc. Neurosci. Abstracts*, **15**(1), 16.

CARPENTER, S.E. & LYNN, B. (1981). Vascular and sensory responses of human skin to mild injury after topical treatment with capsaicin. *Br. J. Pharmacol.*, **73**, 755–758.

COOK, N.S. (1988). The pharmacology of potassium channels and their therapeutic potential. *Trends Pharmacol. Sci.*, **9**, 21–28.

ECKERT, R. & CHAD, J.E. (1984). Inactivation of calcium channels. *Prog. Biophys. Mol. Biol.*, **44**, 215–267.

EWALD, D.A., STERNWEIS, P.C., PANG, I-H. & MILLER, R.J. (1989). Differential G-protein mediated coupling of neurotransmitter receptors to Ca^{2+} channels in rat dorsal root ganglion neurones *in vitro*. *Neuron*, **3**, 1185–1193.

FABATIO, A. & FABATIO, F. (1979). Calculator programs for comput-

ing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J. Physiol. (Paris)*, **75**, 463–505.

FITZGERALD, M. (1983). Capsaicin and sensory neurones. A review. *Pain*, **15**, 109–130.

FOSTER, R.W. & RAMAGE, A.G. (1981). The action of some chemical irritants on somatosensory receptors in the cat. *Neuropharmacology*, **20**, 191–198.

GAMSE, R., LACKNER, D., GAMSE, G. & LEEMAN, S.E. (1981). Effect of capsaicin pretreatment on capsaicin-evoked release of immunoreactive somatostatin and substance P from primary sensory neurons. *Naunyn Schmeidebergs Arch. Pharmacol.*, **316**, 38–41.

GLAUM, S.R., HOLZWARTH, J.A. & MILLER, R.J. (1990). Glutamate receptors activate Ca^{2+} mobilization and Ca^{2+} influx into astrocytes. *Proc. Natl. Acad. Sci., U.S.A.*, **87**, 3454–3458.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

HAMILL, O., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. (1981). Improved patch clamp technique for high resolution current recording from cells and cell free membrane patches. *Pflügers Arch.*, **391**, 85–100.

HESS, P. & TSIEN, R.W. (1984). Mechanism of ion permeation through calcium channels. *Nature*, **309**, 453–456.

HEYMAN, I. & RANG, H.P. (1985). Depolarizing responses to capsaicin in a subpopulation of rat dorsal root ganglion cells. *Neurosci. Lett.*, **56**, 69–75.

JANCSO, G., KIRALY, E., LOO, F., SUCH, G. & NAGY, A. (1985). Selective degeneration of primary sensory neurones in the adult rat. *Neurosci. Lett.*, **59**, 209–214.

MAGGI, C.A., SANTICIOLI, P., GEPPETTI, P., PARLANI, M., ASTOLFI, M., DEL BIANCO, E., PATACCHINI, R., GIULIANI, S. & MELI, A. (1989). The effect of calcium free medium and nifedipine on the release of substance P-like immunoreactivity and contractions induced by capsaicin in the isolated guinea-pig and rat bladder. *Gen. Pharmacol.*, **20**, 445–456.

MILLER, R.J. (1987). Multiple channels and neuronal function. *Science*, **235**, 46–52.

MOORE, C.L. (1971). Specific inhibition of mitochondrial Ca^{2+} transport by ruthenium red. *Biochem. Biophys. Res. Commun.*, **42**, 298–305.

PULLEN, C.J. & WANG, J.H. (1984). Regulation of calcineurin by metal ions. *J. Biol. Chem.*, **259**, 6134–6141.

ROBERTSON, B., DOCHERTY, R.J. & BEVAN, S. (1989). Capsaicin inhibits voltage-activated calcium currents in a sub-population of adult rat dorsal root ganglion neurones. *Neurosci. Abstracts*, **15**, 354.

SZOLCSANYI, J. (1984). Capsaicin: hot new pharmacological tool. *Trends Neurosci.*, **4**, 495–497.

SWANSON, P.D., ANDERSON, L. & STAHL, W.L. (1974). Uptake of calcium ions by synaptosomes from rat brain. *Biochem. Biophys. Acta*, **356**, 174–183.

THAYER, S.A., PERNEY, T.M. & MILLER, R.J. (1988a). Regulation of calcium homeostasis in sensory neurons by bradykinin. *J. Neurosci.*, **8**, 4089–4097.

THAYER, S.A., STUREK, M. & MILLER, R.J. (1988b). Measurement of neuronal Ca^{2+} transients using simultaneous microfluorimetry and electrophysiology. *Pflügers Arch.*, **415**, 216–223.

WOOD, J.N., WINTER, J., JAMES, I.F., RANG, H.P., YEATS, J. & BEVAN, S. (1988). Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture. *J. Neurosci.*, **8**, 3208–3220.

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Impairment of relaxations to acetylcholine and nitric oxide by a phorbol ester in rat isolated aorta

[1]Keith J. Morrison & David Pollock

Department of Pharmacology, University of Glasgow, Glasgow G12 8QQ

1 This study compared the abilities of acetylcholine (ACh) (endothelium-dependent) and nitric oxide (NO) (endothelium-independent and which may be the active component of the endothelium-derived relaxing factor) to relax rat isolated aortic rings contracted with equi-effective concentrations of noradrenaline (NA) or phorbol 12-myristate 13-acetate (PMA).

2 ACh and NO induced concentration-dependent relaxations of aortic rings contracted with NA (EC_{70} value: 0.2 μM). However, relaxations to both ACh and NO were markedly reduced in rings contracted with PMA (EC_{80} value: 0.5 μM). NO-induced relaxations of tissues were not affected by removal of the endothelium, but ACh-induced relaxations were confirmed to be endothelium-dependent.

3 ACh (10 μM) induced a 10 fold increase in guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels above control values in aortic rings contracted with NA (0.2 μM), but did not affect cyclic GMP levels in rings contracted with PMA (0.5 μM).

4 NO (3 μM) induced a 100 fold increase in cyclic GMP levels above control values in aortic rings contracted with NA (0.2 μM), but only an 11 fold increase in tissues contracted with PMA (0.5 μM).

5 It is concluded that the action (s) of EDRF (NO) are impaired in the presence of PMA by a mechanism that may involve the stimulation of protein kinase C in vascular smooth muscle cells.

Introduction

Vascular smooth muscle relaxation induced by endothelium-dependent relaxants and nitrovasodilators is associated with stimulation of soluble guanylate cyclase and elevated tissue content of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Rapoport & Murad, 1983). Furthermore, vascular relaxation is dependent on the nature and concentration of contractile agonist used to raise muscle tone (Furchgott, 1983) and is reflected in altered levels of cyclic GMP (Morrison & Pollock, 1988a). The mechanism (s) underlying this phenomenon remains unclear. This study therefore compared the abilities of acetylcholine (ACh) (endothelium-dependent) and nitric oxide (NO), which may be one of the endothelium-derived relaxing factors (Palmer *et al.*, 1987; Shikano *et al.*, 1988) to increase tissue levels of cyclic GMP and relax rat isolated aortic rings contracted with agonists with different contractile mechanisms. Muscle tone was raised with either noradrenaline (NA) (plasma membrane receptor-mediated response) or the tumour-promoting phorbol ester, phorbol 12-myristate 13-acetate (PMA) (synthetic activator of protein kinase C). These results were presented in preliminary form to the British Pharmacological Society (Morrison & Pollock, 1988b).

Methods

Preparation of aortic rings

Male Wistar rats (250–350 g) were killed by stunning and exsanguination. The thoracic cavity was exposed and the descending thoracic aorta was excised and placed in cold physiological salt solution of the following composition (millimolar): NaCl 118.5, KCl 4.75, $CaCl_2$ 2.5, $MgSO_4 \cdot H_2O$ 1.0, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 22.2. The aorta was cleaned of adherent fat and connective tissue and two pairs of transverse rings (2–3 mm in length) were then prepared from each aorta. Care was taken not to damage the endothelial surface. In certain experiments, in one ring of each pair, the endothelium was removed by inserting a wooden stick into the lumen and rolling the preparation gently over a saline-soaked filter paper. The absence of endothelium in these rings was confirmed by the abolition of the relaxant response to ACh (1 μM) in rings contracted with NA (EC_{50} concentration). Aortic rings were weighed (wet tissue mass) and suspended between stainless steel wire hooks under a resting tension of 2 g in organ chambers containing 25 ml of physiological salt solution, maintained at 37°C and gassed with a mixture of 95% O_2 : 5% CO_2. Isometric tension was recorded with Statham force displacement transducers and displayed on a Linseis recorder. Tension is expressed in g mg^{-1} wet mass of tissue. The tissues were allowed to equilibrate for 60 min before the start of experiments, during which time the tissues were washed at 15 min intervals.

Measurement of cyclic GMP

Tissue content of cyclic GMP was determined in tissues in which mechanical responses had been recorded. At appropriate time intervals following the addition of drugs to the organ chambers, the tissues were rapidly removed, frozen in liquid nitrogen and hand homogenized in Potter Elvehjem glass-glass homogenizers. Cyclic GMP was extracted in trichloroacetic acid (5% w/v) and measured using a radioimmunoassay kit (Amersham U.K.). Levels of cyclic GMP are expressed in pmol mg^{-1} wet mass of tissue.

Experimental protocol

Cumulative and non-cumulative concentration-effect graphs were obtained for NA and PMA respectively, both in the presence and absence of endothelium. In separate experiments, aortic rings were contracted to the same level of tone with NA (EC_{70}: 0.2 μM) or PMA (EC_{80}: 0.5 μM). When muscle tone had stabilised (NA 5 min; PMA 45 min), concentration-effect curves for ACh and NO were constructed by increasing organ chamber concentrations cumulatively.

In experiments in which tissue content of cyclic GMP was determined, aortic rings were contracted with either NA (0.2 μM; 5 min) or PMA (0.5 μM; 45 min) followed by ACh (10 μM) or NO (3 μM). Tissues were frozen in liquid nitrogen at

[1] Author for correspondence at present address: Center for Experimental Therapeutics, Baylor College of Medicine, One Baylor Plaza, Houston TX 77030, U.S.A.

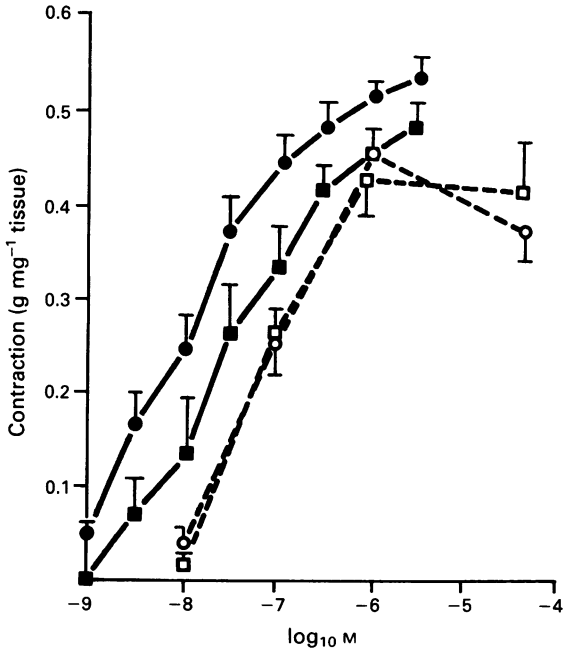


Figure 1 Concentration-effect graph for noradrenaline (NA, in presence of dimethylsulphoxide, 0.05%; closed symbols) and phorbol 12-myristate 13-acetate (PMA, open symbols) in presence (squares) and absence (circles) of endothelium. Contractions are expressed in g mg^{-1} tissue and are shown as mean with s.e.mean shown by vertical bars ($n = 6$).

30 s (ACh) and 20 s (NO) following addition of the relaxants. Preliminary time-course studies showed that maximum cyclic GMP levels were obtained at these times. Control tissues contracted with either NA or PMA were also assayed for cyclic GMP.

Data analysis

Results are expressed as mean ± s.e.mean and were analysed by Student's paired or unpaired t test where appropriate. Significance was accepted when $P < 0.05$. n values refer to the number of rats.

Drugs

The following drugs were used: acetylcholine chloride (Sigma Chemical Company); (−)-noradrenaline bitartrate (Koch-Light); 4α-phorbol 12,13-didecanoate (4αPDD), phorbol 12-myristate 13-acetate (PMA) (Sigma). The cyclic GMP radioimmunoassay kit (TRK. 500) was obtained from Amersham U.K. Drugs were prepared daily in distilled water and kept on ice until used. PMA and 4αPDD were dissolved in dimethylsulphoxide (DMSO) giving stock solutions of 1.6 mM from which appropriate dilutions were made with distilled water. The concentration of DMSO in the organ chamber did not exceed 0.05%. Both PMA and 4αPDD were protected from light. Nitric oxide gas (0.2 ml; BDH) was injected into a rubber-sealed Clinbritic bottle, completely filled with saline (59.6 ml) which had been deoxygenated by bubbling with helium for 2 h. This gave a stock solution of 1.5×10^{-4} M. Concentrations of drugs are expressed as the final organ chamber concentrations (molar).

Results

NA and PMA induced concentration-dependent contractions of aortic rings both in the presence and absence of endothelium (Figure 1). Removal of the endothelium augmented contractions to NA but did not alter responses to PMA. DMSO (0.05%), the vehicle for PMA, did not affect contractions to NA (data not shown).

Contractions to PMA (0.5 μM) stabilized after 45 min but the inactive phorbol ester, 4αPDD (1 μM) did not alter baseline tension (Figure 2). DMSO (0.05%) also did not affect basal tension (data not shown).

ACh and NO induced concentration-dependent relaxations of aortic rings contracted with NA (0.2 μM; 5 min) in the presence of endothelium (Figures 3 and 4). Relaxations to NO were rapid in onset and transient (Figure 3). NO-induced relaxations were not significantly different from those induced by ACh over the same range of concentrations (Figure 4).

Relaxations induced by ACh and NO were similar in rat aortic rings contracted with NA (0.2 μM; 5 min) or NA (0.2 μM; 45 min) (data not shown).

ACh and NO induced significantly smaller relaxations of aortic rings contracted with PMA (0.5 μM; 45 min) in the presence of endothelium (Figures 3 and 4). NO induced slowly developing and transient relaxations (Figure 3). Again, NO-induced relaxations were not significantly different from those induced by ACh over the same range of concentrations (Figure 4).

Removal of the endothelium did not alter the relaxations induced by NO in tissues contracted with either NA or PMA (Figure 5).

ACh (10 μM; 30 s) significantly increased tissue content of cyclic GMP above control values in rings contracted with NA (0.2 μM) (control: 30.8 ± 6.2 pmol g^{-1}; ACh: 282.0 ± 20.8 pmol g^{-1}, $P < 0.001$) (Figure 6). NO (3 μM; 20 s) significantly increased the tissue content of cyclic GMP above control values both in rings contracted with NA (0.2 μM) (control: 30.8 ± 6.2 pmol g^{-1}; NO: 3102.0 ± 475.4 pmol g^{-1}, $P < 0.001$) or PMA (0.5 μM) (control: 22.7 ± 4.9 pmol g^{-1}; NO: 378.4 ± 77.9 pmol g^{-1}, $P < 0.001$) (Figure 6). However, the levels of cyclic GMP induced by ACh or NO were significantly smaller

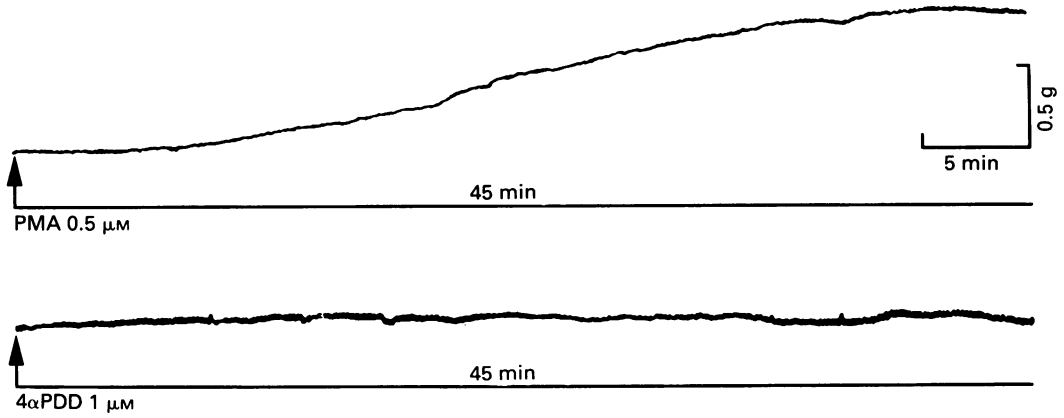
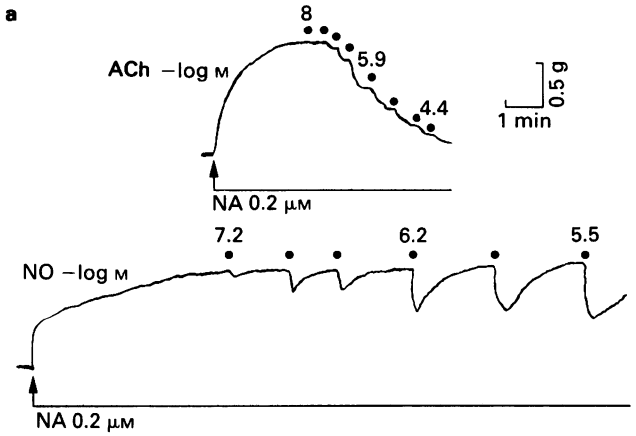


Figure 2 Typical effect of phorbol 12-myristate 13-acetate (PMA, 0.5 μM) and 4α-phorbol 12,13-didecanoate (4αPDD, 1 μM) on baseline tension.



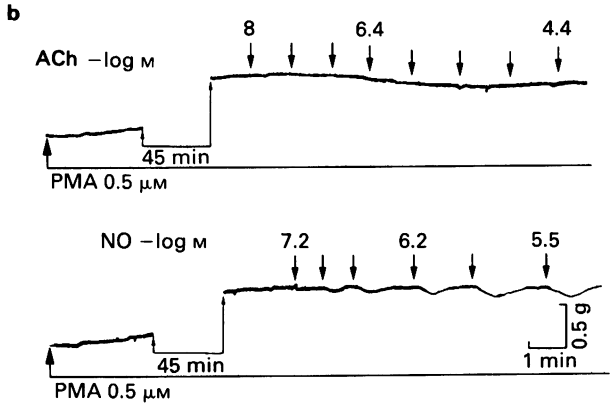


Figure 3 Acetylcholine (ACh)- and nitric oxide (NO)-induced relaxations of rat aortic rings contracted with either NA (0.2 μM; a) or phorbol 12-myristate 13-acetate (PMA, 0.5 μM; b) in the presence of endothelium. Concentrations of ACh, −log M: 8, 7.4, 6.9, 6.4, 5.9, 5.4, 4.9, 4.4; concentrations of NO, −log M: 7.2, 6.9, 6.5, 6.2, 5.9, 5.5, added cumulatively.

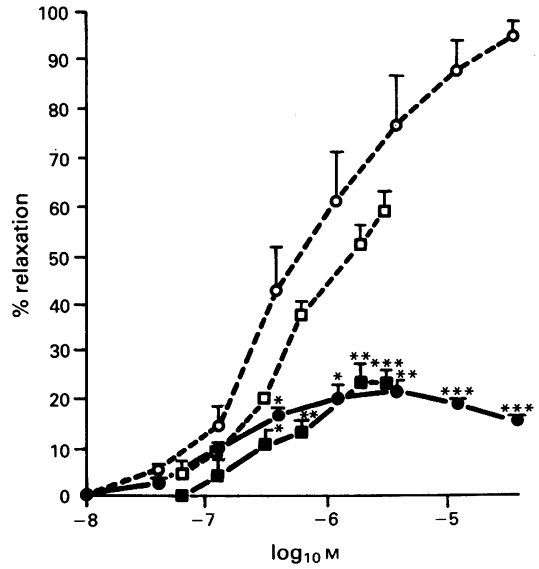


Figure 4 Concentration-effect curves for acetylcholine (ACh, circles) and nitric oxide (NO, squares) in aortic rings contracted with NA (0.2 μM; open symbols) or phorbol 12-myristate 13-acetate (PMA, 0.5 μM; closed symbols) in the presence of endothelium. Relaxations are expressed as a % of the contractions to NA or PMA and are shown as mean with s.e.mean shown by vertical bars. Asterisks indicate significantly smaller relaxations in tissues contracted with PMA than with NA: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ($n = 6$).

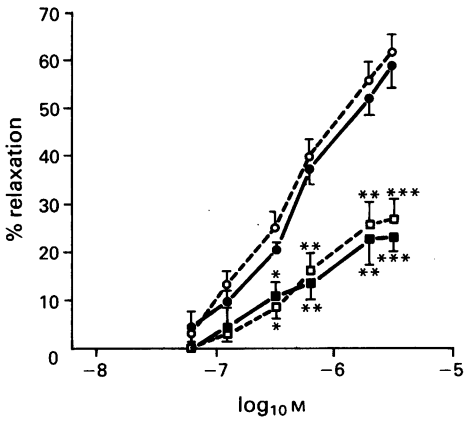


Figure 5 Concentration-effect curves for nitric oxide (NO) in aortic rings contracted with noradrenaline (NA, 0.2 μM; circles) or phorbol 12-myristate 13-acetate (PMA, 0.5 μM; squares) in the presence (open symbols) and absence (closed symbols) of endothelium. Relaxations are expressed as a % of the contractions to NA or PMA and are shown as mean with s.e.mean shown by vertical bars. Asterisks indicate significantly smaller relaxations in tissues contracted with PMA than with NA: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ ($n = 6$).

in tissues contracted with PMA than in tissues contracted with NA ($P < 0.001$) (Figure 6).

Discussion

This study examined the influence of contractile agents used to raise muscle tone, with different mechanisms of action, on the degree and nature of vascular relaxation in rat isolated aortic rings. The results obtained clearly show that relaxations induced by ACh and NO are significantly smaller in tissues contracted with the phorbol ester PMA than in tissues contracted with NA. Furthermore, this effect is associated with significantly smaller increases in tissue content of cyclic GMP.

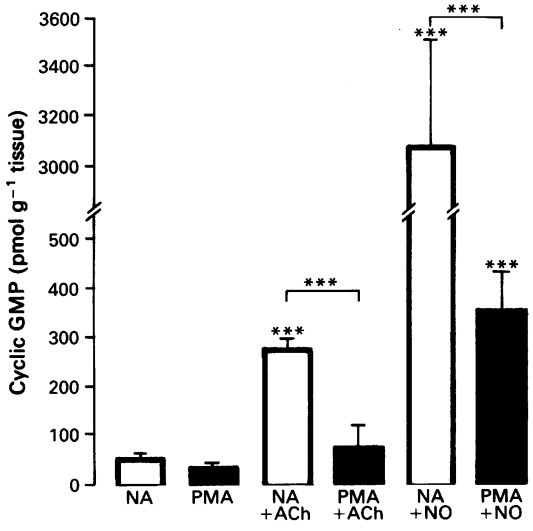


Figure 6 Effects of acetylcholine (ACh, 10 μM; 30 s) and nitric oxide (NO, 3 μM; 20 s) on levels of cyclic GMP in aortic rings contracted with noradrenaline (NA, 0.2 μM) or phorbol 12-myristate 13-acetate (PMA, 0.5 μM). Results are shown as mean with s.e.mean indicated by vertical bars. ACh significantly increased levels of cyclic GMP only in tissues contracted with NA ($^{***}P < 0.001$). NO significantly increased levels of cyclic GMP both in tissues contracted with NA or PMA ($^{***}P < 0.001$). The levels of cyclic GMP induced by ACh and NO were significantly smaller in tissues contracted with PMA than with NA ($^{***}P < 0.001$); ($n = 6$).

In rat aorta, postsynaptic α-adrenoceptors may be classified as a homogeneous population of α_1-adrenoceptors (Beckeringh *et al.*, 1984). Contractions of this tissue to NA may therefore be considered to be mediated solely by α_1-adrenoceptors. Activation of α_1-adrenoceptors results in the accelerated metabolism of membrane phosphoinositides and the rapid generation of 1,2 diacylglycerol (DG) and inositol 1,4,5 trisphosphate (IP_3) (Berridge, 1981; Michell *et al.*, 1981; Villalobos-Molina *et al.*, 1982; Rapoport, 1986). This process appears to be controlled by guanine nucleotide-dependent regulatory proteins (Downes, 1989). The production of DG and IP_3 initiates a bifurcating signal pathway leading to muscle contraction via two synergistic pathways: (i) the Ca^{2+}-calmodulin pathway which is activated by increased intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$; and (ii) activation of protein kinase C. DG is the endogenous activator of protein kinase C and its actions may be mimicked by tumour-promoting phorbol esters such as PMA (Castagna *et al.*, 1982). However, sustained tonic contraction of vascular smooth muscle to α-adrenoceptor agonists may be mediated solely by influx of Ca^{2+} from extracellular sites with only a minor role for protein kinase C suggested (Khalil & Van Breemen, 1988). PMA, unlike NA, will activate protein kinase C without the α_1-adrenoceptor-linked generation of DG and IP_3 via hydrolysis of phosphoinositides. Indeed, the inactive phorbol, 4αPDD did not alter basal tension. Furthermore, PMA did not induce accelerated hydrolysis of phosphoinositides in rat aorta (unpublished data). Therefore, in the present study, relaxations induced by acetylcholine and NO were studied in rings of rat aorta contracted by spasmogens with different mechanisms of action.

ACh, an endothelium-dependent relaxant (Furchgott & Zawadzki, 1980) and NO, which may be one of the endothelium-derived relaxing factors (Palmer *et al.*, 1987; Shikano *et al.*, 1988) induced concentration-dependent relaxations of tissues contracted with NA. Relaxations to NO were transient in nature, possibly due to the inactivation of NO by free radicals such as superoxide present in the organ chambers and tissues. ACh-induced relaxations were more stable in nature since ACh is inactivated more slowly than NO, and is presumably still stimulating endothelial muscarinic receptors leading to the continued production/release of EDRF (s). The relaxations to ACh and NO were associated with significant increases above control values in the tissue content of cyclic GMP. Both NO and the pharmacologically indistinguishable EDRF stimulate soluble guanylate cyclase directly resulting in production of cyclic GMP (Katsuki *et al.*, 1977; Ignarro *et al.*, 1986; 1987). In the present series of experiments, cyclic GMP generated in response to ACh and NO may have inhibited α_1-adrenoceptor-mediated contractions by several mechanisms. Of particular interest is the possibility that cyclic GMP lowered $[Ca^{2+}]_i$ by inhibiting the hydrolysis of phosphoinositides, stimulated by NA, and hence the generation of IP_3 (Rapoport, 1986). This effect may be species-dependent since activation of soluble guanylate cyclase did not inhibit the hydrolysis of phosphoinositides in rabbit aorta (Kajikuri & Kuriyama, 1990).

Relaxations to ACh and NO were significantly reduced in tissues in which muscle tone was raised with an equi-effective concentration of PMA. Moreover, in these tissues, the rate of onset of relaxation appeared to be much slower. This effect may be accounted for by the further generation, by PMA, of free radicals that were able to inactivate NO. Indeed, PMA enhanced the release of superoxide anions from cultured endothelial cells (Matsubara & Ziff, 1986). The reduced ability to relax aortic rings was reflected in smaller increases in levels of cyclic GMP. Since PMA does not stimulate hydrolysis of membrane phosphoinositides, it may be proposed that enhanced hydrolysis of phosphoinositides is first necessary for maximum relaxation of aortic rings to be observed in response to these relaxants. Consistent with this hypothesis is the observation in pig aortic smooth muscle that phosphoinositides were required for maximal stimulation of the plasmalemmal Ca^{2+}-Mg^{2+}-ATPase by protein kinase G, activated by cyclic GMP, in reducing $[Ca^{2+}]_i$ during muscle relaxation (Vrollx *et al.*, 1988). The present results therefore suggest that the mechanism (s) activated during contraction of aortic rings to PMA may not be susceptible or sensitive to the mechanism (s) of relaxation induced by EDRF (NO) and cyclic GMP.

The small relaxations induced by ACh and NO in tissues contracted with PMA may be due to inhibition of myosin light chain kinase by cyclic GMP-dependent protein kinase since contraction is mediated by phosphorylation of the myosin light chains by protein kinase C (Aksoy *et al.*, 1983). This effect is evidently insufficient to mediate a full relaxant response. It is uncertain whether cyclic GMP would affect Ca^{2+} homeostasis in tissues contracted with PMA since there is some doubt as to increased $[Ca^{2+}]_i$ associated with PMA-induced contraction (Sybertz *et al.*, 1986; Khalil & Van Breemen, 1988).

It is interesting to note that the reduced levels of cyclic GMP induced by NO in tissues contracted with PMA were still much greater than those measured in response to a maximal relaxant concentration of ACh in tissues contracted with NA. This result is unexpected if it is assumed that relaxations to both ACh and NO are mediated by cyclic GMP alone. However, this apparent discrepancy may be accounted for by the ACh-induced release of an additional relaxant substance, the endothelium-derived hyperpolarizing factor (Chen *et al.*, 1988; Taylor & Weston, 1988). Thus relaxations to ACh are mediated by at least two factors, and lower levels of cyclic GMP may therefore be required to mediate the physiological response. Furthermore, the ACh-EDRF-cyclic GMP pathway may be more efficiently coupled than the NO-cyclic GMP interaction and so less cyclic GMP is produced to exert relaxation. NO may have induced excess levels of cyclic GMP because this nitrovasodilator stimulates soluble guanylate cyclase directly (Murad *et al.*, 1978), and this action may be less susceptible to physiological manipulation by the contractile agents than the synthesis/release of EDRF (s) that is activated by ACh. Another possibility is that PMA inhibited the relaxant actions of cyclic GMP at the level of the contractile proteins, thus creating the discrepancy between the degree of relaxation and the levels of cyclic GMP obtained in response to NO. However, the close correlation between the size of ACh-induced relaxations and the levels of cyclic GMP argues against this mechanism.

It may be postulated, that in this study, the synthesis and/or release of EDRF was inhibited by PMA. Such a mechanism, involving stimulation of endothelial protein kinase C and phosphorylation of the GTP binding protein G_i, has been observed in guinea-pig pulmonary artery (Weinheimer *et al.*, 1986). Moreover, the release of EDRF, as measured by the production of cyclic GMP, was inhibited by phorbol 12,13 dibutyrate in cultured pig aortic endothelial cells (Smith & Lang, 1990). In the present experiments, however, stimulation of endothelial protein kinase C alone cannot fully explain the results since relaxations to both ACh (endothelium-dependent) and NO were inhibited to the same degree in tissues contracted with PMA. If PMA, via activation of only endothelial protein kinase C, were inhibiting the synthesis and/or release of EDRF (s) stimulated by ACh, then it would be expected that ACh-induced relaxations would be inhibited but relaxations to NO would be unaffected by PMA. The results obtained here therefore suggest that PMA inhibits the action of EDRF (NO) on smooth muscle. This action may occur at the level of soluble guanylate cyclase and involve the phosphorylation of this enzyme (Zwiller *et al.*, 1985). However, the possibility cannot be excluded that PMA inhibited the synthesis/release of EDRF (s) since this action may have been masked by the more predominant effect in vascular smooth muscle. Interestingly, in bioassay experiments, both the release and action of EDRF (s) were inhibited by phorbol 12,13 dibutyrate in canine blood vessels (Rubanyi *et al.*, 1989). The possibility that PMA inhibited the actions of NO in the endo-

thelial cells seems unlikely since the mechanical responses to NO were similar in the presence and absence of endothelium. Furthermore, the ability of NO to stimulate production of cyclic GMP was not affected by removal of the endothelium (data not shown).

In conclusion, relaxations of rat aorta to ACh and NO were markedly less pronounced in tissues contracted with PMA than in tissues contracted with NA. This effect may have been caused by inhibition of the action (s) of EDRF (s) (NO) by a mechanism involving stimulation of protein kinase C in vascular smooth muscle cells.

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References

AKSOY, M.O., MRAS, S., KAMM, K.E. & MURPHY, R.A. (1983). Ca^{2+}, cAMP, and changes in myosin phosphorylation during contraction of smooth muscle. *Am. J. Physiol.*, **245**, C255–C270.

BECKERINGH, J.J., THOOLEN, M.J.M.C., DE JONGE, A., WILFFERT, B., TIMMERMANS, P.B.M.W.M. & VAN ZWEITEN, P.A. (1984). The contractions induced in rat and guinea-pig aortic strips by the α_2-adrenoceptor selective agonists B-HT 920 and UK 14,304 are mediated by α_1-adrenoceptors. *Eur. J. Pharmacol.*, **104**, 197–203.

BERRIDGE, M.J. (1981). Phosphatidylinositol hydrolysis: a multifunctional transducing mechanism. *Mol. Cell. Endo.*, **24**, 115–140.

CASTAGNA, M., TAKAI, Y., KAIBUCHI, K., SANO, K., KIKKAWA, U. & NISHIZUKA, Y. (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.*, **257**, 7847–7851.

CHEN, G., SUZUKI, H. & WESTON, A.H. (1988). Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. *Br. J. Pharmacol.*, **95**, 1165–1174.

DOWNES, C.P. (1989). G protein regulation of phospholipase C. *Trends Pharmacol.*, **10** (suppl): 39–42, 1989.

FURCHGOTT, R.F. (1983). Role of the endothelium in responses of vascular smooth muscle. *Circ. Res.*, **53**, 557–573.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

IGNARRO, L.J., HARBISON, R.G., WOOD, K.S. & KADOWITZ, P.J. (1986). Activation of purified soluble guanylate cyclase by endothelium-derived relaxing factor from intrapulmonary artery and vein: stimulation of acetylcholine, bradykinin and arachidonic acid. *J. Pharmacol. Exp. Ther.*, **237**, 893–900.

IGNARRO, L.J., BYRNS, R.E., BUGA, G.M. & WOOD, K.S. (1987). Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. *Circ. Res.*, **61**, 866–879.

KAJIKURI, J. & KURIYAMA, H. (1990). Inhibitory action of α-human atrial natriuretic peptide on noradrenaline-induced synthesis of myo-inositol 1,4,5-trisphosphate in the smooth muscle cells of rabbit aorta. *Br. J. Pharmacol.*, **99**, 536–540.

KATSUKI, S., ARNOLD, W., MITTAL, C. & MURAD, F. (1977). Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. *J. Cyclic Nucleotide Res.*, **3**, 23–35.

KHALIL, R.A. & VAN BREEMEN, C. (1988). Sustained contraction of vascular smooth muscle: calcium influx or C-kinase activation? *J. Pharmacol. Exp. Ther.*, **244**, 537–542.

MATSUBARA, T. & ZIFF, M. (1986). Superoxide anion release by human endothelial cells: synergism between a phorbol ester and a calcium ionophore. *J. Cell. Physiol.*, **127**, 207–210.

MITCHELL, R.H., KIRK, C.J., JONES, L.M., DOWNES, C.P. & CREBA, J.A. (1981). The stimulation of inositol lipid metabolism that accompanies calcium mobilization in stimulated cells: defined characteristics and unanswered questions. *Phil. Trans. R. Soc. B*, **296**, 123–138.

MORRISON, K.J. & POLLOCK, D. (1988a). Effects of vasodilators on tone, cyclic guanosine monophosphate levels and phosphatidylinositol hydrolysis in rat aortic rings precontracted with noradrenaline, potassium chloride or phorbol 12-myristate 13-acetate. *J. Muscle Res. Cell Motil.*, **9**, 460.

MORRISON, K.J. & POLLOCK, D. (1988b). Effects of acetylcholine and nitric oxide on coupling mechanisms underlying contraction and relaxation in rat isolated aortic rings. *Br. J. Pharmacol.*, **95**, 519P.

MURAD, F., MITTAL, C.K., ARNOLD, W.P., KATSUKI, S. & KIMURA, H. (1978). Guanylate cyclase: activation by azide, nitro compounds, nitric oxide, and hydroxyl radical and inhibition by hemoglobin and myoglobin. *Adv. Cyclic Nucleotide Res.*, **9**, 145–158.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

RAPOPORT, R.M. (1986). Cyclic guanosine monophosphate inhibition of contraction may be mediated through inhibition of phosphatidylinositol hydrolysis in rat aorta. *Circ. Res.*, **58**, 407–410.

RAPOPORT, R.M. & MURAD, F. (1983). Endothelium-dependent and nitrovasodilator-induced relaxation of vascular smooth muscle: role of cyclic GMP. *J. Cyclic Nucleotide Protein Phosphorylation Res.*, **9**, 281–296.

RUBANYI, G.M., DESIDERIO, D., LUISI, A., JOHNS, A. & SYBERTZ, E.J. (1989). Phorbol dibutyrate inhibits release and action of endothelium-derived relaxing factor(s) in canine blood vessels. *J. Pharmacol. Exp. Ther.*, **249**, 858–863.

SHIKANO, K., LONG, C.J., OHLSTEIN, E.H. & BERKOWITZ, B.A. (1988). Comparative pharmacology of endothelium-derived relaxing factor and nitric oxide. *J. Pharmacol. Exp. Ther.*, **247**, 873–881.

SMITH, J.A. & LANG, D. (1990). Release of endothelium-derived relaxing factor from pig cultured aortic endothelial cells, as assessed by changes in endothelial cell cyclic GMP content, is inhibited by a phorbol ester. *Br. J. Pharmacol.*, **99**, 565–571.

SYBERTZ, E.J., DESIDERIO, D.M., TETZLOFF, G. & CHIU, P.J.S. (1986). Phorbol dibutyrate contractions in rabbit aorta: calcium dependence and sensitivity to nitrovasodilators and 8-BR-cyclic GMP. *J. Pharmacol. Exp. Ther.*, **239**, 78–83.

TAYLOR, S.G. & WESTON, A.H. (1988). Endothelium-derived hyperpolarizing factor: a new endogenous inhibitor from the vascular endothelium. *Trends Pharmacol. Sci.*, **9**, 272–274.

VILLALOBOS-MOLINA, R., UC, M., HONG, E. & GARCIA-SAINZ, J.A. (1982). Correlation between phosphatidylinositol labeling and contraction in rabbit aorta: effect of alpha-1 adrenergic activation. *J. Pharmacol. Exp. Ther.*, **222**, 258–261.

VROLLX, M., RAEYMAEKERS, L., WUYTACK, F., EGGERMONT, J., HOFMANN, F. & CASTEELS, R. (1988). Stimulation of $[Ca^{2+} + Mg^{2+}]$ATPase by cGMP-dependent protein kinase may involve phosphoinositides. *J. Muscle Res. Cell Motil.*, **9**, 462.

WEINHEIMER, G., WAGNER, B. & OSSWALD, H. (1986). Interference of phorblesters with endothelium-dependent vascular smooth muscle relaxation. *Eur. J. Pharmacol.*, **130**, 319–322.

ZWILLER, J., REVEL, M.O. & ANANT, N.M. (1985). Protein kinase C catalyses phosphorylation of guanylate cyclase *in vitro*. *J. Biol. Chem.*, **260**, 1350–1353.

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Effects of ω-conotoxin GVIA on autonomic neuroeffector transmission in various tissues

A. De Luca, C.G. Li, M.J. Rand, [1]J.J. Reid, P. Thaina & H.K. Wong-Dusting

Department of Pharmacology, University of Melbourne, Victoria 3052, Australia

1 The effects of ω-conotoxin GVIA (conotoxin), a potent inhibitor of neuronal N-type Ca^{2+} channels, have been examined on responses to stimulation of noradrenergic, cholinergic and non-adrenergic, non-cholinergic (NANC) nerves in a range of isolated tissues to investigate the role of conotoxin-sensitive Ca^{2+} channels in neurotransmission.

2 Contractions elicited by field stimulation of noradrenergic nerves in rat and mouse anococcygeus muscles, rabbit ear artery and rat vas deferens (epididymal portion) were inhibited by conotoxin. Responses to noradrenaline, and to adenosine triphosphate in the vas deferens, were not affected.

3 Positive chronotropic responses to field stimulation of noradrenergic nerves were inhibited by conotoxin in rat and mouse atria, but responses to noradrenaline and tyramine were not affected.

4 The stimulation-induced release of noradrenaline was inhibited by conotoxin in the rabbit ear artery and in rat and mouse atria.

5 Relaxations in response to stimulation of the noradrenergic perivascular mesenteric nerves were reduced or abolished by conotoxin in rat and rabbit jejunum. The response to noradrenaline in rat jejunum was not affected.

6 Contractions elicited by stimulation of cholinergic nerves were inhibited by conotoxin in rat jejunum and mouse ileum (perivascular mesenteric nerves), and in guinea-pig taenia caeci (field stimulation). Responses to acetylcholine in rat jejunum and mouse ileum were not affected.

7 Contractions elicted by stimulation of the cholinergic plus NANC pelvic nerves were inhibited by conotoxin in rabbit colon, and to a lesser extent in guinea-pig colon. The stimulation-induced contraction of the guinea-pig colon was inhibited by conotoxin by a greater proportion in the presence than in the absence of atropine. Responses to acetylcholine were not affected in the rabbit colon but were slightly reduced in the guinea-pig colon.

8 Relaxations in response to field stimulation of NANC nerves were inhibited by conotoxin in guinea-pig taenia caeci and rat gastric fundus strips, and in rat anococcygeus muscle when the tone was raised by guanethidine but not when it was raised by carbachol. The relaxations produced by sodium nitroprusside in the rat gastric fundus and anococcygeus were not affected.

9 Contractions of the rat bladder elicited by stimulation of the peri-urethral nerves, which are NANC- and cholinergically mediated, were relatively insensitive to inhibition by conotoxin. The responses were almost completely abolished by tetrodotoxin.

10 The conotoxin-induced inhibitions of responses to nerve stimulation developed slowly and persisted after removal of conotoxin.

11 The inhibitory effect of conotoxin was inversely proportional to the frequency of stimulation (in several preparations) and to the Ca^{2+} concentration in the bathing solution (in rat vas deferens). These observations suggest that the inhibition by conotoxin of the Ca^{2+} influx required for excitation-secretion coupling in autonomic nerve terminals is not absolute, and can be overcome by repeated stimulation or by raising the Ca^{2+} concentration.

Introduction

Transmitter release from nerve terminals during depolarization produced by invasion of action potentials is thought to depend on the influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels (VSCC) of the N-type (Augustine *et al.*, 1987; Miller, 1987). ω-Conotoxin GVIA (referred to as conotoxin henceforth) is a potent inhibitor of neuronal VSCC (Kerr & Yoshikami, 1984; Cruz *et al.*, 1987; 1988; Miller, 1987; Tsien *et al.*, 1988), but does not affect the propagation of action potentials into the terminals of motoneurones (Kerr & Yoshikami, 1984) or sympathetic neurones (Brock *et al.*, 1989).

Conotoxin has been shown to block responses, in autonomically innervated tissues, to stimulation of noradrenergic (Maggi *et al.*, 1988b; Mohy El-Din & Malik, 1988; Brock *et al.*, 1989; Clasbrummel *et al.*, 1989; Keith *et al.*, 1989; McKnight *et al.*, 1989), cholinergic (Lundy & Frew, 1988; Keith *et al.*, 1989) and non-adrenergic, non-cholinergic (NANC) (Maggi *et al.*, 1988a,b) nerves in a variety of tissues. However, there are some anomalies, since it has been shown that NANC-mediated responses of the rat duodenum (Maggi *et al.*, 1988b) and rat anococcygeus muscle (McKnight *et al.*, 1989) are not sensitive to conotoxin, and the NANC-mediated responses of rat bladder are only slightly inhibited by conotoxin (Maggi *et al.*, 1988b).

It is possible that studies on the inhibition by conotoxin of Ca^{2+} influx through N-type channels might provide new pharmacological and physiological insights into mechanisms of neurotransmission, but further information is needed. Therefore, the aim of the present study was to re-examine some of the apparent anomalies, and to extend the range of tissues used, in an attempt to determine whether or not N-type Ca^{2+} channels are involved in all types of autonomic neurotransmission in all tissues. Preliminary accounts of some of these studies have been communicated to the Australasian Society of Clinical and Experimental Pharmacologists (De Luca & Rand, 1990; De Luca *et al.*, 1990; Li & Rand, 1990a; Reid *et al.*, 1990).

[1] Author for correspondence.

Methods

Sprague-Dawley rats (200–300 g), New Zealand White rabbits (2–4 kg), random-bred Swiss White mice (20–40 g) and Dunkin-Hartley guinea-pigs (400–600 g) were killed by stunning with a blow to the head and exsanguination, and the required tissues were removed as rapidly as possible.

The physiological salt solution (PSS) used for all the isolated tissues had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.45, $NaHCO_3$ 25, KH_2PO_4 1.03, D-(+)-glucose 11.1, disodium edetate 0.067 and ascorbic acid 0.14.

The reservoirs and organ baths containing PSS were gassed continuously with 5% CO_2 and 95% O_2. The PSS passed through a heat exchanger at 37°C before contact with tissues, and organ baths were maintained at 37°C. Organ baths were silicone coated with Coatasil (Ajax Chemicals, Australia) to prevent adsorption of conotoxin. Preparations were allowed to equilibrate for 30–60 min, with frequent changes of PSS for those mounted in organ baths.

Electrical stimulation was with 0.5–1 ms pulses delivered from Grass S88, S44 or S11 stimulators; supramaximal voltages were used for eliciting responses. Other stimulation parameters (frequency and train length) are described below in Methods and in Results. Extrinsic nerves were threaded through bipolar platinum ring electrodes of the type described by Burn & Rand (1960). Intramural nerves were field stimulated through parallel platinum wires on either side of the tissue.

The activity of longitudinally arranged smooth muscle was measured either isometrically with a strain gauge transducer or isotonically with a Ugo Basile 7006 transducer, as stated below, and was displayed on Rikadenki potentiometric recorders. For isotonic recordings, changes in length were magnified either 5 or 12.5 fold for colon preparations and either 10 or 25 fold for other preparations. Calibrations on records of responses of these tissues have been corrected for amplification.

For all preparations, time control studies were carried out; the functional responses to both nerve stimulation and to exogenously added transmitters remained constant over the time course of the experiments.

Preparation of isolated tissues

Rat and rabbit jejunum Preparations were set up as described by De Luca & Rand (1989). Briefly, segments (rat, about 1.5 cm long; rabbit, 2–3 cm long) were taken from the proximal portion of the jejunum together with the branches of the mesenteric blood vessels supplying the segment and mounted in 20 ml organ baths for isotonic recording (tension: rat, 0.5 g wt; rabbit, 0.7 g wt). The main blood vessel (with the accompanying perivascular nerves) was threaded through bipolar platinum ring electrodes.

Mouse ileum Segments about 1 cm long were taken from the distal portion of the ileum together with the branches of the mesenteric blood vessels supplying the segment. The tension was 0.5 g wt. Other details were as for rat and rabbit jejunum.

Rabbit and guinea-pig colon Segments (3–4 cm in length) of distal colon with the parasympathetic pelvic nerves were dissected out as described by Garry & Gillespie (1954) and set up in 100 ml organ baths for isotonic recording under a tension of 3 g wt for rabbit and 1.5 g wt for guinea-pig colon. The pelvic nerves were threaded through bipolar platinum electrodes.

Guinea-pig taenia caeci Preparations were set up as described by Burnstock *et al.* (1966) in a 5 ml organ bath for isometric recording and field stimulation under a tension of 1 g wt.

Rat gastric fundus Strips of the gastric fundus (approximately 15 mm long and 3 mm wide) were set up as described by Vane (1957) in an 8 ml organ bath for isotonic recording under a tension of 1 g wt and field stimulation. The PSS contained atropine (3 μM) and guanethidine (5 μM) to block cholinergic and noradrenergic involvement, and 5-hydroxytryptamine (5 μM) to raise the tone.

Rat and mouse anococcygeus muscles Anococcygeus muscles were set up, as described by Li *et al.* (1988) for rat and by Gibson & Wedmore (1981) for mouse, in 5 ml organ baths for isometric recording and field stimulation (tension: rat, 1 g wt; mouse, 0.3 g wt).

Rat vas deferens Segments about 1 cm long of the epididymal end of vasa deferentia were set up, as described by McCulloch *et al.* (1985), in a 4 ml organ bath for isometric recording with a resting tension of 1 g wt and field stimulation.

Rat urinary bladder Preparations were set up, as described by Hukovic *et al.* (1965), in a 30 ml organ bath for isometric recording under a tension of 1 g wt. The right urethra with the accompanying nerves was threaded through bipolar platinum electrodes.

Rat and mouse atria Atria were set up in 5 ml organ baths for isometric measurement of spontaneous contractions and field stimulation under resting tensions of 1 g wt for rat and 0.25 g wt for mouse atria, as described by Wong-Dusting *et al.* (1989). The force and rate (derived from a tachometer coupler) of contractions were displayed on a Grass polygraph. Atropine (1 μM) was present in the PSS to block responses to cholinergic nerve stimulation.

Rabbit ear artery Segments of artery were set up for perfusion and superfusion at 4 ml min^{-1} under a resting tension of 0.5 g wt, as described by Wong-Dusting & Rand (1988). The perivascular nerves were stimulated through bipolar platinum electrodes. Increases in perfusion pressure, measured with a strain gauge manometer, were used as indices of vasoconstrictor responses.

Labelling of noradrenergic transmitter stores with [^{3}H]-noradrenaline and release of radioactivity

Rat and mouse atria and rabbit ear arteries were incubated in (−)-[7,8-^{3}H]-noradrenaline (atria: 0.27 μM, 4 μCi ml^{-1} for 20 min; arteries: 0.67 μM, 10 μCi ml^{-1} for 60 min) to label transmitter stores. The stimulation-induced release of radioactivity was used as an index of the release of transmitter noradrenaline as described by Wong-Dusting *et al.* (1989).

Drugs

The drugs used and their sources were: acetylcholine perchlorate (BDH); adenosine triphosphate disodium salt (ATP, Sigma); atropine sulphate (Sigma); carbachol (carbamylcholine chloride, Sigma); ω-conotoxin GVIA (conotoxin, Peninsula); guanethidine sulphate (Ciba); 5-hydroxytryptamine creatinine sulphate complex (Sigma); noradrenaline bitartrate (Sigma); (−)-[7,8-^{3}H]-noradrenaline (specific activity 15 Ci $mmol^{-1}$, Amersham); phentolamine mesylate (Ciba); sodium nitroprusside (Sigma); tetrodotoxin (Sigma); tyramine hydrochloride (Sigma).

Statistical analysis

Quantitative data are expressed as means and s.e.mean. Differences between means were assessed by Student's *t* test (two-tailed), or by analysis of variance (ANOVA) followed by planned comparisons, as indicated. Analysis by ANOVA was carried out with the software package CSS (Statsoft). Values

of $P < 0.05$ were taken to indicate statistically significant differences.

Results

Gastrointestinal neuroeffector preparations

Rabbit jejunum Stimulation of the mesenteric perivascular nerves with 30 s trains of pulses at 2–20 Hz produced graded inhibitions of pendulum movements and the higher frequencies also caused relaxation, as shown for stimulation at 4, 8 and 16 Hz in Figure 1. Once the frequency-response relationship had been constructed, repeated responses to stimulation producing 50–70% inhibition of pendulum movements were elicited at 3 min intervals until their reproducibility was established. Conotoxin (50 nM) produced a gradual reduction of these responses, and its effect reached a stable level after 15–20 min exposure. After 30 min exposure to conotoxin, the frequency-response relationship was determined again. Responses to stimulation at 2 and 4 Hz were virtually abolished and those at 8 Hz were significantly reduced to $33 \pm 4\%$ ($n = 3$) of the control value ($P < 0.05$, two-way ANOVA with repeated measures followed by planned comparison) but those at 16 and 20 Hz were not affected (Figure 1).

Rat jejunum The nature of the response to stimulation of the mesenteric perivascular nerves depended on the frequency, as described by De Luca & Rand (1989). Graded contractions were elicited at 0.5, 1 and 2 Hz; there were usually contractions at 5 Hz and relaxations with inhibition of pendulum movements were usually produced at 10 and 20 Hz. Contractions and relaxations elicited in separate preparations by stimulation for 30 s at 5 Hz and 10 Hz, respectively, were greatly attenuated after exposure to 50 nM conotoxin for 15 min, but the corresponding responses to acetylcholine and noradrenaline were not decreased (Figure 2). The residual responses to stimulation were not abolished even when the concentration of conotoxin was increased to 200 nM, but were abolished by tetrodotoxin (1 μM).

Mouse ileum Stimulation of the mesenteric perivascular nerves at 2–20 Hz elicited frequency-dependent contractions (Figure 3). Acetylcholine (1–10 nM) produced contractions in the same range as those produced by nerve stimulation. Atropine (0.1 μM) abolished responses to acetylcholine and greatly reduced the responses to stimulation, indicating that they were predominantly cholinergically mediated; the small residual response to stimulation was abolished by tetrodotoxin (1 μM). After the frequency-response relationship had been established, repeated responses to stimulation at 4 Hz were elicited at 3 min intervals, and in the presence of conotoxin (10 nM) these were gradually reduced and blockade was complete after 30 min. When the frequency-response relationship was again determined after exposure to conotoxin for 45 min, responses to stimulation at 8 Hz or less were abolished and those to 16 and 20 Hz were greatly attenuated (Figure 3).

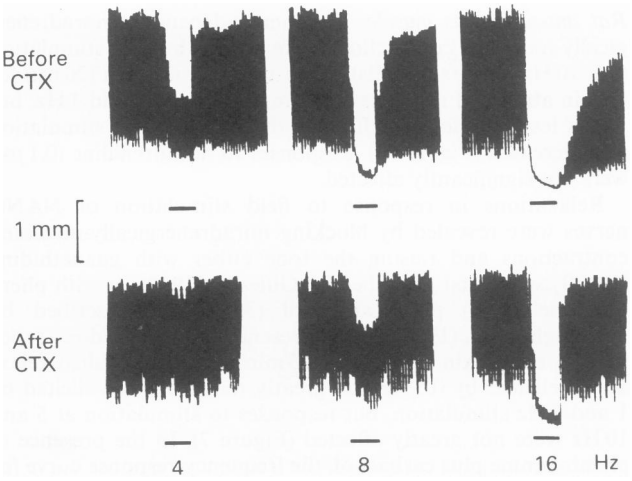

Figure 1 Responses to stimulation of perivascular mesenteric nerves with 30 s trains at 4, 8 and 16 Hz before and 30 min after exposure to 50 nM conotoxin (CTX) in segments of rabbit jejunum. Isotonic recordings were amplified by 25 fold; the vertical scale has been corrected for the amplification factor. Similar results were obtained in 3 separate experiments.

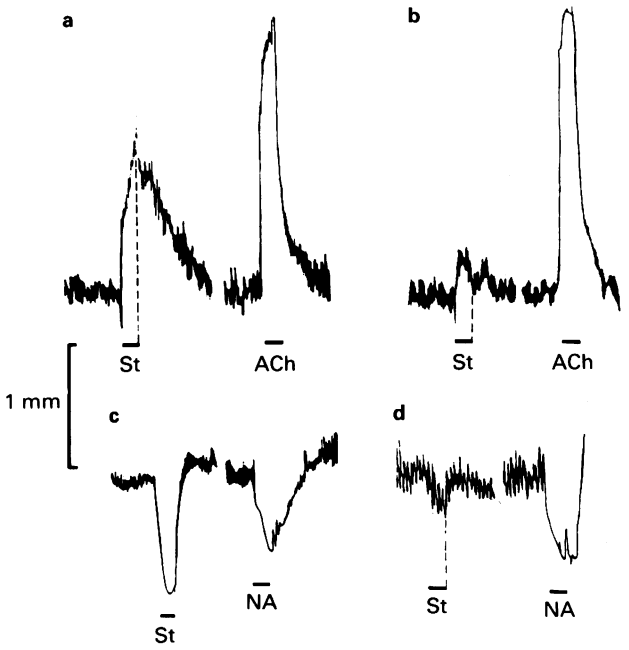

Figure 2 Effect of conotoxin (50 nM) on responses of segments of rat jejunum to stimulation of perivascular mesenteric nerves and to agonists. Contractions were elicited by stimulation (St, 5 Hz, 30 s) and by acetylcholine (ACh, 100 nM for 30 s) before (a) and 15 min after exposure to conotoxin (b). Relaxations were elicited by stimulation (St, 10 Hz, 30 s) and by noradrenaline (NA, 10 μM for 30 s) before (c) and 15 min after exposure to conotoxin (d). Isotonic recordings were amplified by 25 fold; the vertical scale has been corrected for the amplification factor. The dashed lines project from the ends of event markers for stimulation to the records. Similar observations were obtained in 2 or 3 preparations.

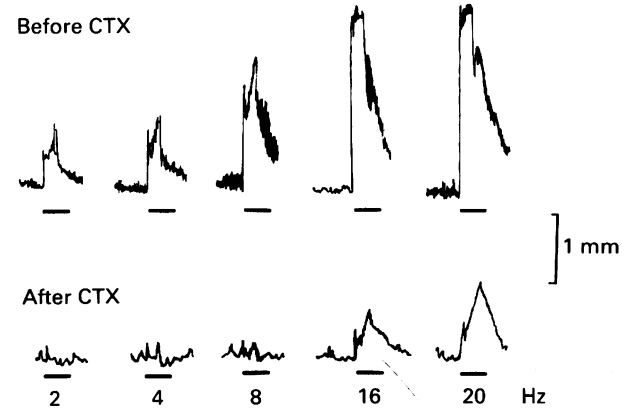

Figure 3 Contractions of a segment of mouse ileum elicited by stimulation of the perivascular mesenteric nerves at 2–20 Hz for 30 s periods before and 45 min after exposure to conotoxin (CTX, 10 nM). Isotonic recordings were amplified by 25 fold; the vertical scale has been corrected for the amplification factor.

With a higher concentration of conotoxin (50 nM), responses to stimulation at 4 Hz were abolished more rapidly and responses to 16 and 20 Hz were barely discernible.

Rabbit colon Graded contractions were elicited by stimulation of the pelvic nerves at 1, 3 and 10 Hz for 30 s periods, and the contractions produced by 0.3–3 μM acetylcholine fell within the range of the responses to stimulation. Exposure to conotoxin (20 nM) resulted in reductions of responses to stimulation that developed more rapidly and were more complete at 1 Hz than at 10 Hz (Figure 4). In these experiments, acetylcholine (3 μM) produced an initial contraction of 9.5 ± 2.6 mm (n = 4). After exposure to conotoxin for 80 min, the responses to acetylcholine were not significantly affected, being 94.3 ± 2.9% of those obtained before conotoxin.

Guinea-pig colon Graded contractions were elicited by stimulation of the pelvic nerves at 1, 3 and 10 Hz for 30 s periods. The guinea-pig colon was more sensitive to acetylcholine than the rabbit colon, and contractions produced by 0.1–0.3 μM acetylcholine fell within the range of the responses to stimulation. Exposure to conotoxin gradually reduced responses to stimulation until they stabilized after 15–30 min to about 75% of control at a concentration of 10 nM, and to about 40% of control at 30 and 100 nM. In contrast to the findings in the rabbit colon, there were no differences in the rate of development or the extent of the inhibitory effect of conotoxin at the different frequencies of stimulation, and responses to acetylcholine in the guinea-pig colon were reduced to 75–80% of control by 30 and 100 nM conotoxin. The addition of atropine (0.1 μM) after conotoxin had produced its full effect resulted in a reduction of about 50% in the remaining response. The residual response in the presence of conotoxin and atropine was abolished by tetrodotoxin (1 μM).

The effect of conotoxin was tested on the non-cholinergic component of the response to stimulation in the presence of atropine (0.1 μM), which abolished responses to acetylcholine and reduced responses to stimulation to about 30% of the initial value; in addition, an initial phase of relaxation was revealed. Exposure to conotoxin (30 nM) reduced the initial relaxation and the contraction (Figure 5), and produced a greater maximum reduction in contractile responses to stimulation (at 10 Hz for 30 s) in the presence of atropine (87% reduction) than in the absence of atropine (64% reduction).

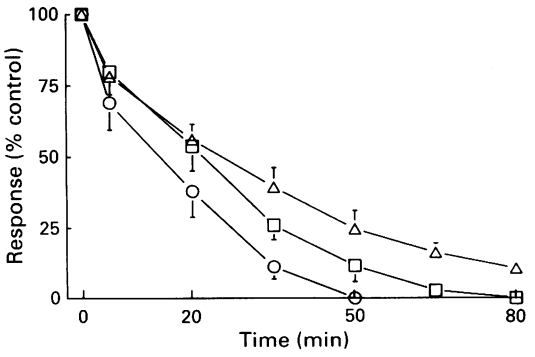

Figure 4 Effect of conotoxin (20 nM) on contractions of rabbit colon elicited by stimulation of the extrinsic pelvic nerves (○ = 1 Hz, □ = 3 Hz, △ = 10 Hz) for 30 s periods. Responses are expressed as percentages of stable control responses before exposure to conotoxin. The horizontal axis is time of exposure to conotoxin. Symbols are means and vertical lines s.e.means (n = 4), which were sometimes smaller than the size of the symbol. The control isotonic contractions (corrected for amplification) were: 1 Hz, 7.8 ± 2.5 mm; 3 Hz, 11.3 ± 2.4 mm; 10 Hz, 12.3 ± 1.6 mm. All responses in the presence of conotoxin were significantly different from the appropriate control responses (P < 0.05, two-way ANOVA with repeated measures followed by planned comparison), except that at 3 Hz for 5 min. The effect of conotoxin was significantly dependent on both time and frequency (P < 0.05, two-way ANOVA with repeated measures).

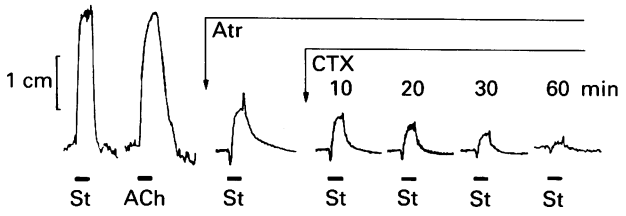

Figure 5 Effect of conotoxin (CTX, 30 nM) on responses of a segment of guinea-pig colon to stimulation (St, 10 Hz, 30 s) of the pelvic nerves in the presence of atropine (Atr, 0.1 μM), which abolished the response to acetylcholine (ACh, 0.1 μM) and reduced the stimulation-induced contraction, revealing an initial relaxation. Numbers above the records are times (in min) of exposure to conotoxin. Isotonic recordings were amplified by 5 fold; the vertical calibration has been corrected for amplification.

Guinea-pig taenia caeci Field stimulation (2 Hz, 5 s) elicited cholinergically-mediated contractions. These were gradually reduced by conotoxin (20 nM for 30 min) to 15.3 ± 1.9% (n = 4) of control responses.

NANC-mediated relaxations in response to field stimulation (0.5 Hz, 5 s) were revealed in the presence of atropine (0.3 μM) and guanethidine (5 μM) (Burnstock *et al.*, 1966). These responses were gradually reduced to 52 ± 4.5% (n = 4) and 20 ± 3.9% (n = 4) of control responses after exposure to conotoxin in concentrations of 20 nM and 100 nM, respectively, for 30 min.

Rat gastric fundus NANC-mediated relaxations were elicited by field stimulation with 60 s trains at 1, 2 and 5 Hz in the presence of atropine (3 μM) and guanethidine (5 μM) to block cholinergic and noradrenergic involvement, and 5-hydroxytryptamine (5 μM) to raise the tone. Exposure to conotoxin (50 nM) for 30 min resulted in loss of the response to stimulation at 1 Hz; responses to stimulation at 2 and 5 Hz were inhibited more slowly to 21.6 ± 3.5% and 44.2 ± 6.6% (n = 3) of control responses, respectively. Relaxations elicited by sodium nitroprusside (50 nM) were not significantly affected, being 92.3 ± 6.2% (n = 3) of initial responses after 30 min exposure to conotoxin (50 nM), and in time-control experiments without conotoxin they were 98.7 ± 4.5% (n = 4) of initial responses.

Pelvic neuroeffector preparations

Rat anococcygeus muscle Frequency-dependent noradrenergically-mediated contractions were elicited by field stimulation (0.5–10 Hz for 10 s periods). Exposure to conotoxin (20 nM) for 15 min abolished responses to stimulation at 0.5 and 1 Hz, but had a lesser inhibitory effect as the frequency of stimulation was increased (Figure 6a). Responses to noradrenaline (0.1 μM) were not significantly affected.

Relaxations in response to field stimulation of NANC nerves were revealed by blocking noradrenergically-mediated contractions and raising the tone either with guanethidine (10–20 μM), as first described by Gillespie (1972), or with phentolamine (3 μM) plus carbachol (30 μM), as described by McKnight *et al.* (1989). In the presence of guanethidine, exposure to conotoxin (20 nM) for 15 min almost abolished relaxations elicited by 0.5 Hz and greatly reduced those elicited by 1 and 2 Hz stimulation, but responses to stimulation at 5 and 10 Hz were not greatly affected (Figure 7). In the presence of phentolamine plus carbachol, the frequency-response curve for relaxations was to the right of that obtained in the presence of guanethidine, and these responses were not significantly reduced by 20 nM conotoxin (Figure 7). Relaxations elicited by sodium nitroprusside (10 nM) were not significantly affected by conotoxin: after 30 min exposure to 20 nM conotoxin and in time-control experiments without conotoxin the relaxations

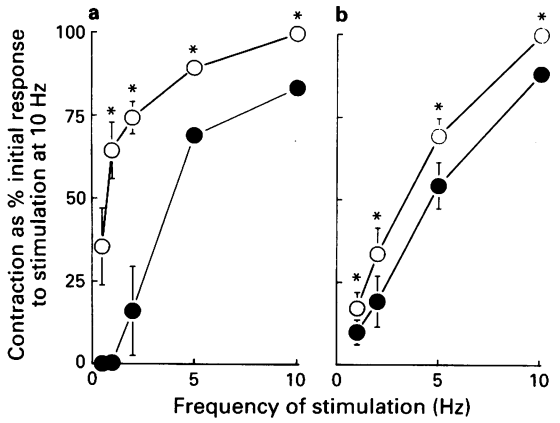

Figure 6 Contractions elicited by field stimulation (0.5–10 Hz for 10 s periods) in rat (a, $n = 5$) and mouse (b, $n = 4$) anococcygeus muscles, before (○) and after (●) 15 min exposure to conotoxin (20 nM). Responses are expressed as percentages of those elicited initially by stimulation at 10 Hz. Symbols are means and vertical lines s.e.means, which were smaller than the size of the symbol in some cases. An asterisk indicates a significant difference between responses obtained before and after exposure to conotoxin ($P < 0.05$, two-way ANOVA with repeated measures followed by planned comparison).

were 103 ± 4.9% ($n = 4$) and 109 ± 7.2% ($n = 5$), respectively, of those obtained initially.

Mouse anococcygeus muscle The effects of exposure to conotoxin (20 nM) for 15 min on noradrenergically-mediated contractions elicited by field stimulation (0.5–10 Hz for 10 s periods) are shown in Figure 6b. Responses to stimulation at all frequencies were significantly reduced.

Rat urinary bladder Frequency-dependent contractions were elicited by stimulation (0.5–50 Hz for 10 s periods) of the peri-urethral nerves. The effects of conotoxin in one experiment are illustrated in Figure 8a. After exposure to conotoxin (20 or 100 nM) for 30 min, responses to stimulation were reduced to an increasing extent by increasing the frequency from 0.5 to 2 Hz, but were reduced to a decreasing extent as the frequency

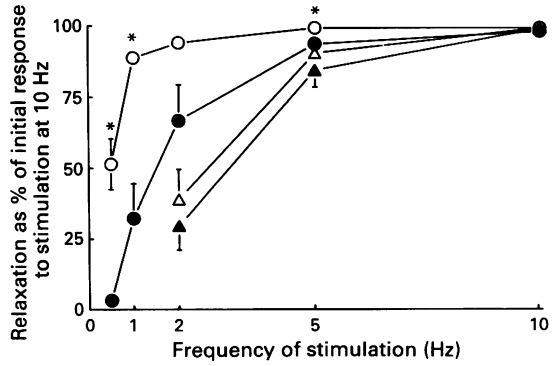

Figure 7 Relaxations of rat anococcygeus muscles elicited by field stimulation (0.5–10 Hz for 10 s periods) in the presence of guanethidine (10–20 μM; ○, ●; $n = 5$) or in the presence of phentolamine plus carbachol (3 μM + 30 μM; △, ▲; $n = 4$), before (○, △) and after (●, ▲) 15 min exposure to conotoxin (20 nM). Responses are expressed as percentages of those elicited initially by stimulation at 10 Hz. Symbols are means and vertical bars show s.e.means, which were smaller than the size of the symbol in some cases. An asterisk indicates a significant difference between responses in the presence of guanethidine obtained before and after exposure to conotoxin ($P < 0.05$, two-way ANOVA with repeated measures followed by planned comparison). Conotoxin had no significant effect on responses obtained in the presence of phentolamine plus carbachol.

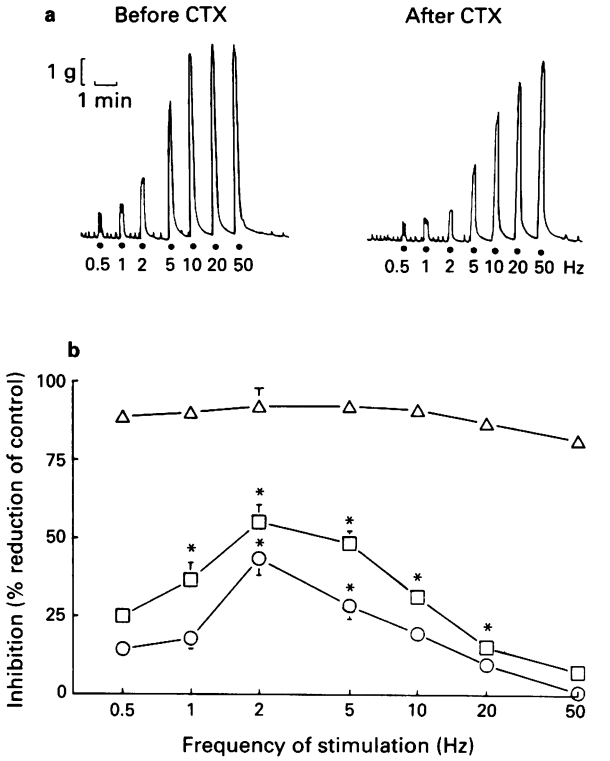

Figure 8 Effect of conotoxin on contractions of rat bladder elicited by stimulation of the peri-urethral nerves (10 s trains at 0.5–50 Hz). (a) Stimulation-induced contractions before and after 30 min exposure to conotoxin (CTX, 20 nM). (b) Inhibition of responses 30 min after exposure to 20 (○) or 100 (□) nM conotoxin or 1 μM tetrodotoxin (△). Inhibitions are expressed as percentage reductions from initial responses at each frequency of stimulation. An asterisk indicates a significant difference between responses obtained in the presence and absence of conotoxin ($P < 0.05$, two-way ANOVA with repeated measures followed by planned comparison). Symbols represent means and vertical lines show s.e.means ($n = 3$ in each case), which were smaller than the size of the symbol in some cases.

was further increased to 50 Hz (Figure 8b). The maximum reduction, which occurred at 2 Hz, was about 50% of the control response. The responses were abolished or reduced to about 10% of the control values by tetrodotoxin (1 μM) (Figure 8b).

Rat vas deferens Contractions of epididymal portions were elicited by field stimulation using a wide range of frequencies and train lengths. Responses to single pulses of stimulation were gradually reduced and abolished after 20 min of exposure to conotoxin (50 nM). Stimulation at frequencies of 0.5–2 Hz elicited responses in which individual contractions were clearly discernible; the first two were abolished and remaining ones were reduced by conotoxin (Figure 9a,b,c). At higher frequencies of stimulation, smooth contractions were elicited (e.g. Figure 9d,e) and these were reduced by conotoxin to extents that were inversely proportional to the train length and frequency of stimulation. Responses to noradrenaline (10 μM) and ATP (3 mM) were not significantly affected by conotoxin.

Changes in the Ca^{2+} concentration of the PSS The effects of conotoxin (50 nM) were investigated in the presence of a raised (5 mM) and lowered (0.625 mM) Ca^{2+} concentration on responses to stimulation that were, respectively, markedly reduced (5 pulses at 5 Hz) and only slightly reduced (10 pulses at 10 Hz) by conotoxin in a PSS with a Ca^{2+} concentration of 2.5 mM. The findings are summarized in Table 1. Compared to its effects in a PSS containing 2.5 mM Ca^{2+}, conotoxin produced a smaller reduction of the control responses when the Ca^{2+} concentration was 5 mM, and a greater reduction of the

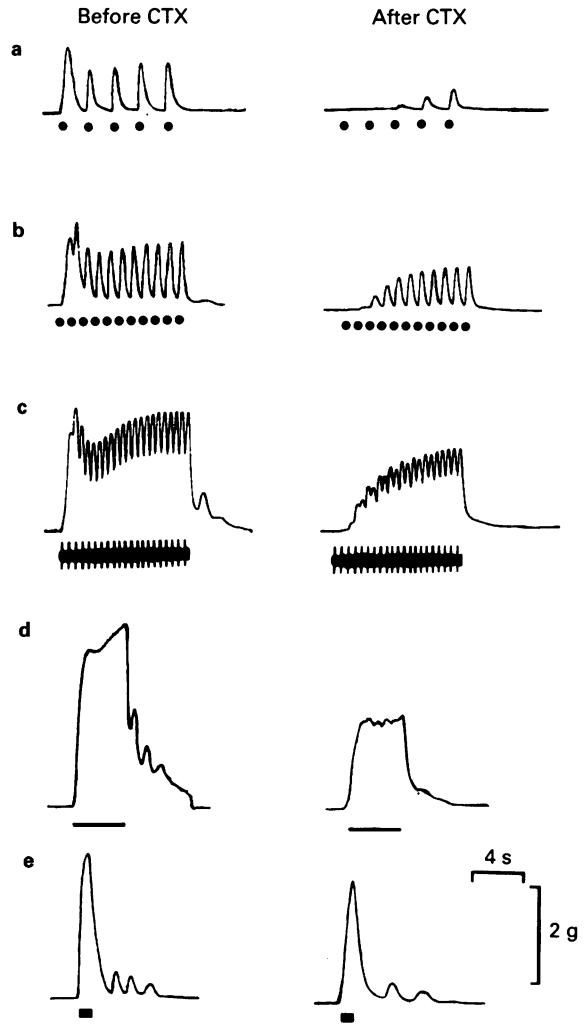

Figure 9 Responses of an epididymal portion of rat vas deferens to field stimulation (a, 5 pulses at 0.5 Hz; b, 11 pulses at 1 Hz; c, 21 pulses at 2 Hz; d, 20 pulses at 5 Hz; e, 10 pulses at 10 Hz) before (left hand records) and after (right hand records) 120 min exposure to conotoxin (CTX, 50 nM). Similar observations were obtained in 3 experiments.

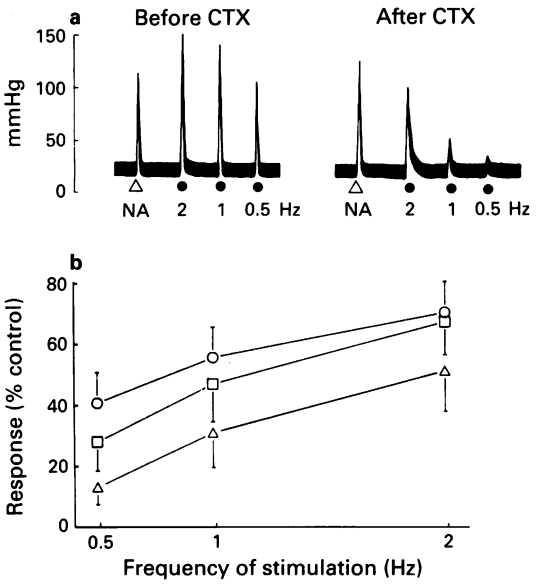

Figure 10 Effect of conotoxin on vasoconstrictor responses elicited by stimulation of the perivascular nerves in the rabbit ear artery. (a) Responses to noradrenaline (NA, 20 ng) and to 10 s trains of pulses at 0.5, 1 and 2 Hz, respectively, before and 60 min after exposure to conotoxin (CTX, 30 nM). (b) Mean results from experiments of the type illustrated in (a) after 60 min exposure to 10 (○), 20 (□) or 30 (△) nM conotoxin. Responses are expressed as percentages of those to each frequency of stimulation before exposure to conotoxin; the mean (± s.e.mean) control increases in perfusion pressure for stimulation at 0.5, 1 and 2 Hz were 28.4 ± 7.1, 46.6 ± 11.3 and 60.8 ± 13.4 mmHg, respectively. Symbols are means and vertical lines show s.e.means ($n = 4$ for each concentration of conotoxin). All responses in the presence of conotoxin were significantly different from the appropriate control responses ($P < 0.05$, two-way ANOVA with repeated measures followed by planned comparison). The effect of conotoxin was significantly dependent on both frequency and concentration of conotoxin ($P < 0.05$, two-way ANOVA with repeated measures).

control responses when the Ca^{2+} concentration was 0.625 mM. Conotoxin had no effect on responses to noradrenaline (10 μM) or ATP (3 mM) in either high or low Ca^{2+} PSS.

Cardiovascular preparations

Rabbit ear artery Vasoconstrictor responses to stimulation of the perivascular sympathetic nerves with 10 s trains at 0.5, 1 and 2 Hz were gradually reduced in the presence of conotoxin (10, 20 and 30 nM), but vasoconstrictor responses to noradrenaline were not reduced (Figure 10). The rate of development of the inhibitory effect of conotoxin on responses to nerve stimulation was concentration-dependent, the effect being fully developed in less than 30 min with 30 nM but only after 45 min with 10 nM conotoxin. The maximal fully developed reductions of responses to nerve stimulation were concentration-dependent and inversely proportional to the frequency of stimulation (Figure 10b). The inhibitory effect persisted for at least 30 min after the conotoxin was removed.

In separate experiments, the stimulation-induced (2 Hz for 10 s) efflux of radioactivity from arteries previously incubated in [^{3}H]-noradrenaline was reduced to about 60% of the corresponding control value by exposure for 30 min to 40 nM conotoxin (Table 2). This reduction in noradrenaline release was in reasonable accordance with the reduction in the vasoconstrictor response to stimulation. The reduction in the stimulation-induced release of radioactivity not only persisted after the removal of conotoxin but was greater, being about 40% of the corresponding control value (Table 2).

Table 1 Effects of changes in the Ca^{2+} concentration of the PSS on the inhibition by conotoxin (50 nM) of field stimulation-induced contractions of rat vasa deferentia

Stimulation		*Extracellular* [Ca^{2+}]	*Response*	
Pulses	*Hz*	(mM)	(% control)	n
5	5	2.5	17.6 ± 8.8	3
5	5	5	59.8 ± 8.0*	3
10	10	2.5	67.3 ± 22.4	3
10	10	0.625	8.0 ± 7.0	4

Responses are expressed as percentages of the peak tension produced by stimulation before exposure to conotoxin. Values shown are means ± s.e.means; n = number of experiments. An asterisk indicates a significant difference from the appropriate control with 2.5 mM Ca^{2+} ($P < 0.05$, unpaired t test).

Table 2 Stimulation-induced effluxes of radioactivity from tissues in which transmitter stores of noradrenaline had been labelled with [^{3}H]-noradrenaline

CTX	n	*First period* (S_1, d.p.m.)	*Second period* (% of S_1)	*Third period* (% of S_1)
Rabbit ear artery				
0	15	3002 ± 312	86.0 ± 6.4	73.1 ± 7.3
40 nM	6	2892 ± 396	50.0 ± 4.4*	29.5 ± 4.1*
Rat atria				
0	4	3965 ± 282	81.2 ± 8.3	76.2 ± 6.6
10 nM	4	4784 ± 409	14.3 ± 2.0*	22.8 ± 1.1*
Mouse atria				
0	4	16452 ± 2341	100.4 ± 3.6	
10 nM	4	16787 ± 1780	36.7 ± 2.6*	

In the rabbit ear artery and rat atria, three periods of stimulation at 2 Hz for 10 s were given. In mouse atria, two periods of stimulation at 5 Hz for 60 s were given. Stimulation periods were 45 min apart. Conotoxin (CTX) was added 30 min before the second period and removed immediately after the last collection of PSS for that period. Effluxes in the second and third periods of stimulation are expressed as percentages of effluxes in the first period of stimulation (S_1). Values are mean ± s.e.mean; n is the number of experiments. An asterisk indicates a significant difference between values obtained from experiments carried out in the presence and absence of conotoxin ($P < 0.05$, unpaired t test).

Mouse and rat atria Conotoxin (10 nM) attenuated the increases in atrial rate caused by field stimulation (0.4 to 8 Hz for 5 s) of the intramural sympathetic nerves (Figure 11). The responses were decreased to a greater extent with the lower than with the higher frequencies of stimulation, and the inhibitory effect persisted for at least 30 min after conotoxin had been removed. The decreases in responses to sympathetic nerve stimulation produced by conotoxin were not greater if the exposure time was increased from 30 to 60 min. Conotoxin (10 nM) had no effect on the increases in mouse or rat atrial rates caused by 0.1 μM noradrenaline or 3 μM tyramine (Table 3).

In atria incubated in [^{3}H]-noradrenaline, conotoxin (10 nM) reduced the stimulation-induced efflux of radioactivity, and the effect in rat atria was shown to persist for 30 min after the removal of conotoxin (Table 2). In the same experiments, the stimulation-induced positive chronotropic responses to sympathetic nerve stimulation were also reduced to approximately the same (rat) or to a lesser (mouse) extent than the effluxes of radioactivity; the increases in atrial rate in the second period of stimulation expressed as percentages of the increase in the first period were 101.0 ± 6.8% (mouse, $n = 3$) and 101.4 ± 1.4% (rat, $n = 4$) in control experiments and 68.5 ± 11.1% (mouse, $n = 4$) and 37.3 ± 4.5% (rat, $n = 4$) in the presence of 10 nM conotoxin.

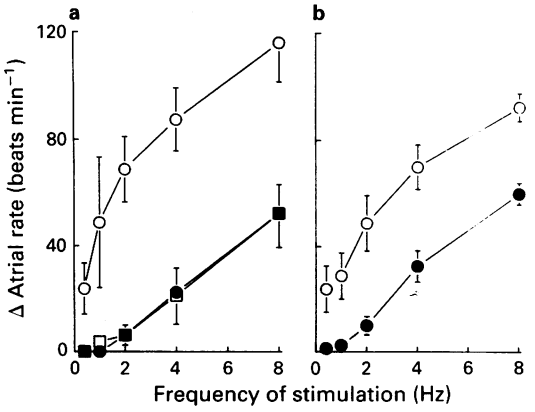

Figure 11 Increases in rate of beating produced by field stimulation (0.4, 1, 2, 4 and 8 Hz for 5 s periods, corresponding to 2, 5, 10, 20 and 40 pulses, respectively) in mouse (a) and rat (b) atria before (○) and after (●) exposure to 10 nM conotoxin for 30 min, and 30 min after the removal of conotoxin from mouse atria (□). Symbols are means and vertical lines show s.e.means ($n = 4$). There was a significant difference between responses obtained before and after exposure to conotoxin for all frequencies of stimulation except 0.4 Hz (two-way ANOVA with repeated measures followed by planned comparisons). The inhibitory effect of conotoxin was significantly dependent on frequency of stimulation ($P < 0.05$, one-way ANOVA with repeated measures). In 2 of the 4 experiments with rat atria, the inhibitory effect was shown to persist unchanged for 30 min after removal of conotoxin (data not illustrated).

Discussion

The results show that conotoxin inhibits responses to stimulation of noradrenergic, cholinergic and NANC nerves, but there are some differences in the sensitivity to conotoxin between tissues and between species. However, no clear pattern emerges from the findings of differential sensitivity to conotoxin on responses mediated by different types of transmitters or on transmitters evoking stimulating as opposed to inhibitory responses of effector tissues.

Effects of conotoxin on noradrenergic transmission

Conotoxin inhibited contractions elicited by field stimulation of noradrenergic nerves in epididymal portions of the rat vas deferens, which are predominantly mediated by noradrenaline (McGrath, 1978; Brown *et al.*, 1983). Maggi *et al.* (1988a) have

Table 3 Increases in atrial rate (beats min^{-1}) produced by 0.1 μM noradrenaline and 3 μM tyramine in rat and mouse isolated atria before and after 30 min exposure to conotoxin (CTX, 10 nM)

	Noradrenaline			*Tyramine*		
	Before CTX	*After CTX*	n	*Before CTX*	*After CTX*	n
Rat	108 ± 15	103 ± 8	4	91 ± 13	98 ± 9	4
Mouse	151 ± 8	158 ± 8	4	100 ± 12	103 ± 13	4

Values are means ± s.e.means; n = number of experiments.

previously shown that conotoxin also inhibits responses to field stimulation in the prostatic portion of the rat vas deferens, where ATP is the predominant transmitter (Sneddon & Westfall, 1984). Noradrenergic nerve-mediated contractions of the rat anococcygeus muscle were inhibited by conotoxin, confirming the finding of McKnight et al. (1989). However, responses in the mouse anococcygeus were appreciably less affected than those in the rat, suggesting a species difference in the sensitivity to conotoxin. Vasoconstrictor responses to noradrenergic nerve stimulation were inhibited by conotoxin in the rabbit ear artery. It has been previously demonstrated that conotoxin inhibits stimulation-induced vasoconstriction in the rat perfused kidney (Mohy El-Din & Malik, 1988) and rat tail artery (Clasbrummel et al., 1989). Relaxations of smooth muscle elicited by noradrenergic nerve stimulation were inhibited by conotoxin in the rat and rabbit jejunum. Positive chronotropic responses to noradrenergic nerve stimulation were inhibited by conotoxin in rat and mouse atria.

The stimulation-induced release of noradrenaline was inhibited by conotoxin (10 nM) in rat and mouse atria by about 80% and 60%, respectively, but the reduction was less in the rabbit ear artery (40%), even though the concentration of conotoxin (40 nM) was higher. These differences in sensitivity to conotoxin do not appear to be related to differences in the stimulation parameters (see Table 2), and presumably reflect differences in the susceptibility to conotoxin between species and tissues. It has been demonstrated previously that conotoxin inhibits electrical stimulation-induced release of noradrenaline from rat perfused kidneys (Mohy El-Din & Malik, 1988), tail artery (Clasbrummel et al., 1989) and anococcygeus muscle (McKnight et al., 1989).

Effects of conotoxin on cholinergic transmission

Conotoxin inhibited contractions elicited by field stimulation of cholinergic nerves of the guinea-pig taenia caeci and cholinergic nerve-mediated components of contractions elicited by extrinsic nerve stimulation in rat jejunum, mouse ileum, and rabbit and guinea-pig colon. The inhibition by conotoxin was less complete in the guinea-pig colon than in the other preparations. It is possible that cholinergic transmission in the guinea-pig colon is relatively conotoxin-resistant. An alternative explanation is that in the guinea-pig colon there may be interactions at postjunctional sites of transmitters with contracting and relaxing actions and at prejunctional sites with transneuronal modulation of transmitter release, and this complexity obscures the effect of conotoxin.

Inhibition of responses to field stimulation of cholinergic nerves has been shown previously in field-stimulated guinea-pig ileum preparations (Lundy & Frew, 1988; Keith et al., 1989).

Effects of conotoxin on NANC transmission

Inhibition by conotoxin of contractions elicited by stimulation of NANC nerves was observed in rabbit and guinea-pig distal colon, and to some extent in the rat bladder. Inhibition of NANC-mediated relaxations by conotoxin was demonstrated in the guinea-pig taenia caeci, and in the rat anococcygeus muscle and gastric fundus.

In the rat anococcygeus, there is evidence indicating that nitric oxide (NO) is involved in the transmission process with low frequencies of stimulation (Li & Rand, 1989b). NANC-mediated relaxations of the rat anococcygeus muscle elicited by low frequencies of field stimulation were inhibited by conotoxin, when they were revealed by blocking the response to stimulation of the noradrenergic nerves and raising the tone with guanethidine. However, when NANC-mediated relaxations were revealed by blocking the response to noradrenergic nerve stimulation with phentolamine and raising the tone with carbachol, they were not significantly affected by conotoxin, as demonstrated previously by McKnight et al. (1989). The NANC relaxations revealed in the presence of guanethidine by stimulation at 0.5 Hz are approximately matched by those revealed in the presence of carbachol by stimulation at 5 Hz. The loss of reactivity to low frequencies of stimulation in the presence of carbachol is due to the inhibition of release of the NANC transmitter by activation of prejunctional muscarinic receptors (Li & Rand, 1989a). The higher frequencies of stimulation necessary to produce relaxations in the presence of carbachol may explain the lack of effect of conotoxin (see below). Alternatively, the prejunctional muscarinic receptors may be coupled to N-type Ca^{2+} channels in such a way that the sensitivity of the channels to conotoxin is diminished when the muscarinic receptors are activated.

Contractions of the rat bladder elicited by stimulation of the peri-urethral nerves, which are NANC- and cholinergically mediated (Maggi et al., 1988b), were relatively insensitive to conotoxin, as was previously found by Maggi et al. (1988b) who elicited contractions by field stimulation of the intramural nerves. Maggi et al. (1988b) showed that responses to stimulation at 0.1 Hz were only reduced by about 25% by 10–300 nM conotoxin, whereas contractions of the guinea-pig bladder were reduced by about 70% by 10 nM to 1 μM conotoxin. In contrast to findings with other tissues in which responses elicited by low frequencies of stimulation were reduced by a greater proportion than those elicited by higher frequencies, the inhibition of stimulation-induced contractions of the rat bladder increased with increasing frequency of stimulation from 0.1 to 5 Hz (where it was about 50%) and only then decreased as the frequency increased, as was also found by Maggi et al. (1988b). It is possible that transmitter release from nerve terminals in the bladder does not only involve Ca^{2+} influx through conotoxin-sensitive channels. An alternative suggestion is that the stimulation-induced contractions of the bladder are the result of the actions of transmitters having contractile and relaxant actions, and that the various transmitters also engage in automodulation and transneuronal modulation of transmitter release, such that reductions in their release do not greatly alter the response. Testing of this suggestion must await further elucidation of the transmitters involved and the development of selective blocking drugs. An example of the complexity of the innervation of the bladder has been demonstrated by Andersson et al. (1989) in the dog, in which hypogastric nerve stimulation produced a small contraction followed by relaxation and pelvic nerve stimulation produced a biphasic contraction, the first phase being NANC-mediated and the second being cholinergic.

In the rabbit colon, the contractions elicited by stimulation of the pelvic nerve supply were reduced by about 20% by 0.1 μM atropine, which completely abolished the response to acetylcholine, and were reduced by about 50% by 300 μM hexamethonium (Garry & Gillespie, 1955; Thaina et al., 1989). Thus, a considerable proportion of the response is due to stimulation of nerve fibres that do not conform to the expectations for pre- or postganglionic cholinergic parasympathetic neurones. Whatever the nature of the NANC transmitter mediating part of the contractile response, the process of transmitter release is conotoxin-sensitive, since stimulation-induced contractions were almost completely abolished.

In the guinea-pig colon, the contractions elicted by stimulation of the pelvic nerves were reduced by 30–50% by 0.1 μM atropine, which completely abolished the response to acetylcholine, and were reduced by about 80% by 300 μM hexamethonium (Thaina et al., 1989). Hence, the component that cannot be attributed to stimulation of pre- or postganglionic cholinergic parasympathetic neurones is less than in the rabbit colon. However, in contrast to the findings with the rabbit colon, conotoxin was relatively ineffective in the guinea-pig colon, in that it only reduced stimulation-induced responses by about 50%. After the cholinergic component of the response had been eliminated with atropine, conotoxin produced a greater reduction in the response. Thus, the conotoxin

insensitivity cannot be assigned to the NANC-mediated component.

Relative sensitivities to conotoxin of different types of transmission in the same organ

In some cases, conotoxin appeared to be equally effective in inhibiting different transmission processes in the same organ. For instance, in the rat jejunum, cholinergically-mediated contractions and noradrenergically-mediated relaxations were equally inhibited, even though the latter were elicited with a higher frequency of stimulation. Furthermore, in the rat anococcygeus there was little difference between the sensitivity to conotoxin of the NANC-mediated relaxations (in the presence of guanethidine) and the noradrenergically-mediated contractions.

However, in other cases there was differential sensitivity to conotoxin between different transmission processes, for example, in rat gastric fundus strips and in guinea-pig taenia caeci.

In the rat gastric fundus, it has been suggested that stimulation-induced relaxations are largely mediated by vasoactive intestinal peptide (VIP) when prolonged trains (5 min) of stimulation at 5 Hz are used (Lefebvre, 1986), but are predominantly mediated by nitric oxide (NO) when lower frequencies or shorter train lengths of stimulation are used (Li & Rand, 1990b). Conotoxin abolished the response to stimulation at 1 Hz and slowed the rate of development of the responses to stimulation at 2 and 5 Hz, suggesting that it was more effective in inhibiting release of NO than VIP. This would be in accord with the findings that VIP release from enteric nerves does not depend on extracellular Ca^{2+} and is not affected by the inorganic Ca^{2+} channel blocker Cd^{2+} (Belai *et al.*, 1987). However, the higher frequency and longer duration of stimulation necessary for producing the VIP-mediated component of the relaxations may be responsible for its relative insensitivity to conotoxin (see below).

In guinea-pig taenia caeci, conotoxin reduced cholinergically- mediated contractions to a greater extent than NANC-mediated relaxations, even though the latter were elicited at a lower frequency of stimulation. Thus, the Ca^{2+} channels linked to neurotransmitter release in the NANC terminals in guinea-pig taenia caeci may not be identical to those in the cholinergic terminals.

Neuronal selectivity of conotoxin

The selectivity of conotoxin for nerve terminals has been demonstrated by the findings that responses of effector tissues to putative transmitters and other agonists are not impaired when stimulation-induced responses are greatly reduced or abolished. Thus, conotoxin did not affect responses of the rabbit ear artery and rat and mouse atria to noradrenaline, of rat jejunum to acetylcholine or noradrenaline, of rabbit colon to acetylcholine, and of rat gastric fundus and anococcygeus muscle to nitroprusside. However, responses of the guinea-pig colon to acetylcholine were reduced by conotoxin. A possible explanation is that acetylcholine may exert part of its action by exciting neurones in the myenteric plexus. It has previously been shown that conotoxin did not affect responses of various tissues to putative transmitters when responses to nerve stimulation were greatly reduced or abolished (Lundy & Frew, 1988; Maggi *et al.*, 1988a,b; Mohy El-Din & Malik, 1988; Clasbrummel *et al.*, 1989; McKnight *et al.*, 1989).

The lack of effect of conotoxin on the response to tyramine in rat and mouse atria demonstrates that its prejunctional inhibitory action is confined to Ca^{2+}-dependent transmitter release.

It has been found that high concentrations of conotoxin (0.3–10 μM) produced contractions of rat gastric fundus and uterus, and enhanced responses of the uterus to acetylcholine and KCl (Ichida *et al.*, 1988). We did not observe any such effects with the lower concentrations of conotoxin used in any of the tissues we studied.

General properties of conotoxin in inhibiting transmitter release

We found that the inhibitory effect of conotoxin on neurotransmission was slow to develop in all the preparations used, as has been noted previously for NANC transmission in the rat heart (Maggi *et al.*, 1988a) and prostatic segments of the rat vas deferens (Maggi *et al.*, 1988b), and for noradrenergic transmission in rat tail artery (Clasbrummel *et al.*, 1989) and rat kidney (Mohy El-Din & Malik, 1988).

It has also been shown in the present study that the rate of development of the inhibitory effect of conotoxin is proportional to its concentration for cholinergic transmission in the mouse ileum and noradrenergic transmission in the rabbit ear artery, as previously demonstrated for NANC transmission in the guinea-pig bladder (Maggi *et al.*, 1988b), for release of tachykinins from sensory terminals in guinea-pig bronchial smooth muscle (Maggi *et al.*, 1988a) and for noradrenergic transmission in the rat tail artery (Clasbrummel *et al.*, 1989). However, this relationship did not occur in the guinea-pig colon.

The extent of the inhibitory effect of conotoxin was concentration-dependent in the field-stimulated guinea-pig ileum (Lundy & Frew, 1988) and rat tail artery (Clasbrummel *et al.*, 1989), and we observed this in the rabbit ear artery. However, the residual responses in the presence of conotoxin in rat jejunum, mouse ileum, guinea-pig colon and rat bladder were not affected by increasing the concentration of conotoxin, although they were tetrodotoxin-sensitive.

A further determinant of the rate of development and of the extent of the inhibitory action of conotoxin is the frequency of stimulation. We have not found a previous statement to this effect in the literature, but we consider it to be an important feature of the action of conotoxin. In some preparations, the inhibitory effect of conotoxin was virtually complete at low frequencies of stimulation but became less as the frequency was increased and in some was virtually absent at a sufficiently high frequency of stimulation. Such effects were observed for noradrenergic transmission in the rabbit jejunum, rabbit ear artery, rat and mouse anococcygeus muscles, rat and mouse atria, and rat vas deferens, for cholinergic transmission in the mouse ileum, and for cholinergic plus NANC transmission in the rabbit colon, and for NANC transmission in the rat anococcygeus muscle. We suggest that the most likely explanation for the frequency-dependent effect of conotoxin is that it reduces the influx of Ca^{2+} into nerve terminals, that follows invasion of the terminals with a single action potential, to below the threshold for activation of excitation-secretion coupling, but the intracellular Ca^{2+} concentration can accumulate to the point that it does trigger excitation-secretion coupling when there are successive invasions of the terminals at a sufficiently high frequency. This explanation fits well for the effect of conotoxin in abolishing the first and second contractions of the rat vas deferens elicited by a train of pulses at low frequencies of stimulation and the gradual increase in size of subsequent contractions (Figure 9). A decrease in the inhibitory effect of conotoxin as the number of pulses in a train was increased was noted by Clasbrummel *et al.* (1989).

Support for the view that conotoxin impedes but does not abolish completely the influx of Ca^{2+} into nerve terminals comes from the finding that its inhibitory effect is inversely proportional to the Ca^{2+} concentration, as demonstrated previously for cholinergic transmission in the frog neuromuscular junction (Kerr & Yoshikami, 1984; Sano *et al.*, 1987) and noradrenergic transmission in the rat tail artery (Clasbrummel *et al.*, 1989), and as found in the present study, for noradrenergic transmission in the rat vas deferens. In frog dorsal root ganglion cells, the depression of the Ca^{2+} current by conotoxin was

partly overcome by increasing the extracellular Ca^{2+} concentration (Oyama *et al.*, 1987).

Another feature of the action of conotoxin is the persistence of its effect after its removal. We observed this in the various preparations we used, and studied it particularly in rat and mouse atria, in which the effect persisted unchanged for at least 30 min, and in the rabbit ear artery, in which the inhibition became even greater after removal of conotoxin. The persistence of the effect of conotoxin has been noted by many other investigators in a variety of systems; for example, inhibition of acetylcholine release from the myenteric plexus of guinea-pig ileum (Lundy & Frew, 1988) and inhibition of neuroeffector transmission in the rat tail artery (Clasbrummel *et al.*, 1989) and vas deferens (Brock *et al.*, 1989).

Conclusions

Conotoxin acts on nerve terminals to inhibit autonomic noradrenergic, cholinergic and NANC neuroeffector transmission in a wide range of tissues, although some are relatively more sensitive than others. The inhibitory effects of conotoxin are inversely proportional to the frequency of stimulation and to the Ca^{2+} concentration of the PSS. These observations suggest that the inhibition by conotoxin of the Ca^{2+} influx required for excitation-secretion coupling in autonomic nerve terminals is not absolute, and can be overcome by repetitive stimulation or by raising the Ca^{2+} concentration. Possible explanations for the relative insensitivity of transmission to conotoxin in some neuroeffector systems may be because (i) they do not require Ca^{2+} influx for excitation-secretion coupling, (ii) Ca^{2+} influx is not through N-type channels, (iii) different types of nerve terminals may be endowed with sub-types of N-type channels having different sensitivities to conotoxin, or (iv) Ca^{2+} influx has already been partly inhibited by autoinhibitory or transneuronal modulation, thereby obscuring the effect of conotoxin.

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References

ANDERSSON, P.-O., SJÖGREN, C., UVNÄS, B. & UVNÄS-MOBERG, K. (1989). The role of vasoactive intestinal polypeptide (VIP) in mediating responses of the urinary bladder to stimulation of the autonomic innervation in anaesthetized dogs. *J. Physiol.*, **418**, 74P.

AUGUSTINE, G.J., CHARLTON, M.P. & SMITH, S.J. (1987). Calcium action in synaptic transmitter release. *Ann. Rev. Neurosci.*, **10**, 633–693.

BELAI, A., RALEVIC, V. & BURNSTOCK, G. (1987). VIP release from enteric nerves is independent of extracellular calcium. *Regul. Peptides*, **19**, 79–89.

BROCK, J.A., CUNNANE, T.C., EVANS, R.J. & ZIOGAS, J. (1989). Inhibition of transmitter release from sympathetic nerve endings by ω-conotoxin. *Clin. Exp. Pharmacol. Physiol.*, **16**, 333–339.

BROWN, C.M., DOCHERTY, J.R., FRENCH, A.M., MACDONALD, A., MCGRATH, J.C. & SCOTT, N.C. (1983). Separation of adrenergic and non-adrenergic contractions to field stimulation in rat vas deferens. *Br. J. Pharmacol.*, **79**, 379–393.

BURN, J.H. & RAND, M.J. (1960). The relation of circulating noradrenaline to the effect of sympathetic stimulation. *J. Physiol.*, **150**, 295–305.

BURNSTOCK, G., CAMPBELL, G. & RAND, M.J. (1966). The inhibitory innervation of the taenia of the guinea-pig caecum. *J. Physiol.*, **182**, 504–526.

CLASBRUMMEL, B., OSSWALD, H. & ILLES, P. (1989). Inhibition of noradrenaline release by ω-conotoxin GVIA in the rat tail artery. *Br. J. Pharmacol.*, **96**, 101–110.

CRUZ, L.J., JOHNSON, D.S. & OLIVERA, B.M. (1987). Characterisation of the ω-conotoxin target. Evidence for tissue specific heterogeneity in calcium channel types. *Biochemistry*, **26**, 820–824.

CRUZ, L.J., JOHNSON, D.S., IMPERIAL, J.S., GRIFFIN, D., LECHEMINANT, G.W., MILJANICH, G.P. & OLIVERA, B.M. (1988). ω-Conotoxins and voltage-sensitive calcium channel subtypes. In *Current Topics in Membranes and Transport*, Vol. 33, *Molecular Biology of Ionic Channels*, ed. Agnew, W.S., Claudio, T. & Sigworth, F.J., pp. 417–429. New York: Academic Press.

DE LUCA, A. & RAND, M.J. (1989). Responses of rat isolated intestine to stimulation of perivascular mesenteric nerves indicate the presence of cholinergic and noradrenergic fibres. *Proc. Aust. Physiol. Pharmacol. Soc.*, **20**, 97P.

DE LUCA, A. & RAND, M.J. (1990). Inhibition by omega-conotoxin GVIA of responses to stimulation of intramural nerves in rat vas deferens: frequency and calcium dependence. *Clin. Exp. Pharmacol. Physiol.*, Suppl. **16**, 83.

DE LUCA, A., THAINA, P. & RAND, M.J. (1990). Effects of omega-conotoxin GVIA on responses to extrinsic nerve stimulation in intestinal nerve-muscle preparations. *Clin. Exp. Pharmacol. Physiol.*, Suppl. **16**, 183.

GARRY, R.C. & GILLESPIE, J.S. (1954). An *in vitro* preparation of the distal colon of the rabbit with orthosympathetic parasympathetic innervation. *J. Physiol.*, **123**, 60P–61P.

GARRY, R.C. & GILLESPIE, J.S. (1955). The responses of the musculature of the colon of the rabbit to stimulation, *in vitro*, of the parasympathetic and of the sympathetic outflows. *J. Physiol.*, **128**, 557–576.

GIBSON, A. & WEDMORE, C.V. (1981). Responses of the isolated anococcygeus muscle of the mouse to drugs and to field stimulation. *J. Auton. Pharmacol.*, **1**, 225–233.

GILLESPIE, J.S. (1972). The rat anococcygeus muscle and its response to nerve stimulation and to some drugs. *Br. J. Pharmacol.*, **45**, 404–416.

HUKOVIC, S., RAND, M.J. & VANOV, S. (1965). Observations on an isolated innervated preparation of rat urinary bladder. *Br. J. Pharmacol.*, **24**, 178–188.

ICHIDA, S., OKA, H., MASADA, A., FUJISUE, T., HATA, T. & MATSUDA, N. (1988). Effects of synthetic ω-conotoxin on the contractile responses of segments of rat ileum, stomach fundus and uterus and guinea pig taenia coli. *Japan J. Pharmacol.*, **48**, 395–405.

KEITH, R.A., MANGANO, T.J., PACHECO, M.A. & SALAMA, A.I. (1989). Characterization of the effects of ω-conotoxin GVIA on the responses of voltage-sensitive calcium channels. *J. Auton. Pharmacol.*, **9**, 243–252.

KERR, L.M. & YOSHIKAMI, D. (1984). A venom peptide with a novel presynaptic blocking action. *Nature*, **308**, 282–284.

LEFEBVRE, R.A. (1986). Study on the possible neurotransmitter of the non-adrenergic non-cholinergic innervation of the rat gastric fundus. *Arch. Int. Pharmacodyn.*, **280** (Suppl.), 110.

LI, C.G., MAJEWSKI, H. & RAND, M.J. (1988). Facilitation of noradrenaline release from sympathetic nerves in rat anococcygeus muscle by activation of prejunctional β-adrenoceptors and angiotensin receptors. *Br. J. Pharmacol.*, **95**, 385–392.

LI, C.G. & RAND, M.J. (1989a). Prejunctional inhibition of non-adrenergic non-cholinergic transmission in the rat anococcygeus muscle. *Eur. J. Pharmacol.*, **168**, 107–110.

LI, C.G. & RAND, M.J. (1989b). Evidence for a role of nitric oxide in the neurotransmitter system mediating relaxation of the rat anococcygeus muscle. *Clin. Exp. Pharmacol. Physiol.*, **16**, 933–938.

LI, C.G. & RAND, M.J. (1990a). Effects of omega-conotoxin GVIA on NANC transmission. *Clin. Exp. Pharmacol. Physiol.*, Suppl. **16**, 184.

LI, C.G. & RAND, M.J. (1990b). Evidence suggesting that nitric oxide (NO) mediates NANC neurotransmission in rat gastric fundus and anococcygeus muscle. *Clin. Exp. Pharmacol. Physiol.*, Suppl. **16**, 86.

LUNDY, P.M. & FREW, R. (1988). Evidence of ω-conotoxin GVIA-sensitive Ca^{2+} channels in mammalian peripheral nerve terminals. *Eur. J. Pharmacol.*, **156**, 325–330.

MAGGI, C.A., PATACCHINI, R., GIULIANI, S., SANTICIOLI, P. & MELI, A. (1988a). Evidence for two independent modes of activation of the 'efferent' function of capsaicin-sensitive nerves. *Eur. J. Pharmacol.*, **156**, 367–373.

MAGGI, C.A., PATACCHINI, R., SANTICIOLI, P., LIPPE, I.T., GIULIANI, S., GEPPETTI, P., DEL BIANCO, E., SELLERI, S. & MELI, A. (1988b). The effect of omega conotoxin GVIA, a peptide modulator of the N-type voltage sensitive calcium channels, on motor responses

produced by activation of efferent and sensory nerves in mammalian smooth muscle. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **338**, 107–113.

McCULLOCH, M.W., PAPANICOLAOU, M. & RAND, M.J. (1985). Evidence for autoinhibition of stimulation-induced noradrenaline release from vasa deferentia of the guinea-pig and rat. *Br. J. Pharmacol.*, **86**, 455–464.

McGRATH, J.C. (1978). Adrenergic and 'non-adrenergic' components in the contractile response of the vas deferens to single indirect stimulus. *J. Physiol.*, **283**, 23–39.

McKNIGHT, A.T., MAGUIRE, J.J. & WOODRUFF, G.N. (1989). Selective block by ω-conotoxin GVIA of motor adrenergic nerves in rat anococcygeus, in vitro. *J. Physiol.*, **409**, 50P.

MILLER, R.J. (1987). Multiple calcium channels and neuronal function. *Science*, **235**, 46–52.

MOHY EL-DIN, M.M. & MALIK, K.U. (1988). Differential effect of ω-conotoxin on release of the adrenergic transmitter and the vasoconstrictor response to noradrenaline in the rat isolated kidney. *Br. J. Pharmacol.*, **94**, 355–362.

OYAMA, Y., TSUDA, Y., SAKAKIBARA, S. & AKAIKE, N. (1987). Synthetic ω-conotoxin: a potent calcium channel blocking neurotoxin. *Brain Res.*, **424**, 58–64.

REID, J.J., WONG-DUSTING, H.K. & RAND, M.J. (1990). Characterization of the effect of omega-conotoxin GVIA (CTX) on noradrenergic transmission in cardiovascular tissues. *Clin. Exp. Pharmacol. Physiol.*, Suppl. **16**, 84.

SANO, K., ENEMOTO, K. & MAENO, T. (1987). Effects of synthetic ω-conotoxin, a new type of Ca^{2+} antagonist, on frog and mouse neuromuscular transmission. *Eur. J. Pharmacol.*, **141**, 235–241.

SNEDDON, P. & WESTFALL, D.P. (1984). Pharmacological evidence that adenosine triphosphate and noradrenaline are co-transmitters in the guinea-pig vas deferens. *J. Physiol.*, **347**, 561–580.

THAINA, P., NOTT, M.W. & RAND, M.J. (1989). Motor responses of rabbit and guinea-pig distal colon to stimulation of the extrinsic pelvic nerves. *Proc. Aust. Physiol. Pharmacol. Soc.*, **20**, 98P.

TSIEN, R.W., LIPSCOMBE, D., MADISON, D.V., BLEY, K.R. & FOX, A.P. (1988). Multiple types of neuronal calcium channels and their selective modulation. *Trends Neurosci.*, **11**, 431–438.

VANE, J.R. (1957). A sensitive method for the assay of 5-hydroxytryptamine. *Br. J. Pharmacol.*, **12**, 344–349.

WONG-DUSTING, H.K. & RAND, M.J. (1988). Pre- and postjunctional effects of neuropeptide Y on the rabbit isolated ear artery. *Clin. Exp. Pharmacol. Physiol.*, **15**, 411–418.

WONG-DUSTING, H.K., REID, J.J. & RAND, M.J. (1989). Paradoxical effects of endothelin on cardiovascular noradrenergic neurotransmission. *Clin. Exp. Pharmacol. Physiol.*, **16**, 229–233.

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5-HT_{1A} agonists increase and 5-HT_3 agonists decrease acetylcholine efflux from the cerebral cortex of freely-moving guinea-pigs

[1]Clementina Bianchi, Anna Siniscalchi & Lorenzo Beani

Department of Pharmacology, University of Ferrara, Via Fossato di Mortara 23, 44100 Ferrara, Italy

1 The influence of 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}), 5-HT_2 and 5-HT_3 agonists and antagonists on acetylcholine (ACh) release from the cerebral cortex was studied in freely moving guinea-pigs.

2 8-Hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, 0.01–1 mg kg^{-1}, s.c.) caused the 5-HT syndrome and dose-dependently increased ACh release. Ru 24969 (1–10 mg kg^{-1}, s.c.) shared the same effects, but it was less potent. (−)-Propranolol (5 mg kg^{-1}) and metitepine (2 mg kg^{-1}) prevented these behavioural and neurochemical responses.

3 (±)-1(4-Iodo-2,5-dimethoxyphenyl)2-aminopropane (DOI) up to 2 mg kg^{-1} did not modify ACh release and ketanserin (0.5 mg kg^{-1}) was ineffective on 5-HT-induced changes of ACh outflow.

4 2-Methyl-5-HT (500 μg, i.c.v.) and 5-HT (500 μg, i.c.v.) plus metitepine (2 mg kg^{-1}, s.c.) inhibited the gross behaviour and ACh release. ICS 205-930 (0.5 mg kg^{-1}) prevented these responses.

5 2-Methyl-5-HT, up to 10 μmol l^{-1}, and 8-OH-DPAT, up to 0.1 μmol l^{-1}, (like 5-HT) did not change [^{3}H]-choline efflux from cerebral cortex slices.

6 These results suggest that exogenous 5-HT and related selective agonists modulate guinea-pig cortical cholinergic structures through 5-HT_{1A} and 5-HT_3 receptors. The stimulation of 5-HT_{1A} autoreceptors may lead to disinhibition of the cholinergic cells, tonically inhibited by the tryptaminergic control. Conversely, the stimulation of 5-HT_3 receptors inhibits ACh release, possibly through an interneurone. No direct 5-HT modulation of the cholinergic nerve endings was found.

Introduction

There is general agreement that 5-hydroxytryptamine (5-HT) exerts an inhibitory control on the cholinergic system in the brain. 5-HT or 5-HT agonists increase acetylcholine (ACh) content *in vivo* and reduce ACh release from different brain areas *in vitro* (Envrard *et al.*, 1977; Samanin *et al.*, 1978; Consolo *et al.*, 1980; Vizi *et al.*, 1981; Gillet *et al.*, 1985; Bianchi *et al.*, 1989).

On the other hand it has been demonstrated that 5-HT, injected into the lateral ventricles, increases ACh release from the cerebral cortex of freely moving guinea-pigs. This effect has been attributed to the activation of 5-HT autoreceptors, because it was prevented by pretreating the animals with 5,7-DHT and was converted into inhibition by metitepine (Bianchi *et al.*, 1986).

5-HT_{1A} and 5-HT_{1B} recognition sites seem to function as autoreceptors on 5-HT neurones ascending from the brain raphe nuclei to the hippocampus and cerebral cortex (Sprouse & Aghajanian, 1987; Sharp *et al.*, 1989a,b) while activation of 5-HT_3 receptors reduces ACh release from the rat entorhinal cortex *in vitro* (Barnes *et al.*, 1989). Thus, experiments have been performed, with the now available selective 5-HT agonists and antagonists, to obtain confirmatory evidence that the autoreceptors are of the 5-HT_{1A} subtype and that 5-HT exerts a tonic inhibitory control on cholinergic cells through 5-HT_3 receptors in the guinea-pig.

Methods

Experiments ex vivo

Guinea-pigs of either sex weighing 400–500 g were anaesthetized with pentobarbitone sodium (30 mg kg^{-1}, i.p.) plus morphine sulphate (2 mg kg^{-1}, i.p.) immediately prior to and 3 h after the barbiturate. The implantation of an epidural cup on the parietal cortex and of a guide cannula in the lateral ventricle (for i.c.v. injections) were made as previously described (Beani *et al.*, 1968; 1978). One day after surgery, the animals were trained to be gently handled. The efflux of ACh from the parietal cortex was measured 2–3 days after surgery by filling the cup with 0.25 ml of Ringer solution containing physostigmine 0.3 mmol l^{-1}. The solution was collected and renewed every 30 min. As a rule the experiments consisted of 3 collection periods before (controls) and 4–6 periods after treatment.

The ACh present in the samples was bioassayed on tetrodotoxin-pretreated guinea-pig ileum (Beani *et al.*, 1978). This method is of high sensitivity (0.5 pmol) and gives results similar to those obtained with mass spectrometry analysis (Beani *et al.*, 1988). The release of ACh from the cerebral cortex was expressed as pmol cm^{-2} 30 min^{-1} and the effect of treatment was evaluated as a percentage of the mean pretreatment value.

Experiments in vitro

In another set of experiments 5-HT agonists and antagonists were tested *in vitro* in superfused slices of parietal cortex preloaded with [^{3}H]-choline (0.1 μmol l^{-1}, specific activity 80 Ci mmol^{-1}) and submitted to electrical stimulation as previously described (Beani *et al.*, 1984). The cortical slices were transferred into perfusion chambers (0.9 ml vol) and perfused at a rate of 0.5 ml min^{-1} with a Krebs solution containing hemicholinium-3 10 μmol l^{-1}, gassed with O_2 (95%) and CO_2 (5%). The tissue was electrically stimulated for 2 min (2 Hz, alternate pulses of 5 ms duration, strength 30 mA cm^{-2}) at 45 min (S_1) and 75 min (S_2) min from the start of the superfusion. Drugs were added 10 min before S_2 and their effects were expressed as changes in the S_2/S_1 ratio (Beani *et al.*, 1984).

Drugs

The compounds used were obtained from the following sources: 5-hydroxytryptamine creatine sulphate, methysergide

[1] Author for correspondence.

maleate and tetrodotoxin (Sigma); metitepine (Hoffman La Roche); (−)-propranolol HCl (ICI); ketanserin (Janssen, Belgium); 8-hydroxy-2-(di-n-propylamino) tetralin HBr (8-OH-DPAT) and (±)-1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane · HCl (DOI) (RBI, U.S.A.); 5-methoxy-3- (1,2,3,6-tetrahydro-4-pyridinyl)-1H-indole (RU24969, Roussel-UCLAF, France); (3a-tropanyl)-1H-indole-3-carboxylic acid ester (ICS 205-930) and 2-methyl-5-HT (Sandoz, Switzerland).

Statistical analysis

The differences in ACh release before and after drug treatment, as well as differences after individual and paired treatments, were analysed by analysis of variance and Student's test or with the Wilcoxon two sample test, depending on the experimental protocol as specified in the table and figure legends.

Results

Effect of 5-HT$_1$ agonists and antagonists

8-OH-DPAT, a selective 5-HT$_{1A}$ receptor agonist, injected i.c.v. or s.c. caused the 5-HT behavioural syndrome (Goodwin *et al.*, 1987) and dose-dependently increased ACh release (Figure 1a,b,d). The effect reached its maximum at 60 min and lasted for more than 2 h. RU-24969, a mixed 5-HT$_{1A}$ and 5-HT$_{1B}$ agonist was also effective but less potent (Figure 1c and d).

(−)-Propranolol, a non selective 5-HT$_1$ antagonist (Nahorski & Willcocks, 1983), had no direct effect on normal behaviour or on ACh release up to 5 mg kg^{-1} i.p. but, as expected, it prevented the facilitatory effect of 8-OH-DPAT both on behaviour and on ACh release (Figure 2a). Pretreatment of guinea-pigs with metitepine (2 mg kg^{-1}) increased ACh release. 8-OH-DPAT, given 30 min after metitepine, did not further increase nor did it inhibit the ACh release (Figure 2b). This result contrasts with the effect of 5-HT which inhibited the increased ACh release caused by metitepine (Figure 2b, see also Bianchi *et al.*, 1986).

The possible role of 5-HT$_{1C}$ receptors in the reduction of ACh outflow caused by 5-HT after metitepine was assessed in mesulergine pretreated guinea-pigs. This 5-HT$_{1C}$ antagonist, (Pazos *et al.*, 1985) up to 0.5 mg kg^{-1} i.p., did not primarily change ACh release (which was 98.6 ± 5.6% of basal values 60 min after drug injection, mean ± s.e.mean of 5 expts.) and did not modify the effect of 5-HT after metitepine. In fact, the percentage changes of transmitter release (with respect to the antagonists alone) calculated 60 min after 5-HT (500 μg, i.c.v.) was 57 ± 10% after metitepine plus mesulergine and 55 ± 8% after metitepine alone (Figure 5).

Effect of 5-HT$_2$/5-HT$_{1C}$ agonists and antagonists

To examine whether the inhibitory modulation of the cholinergic structures by 5-HT might be related to 5-HT$_2$ or 5-HT$_{1C}$ receptor activation, the effect of DOI and ketanserin were tested.

As shown in Figure 3a, DOI, injected s.c. at 2 mg kg^{-1}, did not significantly change ACh release. Ketanserin (0.5 mg kg^{-1}, i.p.), *per se* was ineffective (112% ± 8.8 with respect to pre-drug release 30 min after injection, 5 expts), and did not change the effect of 5-HT either in normal or in metitepine-pretreated guinea-pigs (Figure 3a and Figure 5).

Effect of 5-HT$_3$ agonists and antagonists

In view of the findings that 5-HT$_1$ and 5-HT$_2$/5-HT$_{1C}$ receptors did not seem to be involved in the inhibitory control of the cholinergic neurones, the role of 5-HT$_3$ receptors on ACh release was tested. 2-Methyl-5-HT, a relatively selective 5-HT$_3$ agonist (Richardson & Engel, 1986), injected i.c.v. did not modify ACh release at 250 μg but significantly inhibited it at 500 μg causing behavioural depression (Figure 4). These effects were prevented by ICS 205-930, a selective 5-HT$_3$ antagonist (Richardson *et al.*, 1985), at 0.5 mg kg^{-1} s.c. This drug, *per se*, transiently increased ACh release and caused a delayed ACh

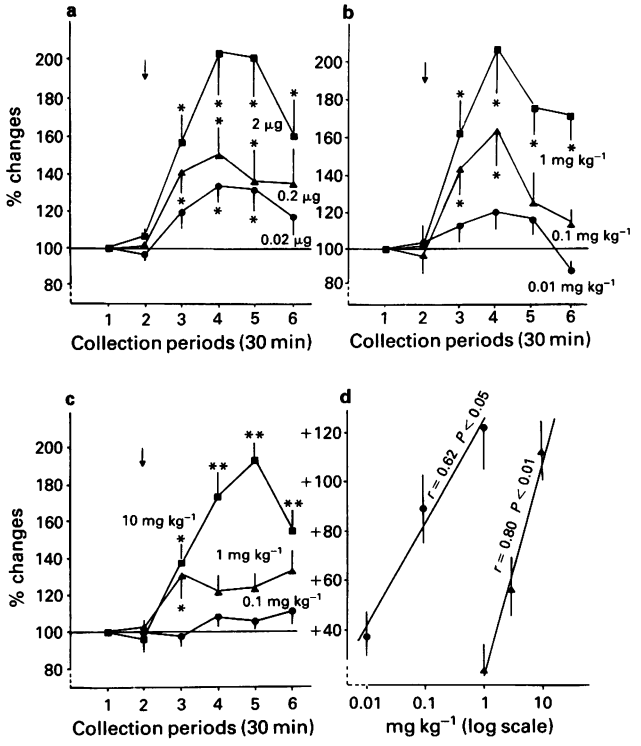

Figure 1 Effect of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) and RU 24969 on acetylcholine (ACh) release from the cerebral cortex of freely moving guinea-pigs. At the arrow: (a) 8-OH-DPAT injected i.c.v.; (b) 8-OH-DPAT injected s.c.; (c) RU 24969 injected s.c.; (d) relationship between s.c. doses and maximum % effect of 8-OH-DPAT (●) and RU 24969 (▲). Each point is the mean of 5–6 values; vertical lines: s.e.mean. The absolute pre-drug mean values of ACh release (pmol cm^{-2} 30 min^{-1}) were: 118.5 ± 2.2. $^*P < 0.05$, $^{**}P < 0.01$, significantly different from pre-drug values, according to ANOVA and Student's *t* test for paired data.

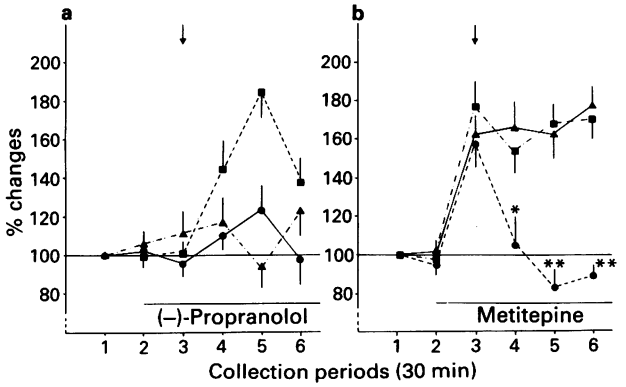

Figure 2 (a) Effect of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) 0.1 mg kg^{-1} s.c. (at the arrow) on acetylcholine (ACh) release from the cerebral cortex of freely moving guinea-pigs pretreated (30 min before) with (−)-propranolol 5 mg kg^{-1} i.p. (●). The effects of 8-OH-DPAT (■) and (−)-propranolol (▲) given alone are also shown. (b) Effect of 5-hydroxytryptamine (5-HT) 500 μg i.c.v. (●) or 8-OH-DPAT 0.1 mg kg^{-1} s.c. (▲) (at the arrow) on ACh release from the cerebral cortex of guinea-pigs pretreated (30 min before) with metitepine 2 mg kg^{-1} i.p. The effect of metitepine given alone (■) is also shown. Each point is the mean of 5–6 values; vertical lines: s.e.mean. The absolute pre-drug mean values of ACh release (pmol cm^{-2} 30 min^{-1}) were: 130 ± 3.2. $^*P < 0.05$, $^{**}P < 0.01$, significantly different from metitepine alone, according to ANOVA and Student's *t* test for non-paired data.

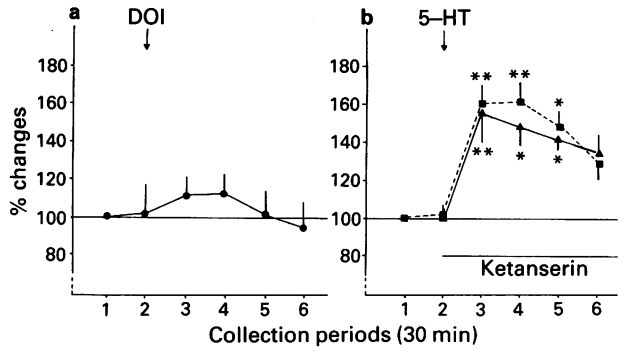

Figure 3 (a) Effect of (±)-1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane (DOI) 2 mg kg^{-1} i.p. (at the arrow) on acetylcholine (ACh) release from the cerebral cortex of freely moving guinea-pigs. (b) Effect of 5-hydroxytryptamine (5-HT) 500 μg i.c.v. (at the arrow) on ACh release from the cerebral cortex of freely moving guinea-pigs pretreated (30 min before) with ketanserin 0.5 mg kg^{-1} i.p. (▲). The effect of 5-HT given alone is also shown (■). Each point is the mean of 5–6 values; vertical lines: s.e.mean. The absolute pre-drug mean values of ACh release (pmol cm^{-2} 30 min^{-1}) were: 133 ± 2.9. *$P < 0.05$ significantly different from ketanserin alone, according to ANOVA and Student's t test for paired data.

increase in the presence of 2-methyl-5-HT (Figure 4). These two effects, however, were not significant. As shown in Figure 5, ICS 205-930, unlike ketanserin and propranolol, fully prevented the inhibition of ACh release caused by 5-HT in metitepine-pretreated animals. 2-Methyl-5HT (500 μg, i.c.v.), like 5-HT, induced a deep depression of transmitter release in metitepine-pretreated guinea-pigs. The percentage changes of ACh release (with respect to antagonist alone) at 60 and 90 min after 2-methyl-5-HT were 50 ± 1.5 and 57 ± 9 respectively (mean ± s.e.mean of 4 animals, $P < 0.05$).

Effect of 5-HT agonists on ACh release from cerebral cortex slices

As shown in Table 1, 2-methyl-5-HT, applied 15 min before S_2, did not change [^{3}H]-choline efflux induced by electrical pulses. Only at 100 μmol l^{-1} did 2-methyl-5-HT moderately, but significantly reduce the S_2/S_1 ratio. However, ICS 205-930

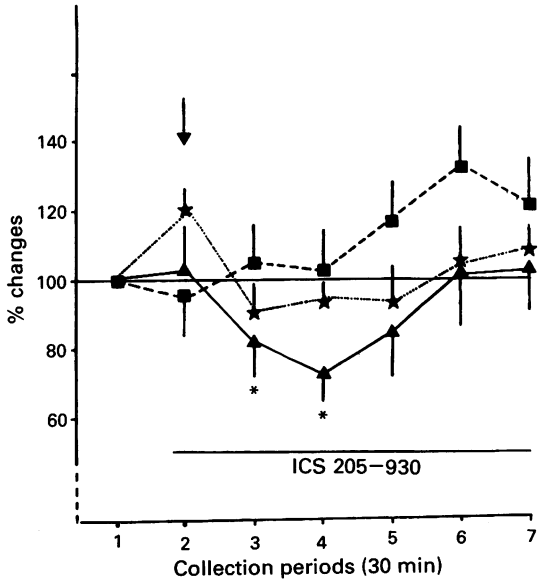

Figure 4 Effect of 2-methyl-5-HT (▲) 500 μg i.c.v. (at the arrow) on acetylcholine (ACh) release from the cerebral cortex of freely moving guinea-pigs and antagonism by ICS 205–930 (■) 0.5 mg kg^{-1} injected s.c. 30 min before. The effect of ICS 205-930 given alone is also shown (★). Each point is the mean of 5 values; vertical lines: s.e.mean. The absolute pre-drug mean values of ACh release (pmol cm^{-2} 30 min^{-1}) were: 118.5 ± 2.5. *$P < 0.05$, significantly different from pre-drug values, according to ANOVA and Student's t test for paired data.

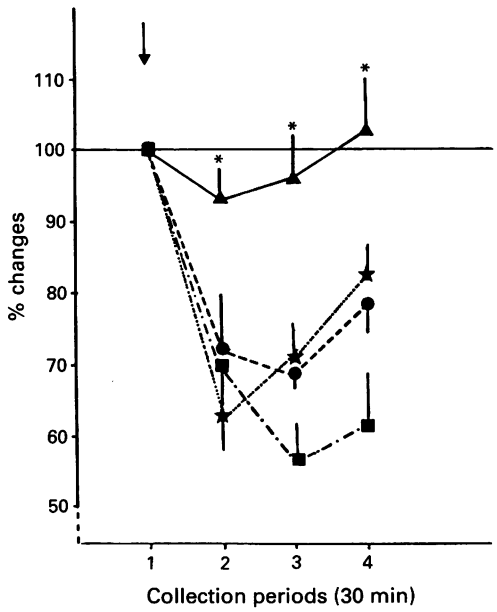

Figure 5 Effect of 5-hydroxytryptamine (5-HT, 500 μg i.c.v., arrow) on acetylcholine (ACh) release from the cerebral cortex of freely moving guinea-pigs pretreated (30 min before) with metitepine 2 mg kg^{-1} i.p. (■); metitepine plus ketanserin 0.5 mg kg^{-1} i.p. (●); metitepine plus (−)-propranolol 5 mg kg^{-1} i.p. (★); metitepine plus ICS 205-930 0.5 mg kg^{-1} s.c. (▲). The values after 5-HT are expressed as % of the effect of the antagonists alone. Each point is the mean of 5–7 values; vertical lines: s.e.mean. The absolute pre-drug mean values of ACh release (pmol cm^{-2} 30 min^{-1}) were: 123 ± 2.7. *$P < 0.05$ significantly different from other treatments, according to ANOVA and Student's t test for non-paired data.

(1 μmol l^{-1}) did not prevent this inhibition. Also in the presence of ritanserin 1 μmol l^{-1}, 2-methyl-5-HT did not inhibit ACh release (Table 1).

In another set of experiments, the effect of 8-OH-DPAT was checked. The results showed that even this 5-HT$_{1A}$ agonist, up to 0.1 μmol l^{-1}, did not modify [^{3}H]-choline efflux (S_2/S_1 ratio: controls, 1.01 ± 0.04; 8-OH-DPAT, 1.02 ± 0.05; mean of 5 experiments ± s.e.mean). Thus any direct action of 2-methyl-5-HT and of 8-OH-DPAT on the electrosecretory coupling process has been ruled out.

Discussion

The present study demonstrates that the administration of 8-OH-DPAT (i.c.v. or s.c.) and RU 24969 (s.c.) increases ACh release and that 2-methyl-5-HT, injected i.c.v., reduces ACh release from the cerebral cortex of freely moving guinea-pigs.

The increased transmitter release induced by 8-OH-DPAT may depend upon the activation of somatodendritic 5-HT$_{1A}$

Table 1 Effect of 2-methyl-5-hydroxytryptamine (2-Me-5-HT) on electrically-evoked (2 Hz) [^{3}H]-choline efflux from parietal cortex slices of the guinea-pig

Experimental conditions	S_2/S_1	*No. of expts.*
Control	0.99 ± 0.03	9
2-Me-5-HT 3 μmol l^{-1}	0.97 ± 0.10	3
2-Me-5-HT 10 μmol l^{-1}	1.00 ± 0.07	3
2-Me-5-HT 100 μmol l^{-1}	0.80 ± 0.02*	5
2-Me-5-HT 100 μmol l^{-1} + ICS 205-930 1 μmol l^{-1}	0.82 ± 0.06*	4
2-Me-5-HT 3 μmol l^{-1} + ritanserin 1 μmol l^{-1}	0.90 ± 0.03	7

2-Me-5-HT was added 10 min before S_2. The antagonists were added to Krebs solution from the beginning of the experiments. The S_2/S_1 ratios in control and treated slices are given.

* Significantly different from controls, $P < 0.05$, according to the Wilcoxon two sample test.

autoreceptors. They are known to reduce the firing rate of 5-HT neurones (De Montigny *et al.*, 1984; Van der Maelen *et al.*, 1986; Blier & De Montigny, 1987; Sprouse & Aghajanian, 1987) and to reduce 5-HT release *in vivo* (Sharp *et al.*, 1989a,b), but less so *in vitro* (Middlemiss, 1984a,b; Engel *et al.*, 1986; Maura & Raiteri, 1986). Consequently, 5-HT_{1A} activation in the whole animal reduces the tonic, inhibitory control on cholinergic cells, as previously inferred from indirect evidence (Bianchi *et al.*, 1986) and leads to increased cortical ACh release, as demonstrated by the present finding.

A direct enhancing effect of 5-HT_{1A} agonists on ACh release from the nerve endings has been ruled out as shown by the negative results from electronically driven neocortical slices, known to contain cholinergic axons. However, a disinhibition of cholinergic transmission via 5-HT_{1A} receptors located on inhibitory cortical interneurones remains a possible mechanism in the whole animal.

The experiments carried out to test whether 5-HT inhibition was due to 5-HT_{1C} or 5-HT_2 receptors proved negative. In fact, DOI, a putative 5-HT_2/5-HT_{1C} agonist (Shannon *et al.*, 1984) did not change ACh release and ketanserin or mesulergine, which have antagonistic activity at both 5-HT_2 and 5-HT_{1C} receptors (Leysen *et al.*, 1983; Pazos *et al.*, 1985), were ineffective against either inhibited or stimulated ACh release induced by 5-HT.

In contrast, 5-HT_3 receptors are clearly involved in the inhibition of ACh release. 2-Methyl-5-HT, a selective 5-HT_3 agonist (Richardson & Engel, 1986) inhibits ACh release both in normal and metitepine-pretreated guinea-pigs and ICS 205-930, a selective 5-HT_3 antagonist (Richardson *et al.*, 1985), prevents both 2-methyl-5-HT as well as 5-HT plus metitepine-induced inhibition. As regards the localization of this inhibitory control, it is worth noting that 2-methyl-5-HT alone or plus ritanserin, tested on guinea-pig cortical slices showed a slight inhibition but only at high concentrations and ICS 205-930 did not prevent it. These findings differ from the recent report of Barnes and coworkers (1989) demonstrating that low 2-methyl-5-HT concentrations (plus ritanserin) inhibited ACh release from slices of rat entorhinal cortex. The differences in animal species, cerebral area studied and type of stimulation (KCl versus electrical stimulation) are probably responsible for this discrepancy. In any case, since 2-methyl-5-HT is ineffective in guinea-pig cortical slices, it is conceivable that 5-HT inhibits, through 5-HT_3 receptors, the cholinergic cells either directly or via interneurones.

It is generally accepted that 5-HT_3 receptors exert stimulatory effects on the target neurones and facilitate the release of transmitters (Gaddum & Piccarelli, 1957; Fozard & Mobarak Ali, 1978; Hagan *et al.*, 1987; Carboni *et al.*, 1989). This evidence opens the question as to whether 5-HT_3-induced ACh inhibition is direct or mediated through other neuronal pools. The available data lead one to rule out dopamine involvement. Previous studies, in fact, have demonstrated that dopamine release is associated with increased ACh outflow from the cortex (Bianchi *et al.*, 1979). Experiments are in progress to ascertain whether other neuronal systems known to inhibit ACh release, such as the noradrenergic, the GABAergic and enkephalinergic neurones, are involved (but see, Barnes *et al.*, 1989).

In conclusion, exogenous 5-HT can modulate the guinea-pig corticipetal cholinergic structures: (i) by stimulating 5-HT_{1A} somatodendritic autoreceptors: this leads to disinhibition of the cholinergic cells and increased ACh release; (ii) by activating 5-HT_3 receptors: they probably trigger other neuronal pools which, in turn, inhibit the cholinergic cells. Since the subclasses of 5-HT receptors are topographically and functionally different in different animals (Peroutka, 1988; Hoyer, 1989; Hoyer & Middlemiss, 1989; Burridge *et al.*, 1989; Siniscalchi *et al.*, 1990), caution must be taken in extrapolating these conclusions to other species, including man. In any case, the present data stress the physiological inhibitory role played by 5-HT through 5-HT_3 receptors on the cholinergic system projecting into the cerebral cortex.

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References

BARNES, J.M., BARNES, N.M., COSTALL, B., NAYLOR, R.J. & TYERS, M.B. (1989). 5-HT_3 receptors mediate inhibition of acetylcholine release in cortical tissue. *Nature*, **338**, 762–763.

BEANI, L., BIANCHI, C., SANTINOCETO, L. & MARCHETTI, P. (1968). The cerebral acetylcholine release in conscious rabbits with semipermanently implanted epidural cups. *Int. J. Neuropharmacol.*, **7**, 469–481.

BEANI, L., BIANCHI, C., GIACOMELLI, A. & TAMBERI, F. (1978). Noradrenaline inhibition of acetylcholine release from guinea-pig brain. *Eur. J. Pharmacol.* **48**, 179–193.

BEANI, L., BIANCHI, C., SINISCALCHI, A., SIVILOTTI, L., TANGANELLI, S. & VERATTI, E. (1984). Different approaches to study acetylcholine release: endogenous Ach versus tritium efflux. *Naunyn Schmiedebergs Arch. Pharmacol.*, **328**, 119–126.

BEANI, L., BIANCHI, C., TANGANELLI, S., ANTONELLI, T., SIMONATO, M. & RANDO, S. (1988). Inversion of the ALPHA-2 and ALPHA-1 noradrenergic control of the cortical release of acetylcholine and γ-aminobutyric acid in morphine-tolerant guinea-pigs *J. Pharmacol. Exp. Ther.*, **247**, 294–301.

BIANCHI, C., TANGANELLI, S. & BEANI, L. (1979). Dopamine modulation of acetylcholine release from the guinea pig brain. *Eur. J. Pharmacol.*, **58**, 235–246.

BIANCHI, C., SINISCALCHI, A. & BEANI, L. (1986). The influence of 5-hydroxytryptamine on the release of acetylcholine from guinea-pig brain ex vivo and in vitro. *Neuropharmacology*, **25**, 1043–1049.

BIANCHI, C., SINISCALCHI, A. & BEANI, L. (1989). Effect of 5-hydroxytryptamine on [^{3}H]-acetylcholine release from guinea-pig striatal slices. *Br. J. Pharmacol.*, **97**, 213–221.

BLIER, P. & DE MONTIGNY, C. (1987). Modification of 5-HT neuron properties by sustained administration of the 5-HT_{1A} agonist gepirone: electrophysiological studies in the brain. *Synapse*, **1**, 470–480.

BURRIDGE, J., BUTLER, A. & KILPATRICK, G.J. (1989). 5-HT_3 receptors mediate depolarization of guinea-pig isolated vagus nerve. *Br. J. Pharmacol.*, **96**, 269P.

CARBONI, E., ACQUAS, E., FRAU, R. & DI CHIARA, G. (1989). Differential inhibitory effects of a 5-HT_3 antagonist on drug-induced stimulation of dopamine release. *Eur. J. Pharmacol.*, **164**, 515–519.

CONSOLO, S., LADINSKY, H., FORLANI, G.L., TIRELLI, A.S. & GARATTINI, S. (1980). Comparison of the effects of stereoisomers of fenfluramine on the acetylcholine content of the rat striatum, hippocampus and nucleus accumbens. *J. Pharm. Pharmacol.*, **32**, 202–203.

DE MONTIGNY, C., BLIER, P. & CHAPUT, Y. (1984). Electrophysiologically-identified serotonin receptors in rat CNS: effect of antidepressant treatment. *Neuropharmacology*, **23**, 1511–1520.

ENGEL, G., GOTHERT, M., HOYER, D., SCHLICKER, E. & HILLENBRAND, K. (1986). Identity of inhibitory presynaptic 5-hydroxytryptamine (5-HT) autoreceptors in the brain cortex with 5-HT_{1B} binding sites. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **332**, 1–7.

ENVRARD, C., JAVOY, F., HERBERT, A. & GLOWINSKI, J. (1977). Effect of quipazine, a serotonin-like drug on striatal cholinergic interneurones. *Eur. J. Pharmacol.*, **41**, 281–289.

FOZARD, J.R. & MOBARAK ALI, A.T.M. (1978). Receptors for 5-hydroxytryptamine on the sympathetic nerves of rabbit heart. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **301**, 223–235.

GADDUM, J.H. & PICARELLI, Z.P. (1957). Two kinds of tryptamine receptor. *Br. J. Pharmacol. Chemother.*, **12**, 323–328.

GILLET, G., AMMOR, S. & FILLION, G. (1985). Serotonin inhibits acetylcholine release from rat striatum slices: evidence for a presynaptic receptor-mediated effect. *J. Neurochem.*, **45**, 1687–1691.

GOODWIN, G.M., DE SOUZA, R.J., HEAL, D.J. & GREEN, A.R. (1987). The pharmacology of the behavioural and hypothermic responses of rats to 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT). *Psychopharmacology*, **91**, 506–511.

HAGAN, R.M., BUTLER, A., HILL, J.M., JORDAN, C.C., IRELAND, S.J. & TYERS, M.B. (1987). Effect of the 5HT_3 receptor antagonist, GR38032F, on responses to injection of a neurokinin agonist into

the ventral tegmental area of the rat brain. *Eur. J. Pharmacol.*, **138**, 303–305.

HOYER, D. & MIDDLEMISS, D.N. (1989). Species differences in the pharmacology of terminal 5-HT autoreceptors in mammalian brain. *Trends Pharmacol. Sci.*, **10**, 130–133.

LEYSEN, J.E., VAN GOMPEL, P., VERWIMP, M. & NIEMEGEERS, C.J.E. (1983). Role and localization of serotonin$_2$ (S_2)-receptor-binding sites: effects of neuronal lesions. In *CNS Receptors. From Molecular Pharmacology to Behaviour.* ed. Mandel, P. & De Feudis, F.V. pp. 373–383. New York: Raven Press.

MAURA, G. & RAITERI, M. (1986). Cholinergic terminals in rat hippocampus possess 5-HT_{1B} receptors mediating inhibition of acetylcholine release. *Eur. J. Pharmacol.*, **129**, 333–337.

MIDDLEMISS, D.N. (1984a). The putative 5-HT_1 receptor agonist RU 24969 inhibits the efflux of 5-hydroxytryptamine from rat frontal cortex slices by stimulation of the 5-HT autoreceptor. *J. Pharm. Pharmacol.*, **37**, 434–437.

MIDDLEMISS, D.N. (1984b). 8-Hydroxy-2-(-di-n-propylamino) tetralin is devoid of activity at the 5-hydroxytryptamine autoreceptor in rat brain. Implication for the proposed link between the autoreceptor and the [^{3}H]-5-HT recognition site. *Naunyn Schmiedebergs Arch. Pharmacol.*, **327**, 18–22.

NAHORSKI, S.R. & WILLCOCKS, A.L. (1983). Interactions of β-adrenoceptor antagonists with 5-hydroxytryptamine receptor subtypes in rat cerebral cortex. *Br. J. Pharmacol.*, **78**, 107P.

PAZOS, A., HOYER, D. & PALACIOS, J.M. (1985). The binding of serotonergic ligands to the porcine choroid plexus: characterization of a new type of serotonin recognition site. *Eur. J. Pharmacol.*, **106**, 539–546.

PEROUTKA, J.S. (1988). Species variations in 5-HT_3 recognition sites labelled by ^{3}H-quipazine in the central nervous system. *Naunyn-Schmiedebergs Arch Pharmacol.*, **338**, 472–475.

RICHARDSON, B.P., ENGEL, G., DONATSCH, P. & STADLER, P.A. (1985). Identification of serotonin M-receptor subtypes and their specific blockade by a new class of drugs. *Nature*, **316**, 126–131.

RICHARDSON, B.P. & ENGEL, G. (1986). The pharmacology and function of 5-HT_3 receptors. *Trends Neurosci.*, **9**, 424–428.

SAMANIN, R., QUATTRONE, A., CONSOLO, S., LADINSKI, H. & ALGERI, S. (1978). Biochemical and pharmacological evidence of the interaction of serotonin with other aminergic system in the brain. In *Interactions between Putative Neurotransmitters in the Brain.* ed. Garattini, S., Pujol, J.F. & Samanin, R. pp. 383–399. New York: Raven Press.

SHANNON, M., BATTAGLIA, G., GLENNON, R.A. & TITELER, M. (1984). 5-HT_1 and 5-HT_2 binding properties of derivatives of the hallucinogen L-(2,5-dimethoxyphenyl)-2-aminopropane (2,5-DMA). *Eur. J. Pharmacol.*, **102**, 23–29.

SHARP, T., BRAMWELL, S.R. & GRAHAM-SMITH, D.G. (1989a). 5-HT_1 agonists reduce 5-hydroxytryptamine release in rat hippocampus in vivo as determined by brain microdialysis. *Br. J. Pharmacol.*, **96**, 283–290.

SHARP, T., BRAMWELL, S.R., CLARK, D. & GRAHAM-SMITH, D.G. (1989b). In vivo measurement of extracellular 5-hydroxytryptamine in hippocampus of the anaesthetized rat using microdialysis: changes in relation to 5-hydroxytryptaminergic neuronal activity. *J. Neurochem.*, **53**, 234–240.

SINISCALCHI, A., BEANI, L. & BIANCHI, C. (1990). Different effects of 8-OH-DPAT, a selective 5-HT_{1A} receptor agonist, on cortical acetylcholine release, electrocorticogram and body temperature in guinea pigs and rats. *Eur. J. Pharmacol.*, **175**, 219–223.

SPROUSE, J.S. & AGHAJANIAN, G.K. (1987). Electrophysiological responses of serotonergic dorsal raphe neurones to 5-HT_{1A} and 5-HT_{1B} agonists. *Synapse*, **1**, 3–9.

VAN DER MAELEN, C.P., MATHESON, G.K., WILDERMAN, R.C. & PATTERSON, L.A. (1986). Inhibition of serotonergic dorsal raphe neurones by systemic and iontophoretic administration of buspirone, a non-benzodiazepine anxiolytic drug. *Eur. J. Pharmacol.*, **129**, 123–130.

VIZI, E.S., HARSING, L.G. & ZSILLA, G. (1981). Evidence of the modulatory role of serotonin in acetylcholine release from striatal interneurones. *Brain Res.*, **212**, 89–99.

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The inhibitory adenosine receptor at the neuromuscular junction and hippocampus of the rat: antagonism by 1,3,8-substituted xanthines

A.M. Sebastião, T.W. Stone* & J.A. Ribeiro

Laboratory of Pharmacology, Gulbenkian Institute of Science, 2781 Oeiras, Portugal, and *Department of Pharmacology, University of Glasgow, Glasgow G12 8QQ

1 The ability of 1,3,8-substituted xanthines to antagonize the inhibitory effects of adenosine receptor agonists on the amplitude of nerve-evoked twitches of the rat phrenic-diaphragm and on the amplitude of orthodromically-evoked population spikes, recorded from the CA_1 pyramidal cells of rat hippocampal slices, was investigated.

2 1,3-Dipropyl-8-cyclopenthylxanthine (DPCPX), 1,3-dipropyl-8-(carboxymethyloxyphenyl)xanthine (XCC), 1,3-dipropyl-8-(4-((2-aminoethyl)amino)carbonylmethyloxyphenyl)xanthine (XAC), 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX), 8-phenyltheophylline (8-PT), 1,3-diethyl-8-phenylxanthine (DPX) and PD 115,199, in concentrations virtually devoid of effect on neuromuscular transmission, shifted to the right in a near parallel manner the log concentration-response curve for the inhibitory effect of 2-chloroadenosine (CADO) on nerve-evoked twitches of the phrenic-diaphragm. Linear Schild plots with slopes near to unity were obtained for all the xanthines.

3 The order of potency of the xanthines as antagonists of the effect of CADO in the phrenic-diaphragm was DPCPX (K_i = 0.54 nM) > XCC (K_i = 10 nM), XAC (K_i = 11 nM), PACPX (K_i = 13 nM) > DPX (K_i = 22 nM), 8-PT (K_i = 25 nM) > PD 115,199 (K_i = 57 nM). The potency of DPCPX in antagonizing the inhibitory effects of **R**-N^6-phenylisopropyladenosine (**R**-PIA) and 5'-N-ethylcarboxamide adenosine (NECA) on nerve-evoked twitch responses was not statistically different from its potency in antagonizing the inhibitory effect on CADO.

4 In the hippocampal slices, DPCPX, XCC and XAC, used in concentrations virtually devoid of effect on population spike amplitude, shifted to the right in a near parallel manner the log concentration-response curve for the inhibitory effect of CADO on the amplitude of the population spikes. The Schild plots were linear with slopes near unity.

5 The potencies of DPCPX (K_i = 0.45 nM) and XAC (K_i = 11 nM) in antagonizing the inhibitory adenosine receptor at the hippocampus were similar to their potencies for antagonism of the inhibitory adenosine receptor at the phrenic-diaphragm. XCC was only slightly more potent (K_i = 5.4 nM) as an antagonist of the adenosine receptor in the hippocampus than in the phrenic-diaphragm.

6 The results suggest that the inhibitory adenosine receptors in the phrenic-diaphragm and in the hippocampus of the rat are similar, and that according to the antagonist potencies, these receptors belong to the A_1-adenosine receptor subtype.

Introduction

Adenosine receptors of the P_1-purinoceptor type (Burnstock, 1978) have been classified into A_1 and A_2 subtypes according to the relative potency of agonists and their ability to increase or decrease adenylate cyclase activity (Van Calker *et al.*, 1979; Londos *et al.*, 1980). The recent development of xanthine derivatives with antagonist selectivity for the A_1- and A_2-adenosine receptors (e.g. Daly & Jacobson, 1989) allows the tentative classification of the adenosine receptors on the basis of the potency of the antagonists.

The P_1-purinoceptor mediating presynaptic inhibition of transmission at the frog neuromuscular junction operates through a mechanism which does not appear to involve the adenylate cyclase system (Sebastião & Ribeiro, 1990a), and has agonist (Ribeiro & Sebastião, 1985) and antagonist (Sebastião & Ribeiro, 1989) profiles distinct from those of the A_1- and A_2-adenosine receptors. In the rat hippocampus early work with adenosine receptor agonists suggested the involvement of A_1-receptors in mediating the presynaptic inhibition of transmitter release. This effect also appears to be independent of adenylate cyclase although it is sensitive to blockade by N-ethylmaleimide, suggesting the involvement of an atypical G-protein system (Fredholm & Lindgren, 1987).

In the present work we investigated the potency of several 1,3,8-substituted xanthines as antagonists of the adenosine receptor mediating inhibition of synaptic transmission at the neuromuscular junction and hippocampus of the rat, to compare the antagonist profile of inhibitory adenosine receptors in the peripheral and central nervous systems of the same animal species.

Brief accounts of some of the results have already been presented (Ribeiro & Sebastião, 1989; Sebastião *et al.*, 1989; Sebastião & Ribeiro, 1990b).

Methods

The experiments were carried out on phrenic nerve-hemidiaphragm preparations and on hippocampal slices of the rat. Male rats of not more than 250 g in weight were used.

Phrenic nerve-hemidiaphragm preparations

Innervated strips (approx. 10 mm wide) of the left hemidiaphragm were set up in a 25 ml bath at 37°C, through which the solutions flowed continuously with the aid of a roller pump. The solutions were changed by transferring the inlet tube of the pump from one flask to another. The flow rate was 50 ml min^{-1} during the first 2 min after the solutions had been changed and 10 ml min^{-1} until the next changeover of solutions. Throughout the experiments the solutions were continuously gassed with 95% O_2 and 5% CO_2. The nerve was stimulated supramaximally with rectangular pulses of 100 μs

duration applied once every 5 s. The twitch responses to nerve stimulation were recorded isometrically at a resting tension of 50 mN with a Sanborn transducer and displayed on a Hewlett-Packard recorder. The bathing solution (pH 7.4) contained (mM): NaCl 117, KCl 5, $NaHCO_3$ 25, NaH_2PO_4 1.2, glucose 11, $CaCl_2$ 2.5 and $MgCl_2$ 1.2. In order to reduce the safety margin of neuromuscular transmission (Paton & Waud, 1967) submaximal concentrations (0.8–1.1 μM) of tubocurarine were added to the bath. The concentration of tubocurarine was constant throughout each experiment and was chosen as that causing about 50% decrease in the amplitude of nerve-evoked twitch responses. The averaged amplitude of the nerve-evoked twitches in the experiments described in this paper was 76 ± 1.8 mN in the normal bathing solution and 32 ± 1.1 mN in the presence of tubocurarine (0.8–1.1 μM).

Hippocampal slices

The hippocampi were removed into ice-cold bathing solution of the following composition (mM): NaCl 115, KCl, 2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 2.2, $NaHCO_3$ 25 and glucose 10. Slices were transversely cut 400–500 μm thick on a McIlwain tissue chopper and stored immersed in the bathing solution at room temperature under an atmosphere of water-saturated 95% O_2 and 5% CO_2. After a minimum recovery period of 1 h, individual slices were transferred to a 1 ml perfusion chamber through which the solutions flowed continuously at a rate of 3 ml min^{-1}. The slices were completely submerged in the bathing solution, which was kept at 28–30°C, unless otherwise stated. Occasionally, experiments were performed at room temperature (22–25°C). The solutions were continuously gassed with 95% O_2 and 5% CO_2 and were changed by transferring the inlet tube of the perfusion system from one flask to another. The bath level was kept constant by suction.

Orthodromic population spikes were recorded in the pyramidal layers of the CA_1 region through a single glass microelectrode filled with 3 M KCl or 1 M NaCl and with 5–10 MΩ resistance. The Schaffer collateral and commisural afferents to the CA_1 pyramids were stimulated with rectangular pulses of 100 μs duration delivered once every 10 s through a bipolar concentric electrode placed in the stratum radiatum. The intensity of the stimuli (30–200 μA) was set so that the amplitude of the recorded population spikes was about 80% of the population spike amplitude obtained with supramaximal stimulation. Evoked responses were either displayed on a digital oscilloscope or 8 or 16 consecutive responses were averaged with a Datalab DL-4000 computer. The output of the oscilloscope or computer was coupled to a pen recorder. The usual procedure was to continue to record potentials in the same solution until a stable value was obtained.

Concentration-response curves and Schild regressions

2-Chloroadenosine (CADO) was usually used as the adenosine receptor agonist because this adenosine analogue is a full agonist of the adenosine receptor at the rat phrenic-diaphragm (Sebastião & Ribeiro, 1988) and hippocampus (Dunwiddie & Fredholm, 1984), and is easily washed out. In some experiments 5'-N-ethylcarboxamide adenosine (NECA) or **R**-N^6-phenylisopropyladenosine (**R**-PIA) were used as the agonists for comparison with the results obtained with CADO. In each experiment a concentration-response curve to the agonist was obtained first in the absence of the antagonist. The preparation was then equilibrated with the antagonist, in the absence of the agonist, for at least 60 min, and a second concentration-response curve for the agonist, still in the presence of the antagonist, was then performed. In separate experiments it was observed that two consecutive agonist concentration-response curves in the absence of antagonist did not differ, as assessed by the ratios (0.99 ± 0.07, $n = 10$) between the EC_{50} values for the agonist during the 2nd and the 1st applications.

In the rat phrenic-diaphragm preparations the EC_{50} value for the agonist was considered to be the concentration of agonist causing a 45% decrease in the nerve-evoked twitch amplitude, since the averaged maximal effect of the adenosine receptor agonists in this preparation is about 90% decrease in twitch amplitude (cf. Sebastião & Ribeiro, 1988). In the rat hippocampus the EC_{50} value for the agonist was considered to be the concentration of agonist causing a 50% decrease in the amplitude of orthodromic population spikes since they could be inhibited 100% by the agonist. The EC_{50} values for the agonist were calculated in each experiment by regression analysis of the linear part (between 20% and 80% effect) of the concentration-response curves for the agonist. The ratios between the EC_{50} values for the agonist (CR) in the presence and in the absence of antagonist were used in the Schild equation (Arunlakshana & Schild, 1959)

$$\log (CR - 1) = n \log [B] - \log K_i$$

where [B] is the molar concentration of antagonist, K_i the equilibrium dissociation constant of the antagonist for the receptor, and n the slope of the regression of log (CR − 1) upon log [B]. pA_2 values were determined on the abscissae intercept of the regression of log (CR − 1) upon log [B]. Where the regression was linear and the slope approximated to unity, the K_i value was taken as the negative antilog of the pA_2 value.

Drugs

Drugs used were: 2-chloroadenosine, (+)-tubocurarine chloride (Sigma); 1,3-diethyl-8-phenylxanthine (DPX), 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX), 1,3-dipropyl - 8 - (4 - ((2 - aminoethyl)amino)carbonylmethy - loxyphenyl)xanthine (XAC), 1,3 - dipropyl - 8 - cyclopentyl - xanthine (DPCPX), 5'-N-ethylcarboxamide adenosine (NECA), **R**-N^6-phenylisopropyladenosine (**R**-PIA), 8-phenyltheophylline (8-PT) (Research Biochemicals Inc.); 1,3-dipropyl-8-(carboxymethyloxyphenyl)xanthine (XCC) was kindly supplied by Dr K.A. Jacobson (National Institute of Health, Bethesda, U.S.A.) and PD 115,199 (N-(2-(dimethylamino)ethyl) - N - methyl - 4 - (2,3,6,7 - tetrahydro - 2,6-dioxo - 1,3-dipropyl-1H-purin-8-yl)-benzenesulphonamide) was a gift from Dr R.F. Bruns and Warner-Lambert/Parke-Davis (Ann Arbor, U.S.A.). **R**-PIA was made up in a 50 mM stock solution in dimethylsulphoxide (DMSO). Stock solutions of DPX (2.5 mM), PD 115,199 (1 mM) and 8-PT (10 mM) were made up in 80% methanol/20% M NaOH (v/v); stock solutions of DPCPX (50 μM) and PACPX (100 μM) were made up in 99% DMSO/1% M NaOH (v/v); stock solutions of XAC (80 μM) and XCC (100 μM) were made up in DMSO. In each experiment all the solutions without the antagonist contained the same amount of solvent as the solutions with the antagonist.

Statistics

Data are expressed as mean ± s.e.mean, and n represents the number of experiments. The significance of the difference between the means was calculated by Student's t test. P values of 0.05 or less were considered to represent significant differences.

Results

Phrenic nerve-diaphragm

Figure 1 shows the effects of 2-chloroadenosine (CADO), **R**-N^6-phenylisopropyladenosine (**R**-PIA) and 5'-N-ethylcarboxamide adenosine (NECA) on the amplitude of nerve-evoked twitch responses of the diaphragm in the absence and in the presence of 1,3-dipropyl-8-cyclopenthylxanthine (DPCPX, 1–5 nM). CADO (0.3–30 μM), **R**-PIA (0.01–0.3 μM) and NECA (0.01–1 μM) decreased, in a concentration-

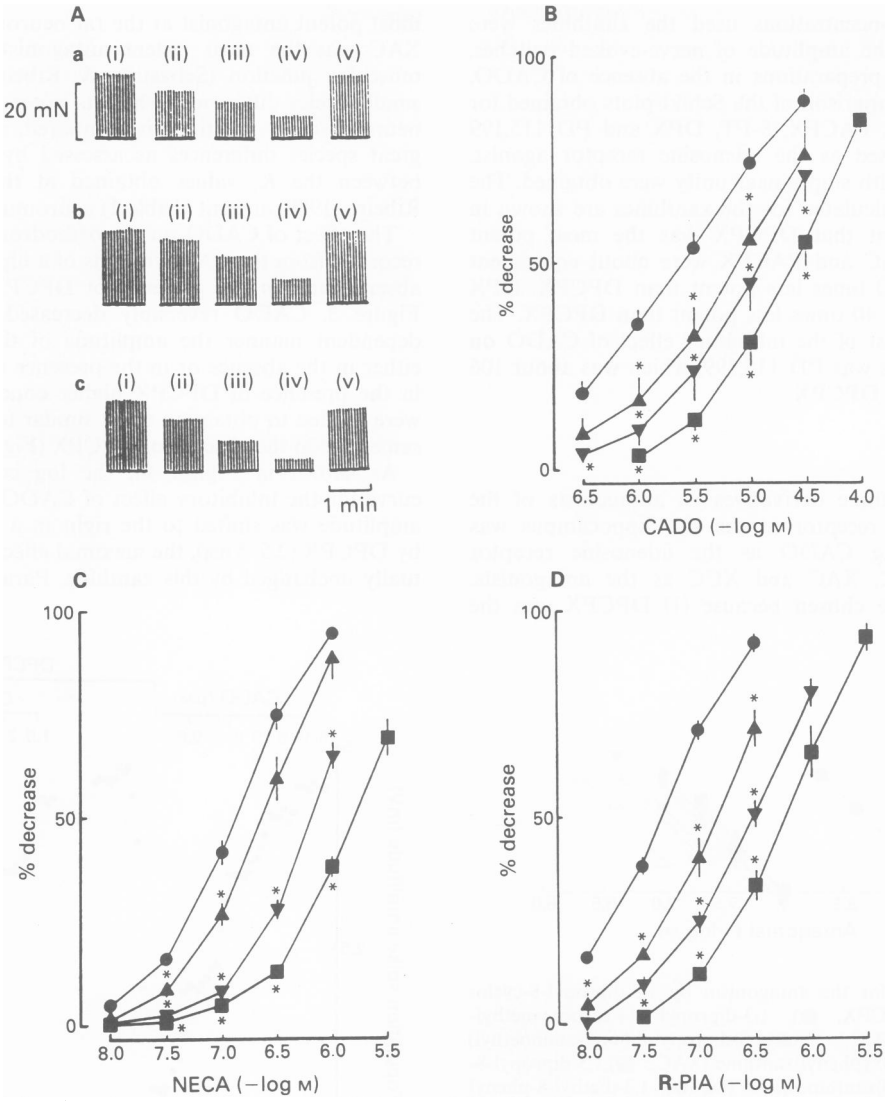

Figure 1 Effects of 2-chloroadenosine (CADO), 5'-N-ethylcarboxamide adenosine (NECA) and R-N^6-phenylisopropyladenosine (R-PIA) in the absence and in the presence of 1,3-dipropyl-8-cyclopenthyl xanthine (DPCPX) on the amplitude of nerve-evoked twitch responses of the rat phrenic-diaphragm. (A) Pen-recorder traces of nerve-evoked twitches; (a) effect of CADO; responses recorded: (i) in the control bathing solution before applying CADO, and after 12 min in (ii) 1 μM CADO, (iii) 3 μM CADO, (iv) 10 μM CADO, and (v) 75 min after the washing out of CADO had started; (b) effect of NECA; responses recorded: (i) in the control bathing solution before applying NECA, and after 10 min in (ii) 30 nM NECA, (iii) 100 nM NECA, (iv) 300 nM NECA, and (v) 60 min after commencement of the washing out of NECA; (c) effect of R-PIA; responses recorded: (i) in the control bathing solution before applying R-PIA, and after 15 min in (ii) 30 nM R-PIA, (iii) 100 nM R-PIA, (iv) 300 nM R-PIA, and (v) 120 min after commencement of the washing out of R-PIA. The full effect of each concentration of agonist is shown. (B to D) The action of DPCPX on the log concentration-response curves for the effects of CADO (B), NECA (C) and R-PIA (D); the ordinates show the percentage decreases in twitch amplitude caused by the agonists in the absence (control, ●, n = 10–11), and in the presence of 1 nM (▲, n = 3–4), 2.5 nM (▼, n = 3–4), and 5 nM (■, n = 3–4) DPCPX. Zero % is the nerve-evoked twitch amplitude in the control bathing solution and 100% represents a complete inhibition of the twitches. The vertical bars represent s.e.mean and are shown when they exceed the symbols in size. *P < 0.05 (Student's t test) as compared with controls. All solutions without DPCPX contained its solvent (99% DMSO/1% M NaOH) in the same concentration (0.002–0.01% v/v) as that present in the DPCPX solutions. The solutions contained tubocurarine (0.85–1.1 μM) which decreased the nerve-evoked twitch amplitude to 43 ± 1.7% of its value (84 ± 2.2 mN) in the normal bathing solution.

dependent manner, the amplitude of the nerve-evoked twitches. DPCPX (1–5 nM) used alone was virtually devoid of effect on nerve-evoked twitches, but shifted to the right the concentration-response curves for CADO (Figure 1B), NECA (Figure 1C) and R-PIA (Figure 1D), the shift being greater the higher the concentration of DPCPX. The slope of the regression line of the linear part of the log concentration-response curves for the agonists was not significantly modified by DPCPX (1–5 nM). The Schild regressions for DPCPX, obtained from the data in Figure 1, were linear (correlation coefficients with P < 0.05) and had a slope near unity (1.01 with CADO, 1.02 with NECA and 1.03 with R-PIA). The pA_2 value for DPCPX obtained with CADO as the agonist was 9.27 ± 0.05 (n = 11). This value was not significantly different (P > 0.05) from the pA_2 values obtained with NECA (pA_2 = 9.20 ± 0.03, n = 10) or R-PIA (pA_2 = 9.30 ± 0.03, n = 10) as the adenosine receptor agonists.

Concentration-dependent shifts to the right of the concentration-response curves for the inhibitory effect of CADO on nerve-evoked twitches were also observed with 1,3-dipropyl-8-(carboxymethyloxyphenyl)xanthine (XCC, 10–100 nM), 1,3-dipropyl-8-(4-((2-aminoethyl)amino)-carbonylmethyloxyphenyl)xanthine (XAC, 10–50 nM), 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX, 10–100 nM), 8-phenyltheophylline (8-PT, 50–250 nM), 1,3-diethyl-8-phenylxanthine (DPX, 50–250 nM) and PD 115,199 (100–500 nM). The slope of the linear part of the log concentration-response curves for CADO was not appreciably modified by these

xanthines. In the concentrations used the xanthines were devoid of effect on the amplitude of nerve-evoked twitches, when applied to the preparations in the absence of CADO. Figure 2 shows a comparison of the Schild plots obtained for DPCPX, XCC, XAC, PACPX, 8-PT, DPX and PD 115,199 when CADO was used as the adenosine receptor agonist. Linear Schild plots with slopes near unity were obtained. The pA_2 and K_i values calculated for the xanthines are shown in Table 1. It is evident that DPCPX was the most potent antagonist, XCC, XAC and PACPX were about equipotent and approximately 20 times less potent than DPCPX. DPX and 8-PT were about 40 times less potent than DPCPX. The least potent antagonist of the inhibitory effect of CADO on nerve-evoked twitches was PD 115,199, which was about 106 times less potent than DPCPX.

Hippocampal slices

The potency of xanthine derivatives as antagonists of the inhibitory adenosine receptor at the rat hippocampus was investigated by using CADO as the adenosine receptor agonist and DPCPX, XAC and XCC as the antagonists. These xanthines were chosen because (1) DPCPX was the most potent antagonist at the rat neuromuscular junction, (2) XAC was the most potent antagonist at the frog neuromuscular junction (Sebastião & Ribeiro, 1989) and shows small species differences when its potency at the rat and frog neuromuscular junctions is compared, and (3) XCC exhibits great species differences as assessed by the high ratio (190) between the K_i values obtained at the frog (Sebastião & Ribeiro, 1989) and rat (Table 1) neuromuscular junctions.

The effect of CADO on the orthodromic population spikes recorded from the CA_1 pyramids of a hippocampal slice in the absence and in the presence of DPCPX (5 nM) is shown in Figure 3. CADO reversibly decreased in a concentration-dependent manner the amplitude of the population spikes, either in the absence or in the presence of DPCPX. However, in the presence of DPCPX higher concentrations of CADO were needed to obtain an effect similar to that observed in the same slice in the absence of DPCPX (Figure 3a).

As shown in Figure 4a, the log concentration-response curve for the inhibitory effect of CADO on population spike amplitude was shifted to the right in a near parallel manner by DPCPX (2.5–5 nM), the maximal effect of CADO being virtually unchanged by this xanthine. Parallel shifts to the right

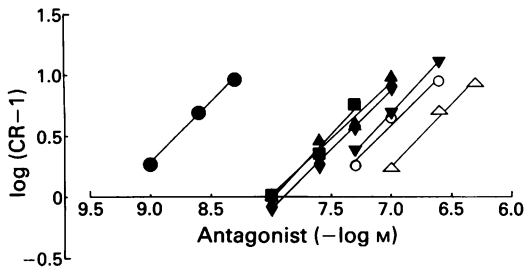

Figure 2 Schild plots for the antagonism by 1,3-dipropyl-8-cyclopenthyl xanthine (DPCPX, ●), 1,3-dipropyl-8-(4-carboxymethyloxyphenyl)xanthine (XCC, ▲),1,3-dipropyl-8-(4-((2-aminoethyl) amino) carbonylmethyloxyphenyl)xanthine (XAC, ■),1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX, ◆), 1,3-diethyl-8-phenyl xanthine (DPX, ▼), 8-phenyltheophylline (8-PT, ○) and PD 115,199 (△) of the inhibitory effect of 2-chloroadenosine (CADO) on nerve-evoked twitch responses of the rat phrenic-diaphragm. Each point is the average of the concentration-ratio (CR) values obtained in 3–4 experiments. The regression lines were calculated by the method of least squares using individual CR values; the correlation coefficients ranged from 0.955 to 0.819 ($P < 0.05$ in all cases). CR values were calculated at the level of 45% decrease in twitch amplitude caused by CADO, which was considered to be its EC_{50} value since the averaged maximal effect of adenosine receptor agonists in the rat phrenic-diaphragm is about 90% decrease in nerve-evoked twitch amplitude (cf. Sebastião & Ribeiro, 1988). Averaged EC_{50} value for CADO in the absence of the antagonists: 2.5 ± 0.18 μM ($n = 77$).

Table 1 Potencies of xanthine derivatives as antagonists of the inhibitory effect of 2-chloroadenosine on nerve-evoked twitch amplitude in the rat phrenic-diaphragm

	pA_2	Slope*	K_i (nM)	n
DPCPX	9.27 ± 0.05	1.01	0.54	11
XCC	8.01 ± 0.03*	0.93	10	12
XAC	7.97 ± 0.07	1.09	11	10
PACPX	7.90 ± 0.04	0.98	13	12
DPX	7.66 ± 0.04**	1.06	22	9
8-PT	7.61 ± 0.05	0.98	25	12
PD 115,199	7.24 ± 0.04***	1.04	57	11

*Slope of the regression line of the Schild plot shown in Figure 2. *$P < 0.05$ as compared with DPCPX; **$P < 0.05$ as compared with PACPX; ***$P < 0.05$ as compared with 8-PT (Student's t test). For key to abbreviations used see legend to Figure 2.

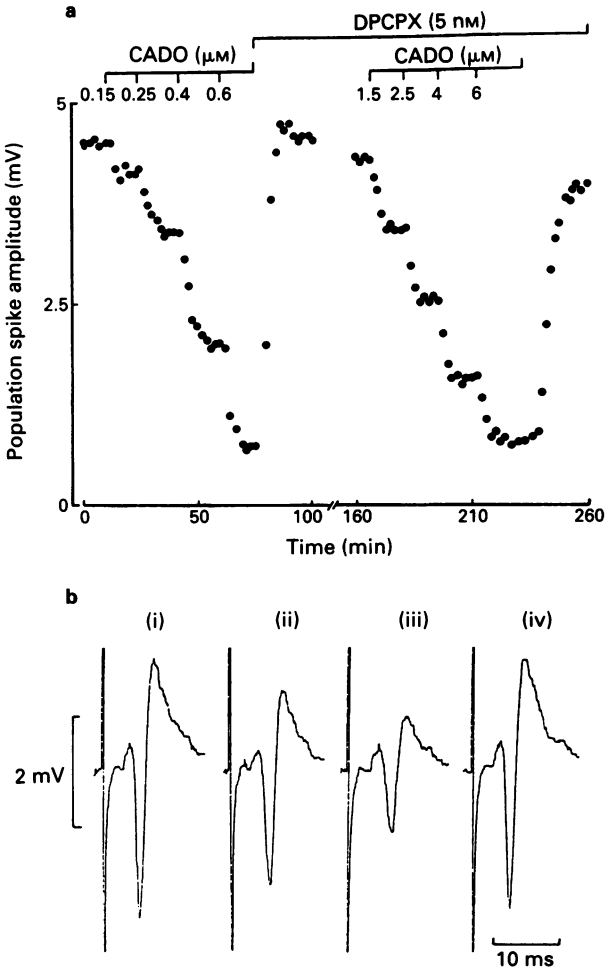

Figure 3 Effect of 2-chloroadenosine (CADO) in the absence and in the presence of 1,3-dipropyl-8-cyclopenthylxanthine (DPCPX, 5 nM) on the averaged amplitude of orthodromically-evoked population spikes recorded from CA_1 pyramidal cells of a rat hippocampal slice. (a) Time course of the effect of CADO. The ordinates show the amplitude of the computed averages of 8 successive population spikes and the abscissae the times averaging began. The horizontal bars represent the period of perfusion of the drugs. (b) Pen-recorder traces of averaged population spikes corresponding to (i) 10 min, (ii) 40 min, (iii) 60 min, and (iv) 85 min in (a); each population spike is preceded by the presynaptic volley and the stimulus artifact. All solutions without DPCPX contained a similar concentration (0.1% v/v) of its solvent (99% DMSO/1% M NaOH).

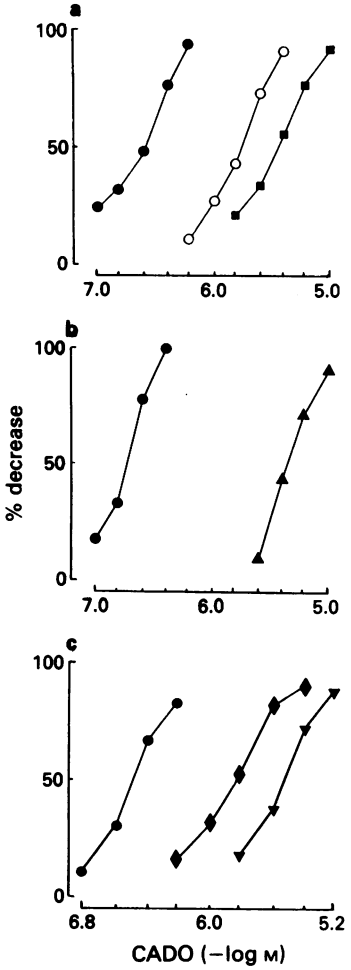

Figure 4 Actions of (a) 1,3-dipropyl-8-cyclopenthyl xanthine (DPCPX), (b) 1,3-dipropyl-8-carboxymethyloxyphenyl)xanthine (XCC), and (c) 1,3-dipropyl-8-(4-((2-aminoethyl)amino)-carbonylmethyloxyphenyl)xanthine (XAC) on the log concentration-response curves for the inhibitory effect of 2-chloroadenosine (CADO) on orthodromically-evoked population spikes recorded from the CA_1 pyramidal cells of rat hippocampal slices. The ordinates show the percentage decreases in population spike amplitude caused by CADO in the absence (●) and in the presence of: (a) 2.5 nM (○) or 5 nM (■) DPCPX; (b) 100 nM (▲) XCC; (c) 25 nM (◆) or 100 nM (▼) XAC. Zero % is the population spike amplitude before applying CADO and 100% represents a complete inhibition of the population spikes. Each panel represents the results obtained in a single experiment where the effect of CADO in the absence and in the presence of the antagonist was compared in the same slice. Population spike amplitude in the control bathing solution 4.7 mV in (a), 2.3 mV in (b) and 2.7 mV in (c).

of the log concentration-response curve for the effect of CADO on population spikes were also obtained with XCC (Figure 4b) and XAC (Figure 4c).

The Schild plots for DPCPX (1–5 nM), XCC (25–100 nM) and XAC (25–100 nM) obtained from pooled data from different slices were linear with slopes near unity (Figure 5). In the concentrations used the xanthines did not modify in an appreciable manner the amplitude of the orthodromic population spikes, when applied to the slices in the absence of CADO. The pA_2 and K_i values obtained for DPCPX, XCC and XAC at the hippocampal slices are compared in Table 2. DPCPX was the most potent antagonist and was about 10 times more potent than XCC, which was about 2 times more potent than XAC. The pA_2 and K_i values obtained for XAC in experiments performed at room temperature are also shown in Table 2. No statistically significant ($P > 0.05$) differences were found between the pA_2 values obtained for this xanthine in the experiments performed at 28–30°C or at room temperature (22–25°C).

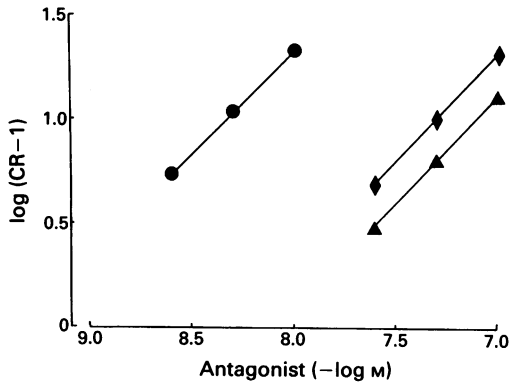

Figure 5 Schild plots for the antagonism by 1,3-dipropyl-8-cyclopenthyl xanthine (DPCPX, ●), 1,3-dipropyl-8-carboxymethyloxyphenyl)xanthine (XCC, ◆), and 1,3-dipropyl-8-(4-((2-aminoethyl)amino)carbonylmethyloxyphenyl)xanthine (XAC, ▲) of the inhibitory effect of 2-chloroadenosine (CADO) on the amplitude of orthodromically evoked population spikes recorded from CA_1 pyramidal cells in rat hippocampal slices. Each point is the average of the concentration-ratio (CR) values obtained in 2–4 experiments. The regression lines were calculated by the method of the least squares using individual CR values; correlation coefficients ranged from 0.943 to 0.894 ($P < 0.05$ in all cases). CR values were calculated at the level of 50% decrease in population spike amplitude (EC_{50}) caused by CADO. Averaged EC_{50} value for CADO in the absence of the antagonists: 283 ± 30 nM ($n = 22$).

The ratios between the K_i values obtained for DPCPX, XCC and XAC at the phrenic-diaphragm (Table 1) and at the hippocampus (Table 2) were below 2 (1.2 for DPCPX, 1.9 for XCC and 1.0–1.3 for XAC). Only in the case of XCC did the difference between the pA_2 values in the two preparations reach statistical significance ($P < 0.05$, Student's t test).

Discussion

The present results show that several 1,3,8-substituted xanthine derivatives antagonize the inhibitory effects of adenosine receptor agonists on the amplitude of nerve-evoked twitches of the rat diaphragm and on the amplitude of orthodromically-evoked population spikes recorded from the CA_1 pyramidal cells of the rat hippocampus. The rank order of potencies of the xanthines for the adenosine receptor in the innervated rat diaphragm was DPCPX > XCC, XAC, PACPX > DPX, 8-PT > PD 115,199. The potencies of DPCPX and XAC as antagonists of the adenosine receptor in the rat hippocampal slices were similar to their potencies at the innervated rat diaphragm; XCC was only slightly more potent as an antagonist of the adenosine receptor in the hippocampus than in the phrenic-diaphragm.

In concentrations similar to those used in the present study, the adenosine receptor agonists CADO, NECA and R-PIA

Table 2 Potencies of xanthine derivatives as antagonists of the inhibitory effect of 2-chloroadenosine on orthodromically-evoked population spike amplitude in the rat hippocampus

	pA_2	*Slope*[a]	K_i (nM)	n	*Temperature*
DPCPX	9.35 ± 0.03	0.99	0.45	7	28–30°C
XCC	8.27 ± 0.04*	1.03	5.4	11	28–30°C
XAC	7.94 ± 0.04**	1.06	11	5	28–30°C
XAC	8.08 ± 0.04**	1.02	8.3	6	22–25°C

[a]Slope of the regression line of the Schild plot shown in Figure 5. *$P < 0.05$ as compared with DPCPX; **$P < 0.05$ as compared with XCC (Student's t test). For key to abbreviations used see legend to Figure 5.

are devoid of effect on the amplitude of twitches evoked by direct muscle stimulation of phrenic-diaphragm preparations in the presence of supramaximal concentrations of tubocurarine (Sebastião & Ribeiro, 1988), and thus the effect of these adenosine analogues on the amplitude of nerve-evoked twitches is due to inhibition of neuromuscular transmission rather than to a direct action on contractile mechanisms. The inhibition of neuromuscular transmission caused by the adenosine receptor agonists in the phrenic-diaphragm preparation should be attributed to a presynaptic action of these substances, i.e. to a decrease in the evoked release of acetylcholine from the phrenic nerve endings, since adenosine and its analogues decrease the quantal content of endplate potentials without affecting the amplitude of miniature endplate potentials (Ginsborg & Hirst, 1972; Ribeiro & Walker, 1975; Singh *et al.*, 1986). In the rat hippocampus adenosine receptor agonists have pre- post- and non-synaptic inhibitory actions, but only the presynaptic actions are dependent on the presence of magnesium ions (Bartrup & Stone, 1988a; Schubert & Mager, 1989). In the absence of magnesium the inhibitory effect of CADO on the amplitude of orthodromically-evoked population spikes of CA_1 pyramidal cells of the rat hippocampus is markedly reduced, or even abolished, at concentrations of CADO below 1 μM (Bartrup & Stone, 1988b). This indicates that in the concentrations used in the present work, the main effect of CADO on population spike amplitude has a presynaptic origin.

In both the phrenic-diaphragm preparation and the hippocampal slices, the 1,3,8-substituted xanthines appear to antagonize a homogeneous population of adenosine receptors since the Schild plots were linear at low concentration-ratios (cf. Kenakin, 1987). A further indication that in the phrenic-diaphragm DPCPX antagonizes a homogeneous population of adenosine receptors, which can be activated by CADO, NECA and R-PIA, comes from the observation that the potency of DPCPX in antagonizing the inhibitory effect of CADO did not differ from the potency of the xanthine in antagonizing the inhibitory effects of NECA or R-PIA.

A narrow range of antagonist concentrations was used in the present work to avoid the use of high concentrations of agonist which could act on other types of adenosine receptor eventually present in the preparations. Though the low concentration-ratios obtained might not be sufficient to characterize the nature of the antagonism, the finding that the slopes of the Schild plots were near unity is an indication that the inhibitory adenosine receptors in the phrenic-diaphragm and hippocampus are competitively antagonized by the 1,3,8-substituted xanthines.

The similarity between the K_i values for DPCPX, XCC and XAC in the rat phrenic-diaphragm and in the rat hippocampus indicates that no marked differences exist between the presumed presynaptic adenosine receptor in both preparations. These observations suggest that presynaptic adenosine receptors in the peripheral and in the central nervous systems of the same animal species are similar in relation to the antagonist potencies.

The reported K_i values of DPCPX for the A_1-adenosine receptor in whole brain membranes of the rat, determined from the ability of the xanthine to inhibit binding of [^{3}H]-N^6-cyclohexyladenosine or [^{3}H]-R-PIA, range from 0.3 nM to 1.2 nM (Ukena *et al.*, 1986a; Bruns *et al.*, 1987; Lohse *et al.*, 1987). A much higher K_i value for DPCPX (340 nM) was obtained in relation to its ability to inhibit binding of [^{3}H]-NECA to rat striatal membranes (Bruns *et al.*, 1987; Lohse *et al.*, 1987), which possess A_2-adenosine receptors. Intermediate K_i values for DPCPX (8–35 nM) were found in relation to its ability to antagonize the adenosine receptor coupled to inositol phospholipid metabolism in mammalian cerebral cortical slices (Alexander *et al.*, 1989), or to antagonize the adenosine receptor mediating presynaptic inhibition of transmission at the frog neuromuscular junction (Sebastião & Ribeiro, 1989), which has been classified as an A_3-adenosine receptor subtype (Ribeiro & Sebastião, 1986) of the P_1-purinoceptors and also appears to be coupled to the phosphoinositide transducing system (Sebastião & Ribeiro, 1990a). The K_i values for DPCPX found in the present work (0.45–0.54 nM) are consistent with a classification of the inhibitory adenosine receptors in the rat phrenic-diaphragm and rat hippocampus as A_1-adenosine receptors. Furthermore, the potencies of the 1,3,8-substituted xanthines as antagonists of the inhibitory adenosine receptor at the phrenic-diaphragm correlate with their potencies as antagonists of the A_1 adenosine receptor in rat brain membranes (Figure 6a) or rat adipocytes (Figure 6b), but do not correlate with their potencies as antagonists of the

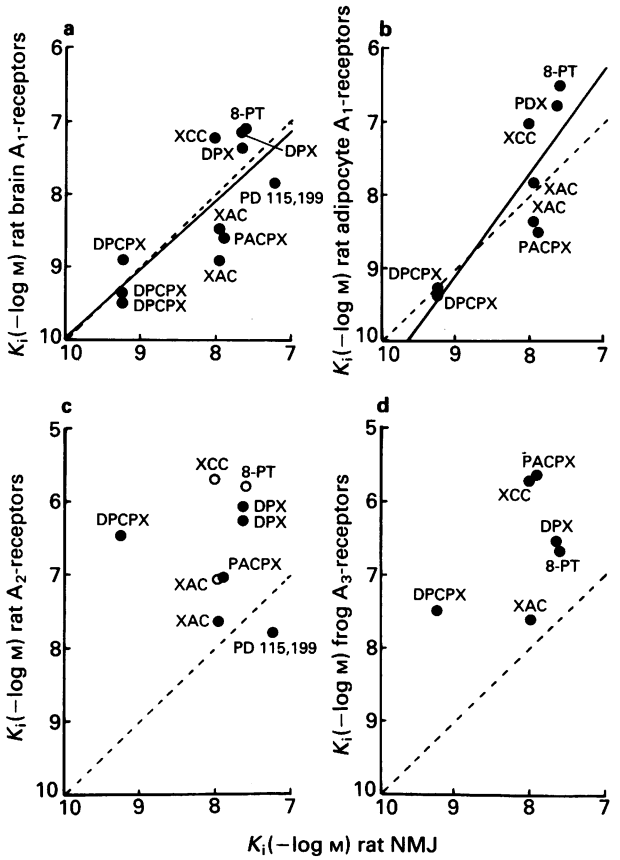

Figure 6 Scatter diagram comparing the affinities of 1,3,8-substituted xanthines for the adenosine receptor at the rat neuromuscular junction (NMJ) (expressed in the abscissae as the K_i values shown in Table 1) and for (a) the A_1-adenosine receptor in whole rat brain membranes, determined from inhibition of [^{3}H]-R-N^6-phenylisopropyladenosine [^{3}H]-R-PIA) or [^{3}H]-cyclohexyladenosine ([^{3}H]-CHA) binding (Ukena *et al.*, 1986a; Bruns *et al.*, 1987; Daly *et al.*, 1987; Lohse *et al.*, 1987); (b) the A_1-adenosine receptor in rat adipocytes, determined from antagonism of R-PIA-induced inhibition of adenylate cyclase activity (Ukena *et al.*, 1986b; Lohse *et al.*, 1987; Martinson *et al.*, 1987); (c) the A_2-adenosine receptors in (●) rat striatal membranes, determined from inhibition of [^{3}H]-5'-N-ethylcarboxamideadenosine [^{3}H]-NECA) binding (Bruns *et al.*, 1987; Lohse *et al.*, 1987), or (○) rat phaeochromocytoma cells, determined from antagonism of NECA-induced stimulation of adenylate cyclase activity (Ukena *et al.*, 1986b); (d) the A_3 adenosine receptor at the frog neuromuscular junction, determined from antagonism of 2-chloroadenosine (CADO)-induced inhibition of transmission (Sebastião & Ribeiro, 1989). The correlation coefficients (method of the least squares) were: (a) $r = 0.775$, $P < 0.01$; (b) $r = 0.839$, $P < 0.01$; (c) $r = -0.154$, $0.6 < P < 0.7$; (d) $r = 0.441$, $0.3 < P < 0.4$. The regression line is indicated in the cases where the correlation coefficient was statistically significant (a and b). The broken line indicates a gradient of unity. When different K_i values for the same xanthine in the same preparation have been shown they are all represented (e.g. DPCPX, XAC and XCC in (a), XAC in (b), DPX in (c)) and used in the calculation of the correlation coefficients. For key to abbreviations used see legend to Figure 2. Note the significant correlation between the affinities of the xanthines for the adenosine receptor at the rat neuromuscular junction and for the A_1-adenosine receptors.

A_2-adenosine receptor in rat striatal membranes or phaeochromocytoma cells (Figure 6c), or as antagonists of the A_3-adenosine receptor in the frog neuromuscular junction (Figure 6d).

In summary, the present results indicate that the inhibitory adenosine receptors in a peripheral nervous system preparation, the rat phrenic-diaphragm, and in a central nervous system preparation, the rat hippocampus, are similar in relation to the antagonist profile. According to the potencies of 1,3,8-substituted xanthines these receptors belong to the A_1-adenosine receptor subtype.

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References

ALEXANDER, S.P.H., KENDALL, D.A. & HILL, S.J. (1989). Differences in the adenosine receptors modulating inositol phosphates and cyclic AMP accumulation in mammalian cerebral cortex. *Br. J. Pharmacol.*, **98**, 1241–1248.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BARTRUP, J.T. & STONE, T.W. (1988a). Presynaptic actions of adenosine are magnesium-dependent. *Neuropharmacology*, **27**, 761–763.

BARTRUP, J.T. & STONE, T.W. (1988b). Interactions between adenosine and magnesium on rat hippocampal slices. *Brain Res.*, **463**, 374–379.

BRUNS, R.F., FERGUS, J.H., BADGER, E.W., BRISTOL, J.A., SANTAY, L.A., HARTMAN, J.D., HAYS, S.J. & HUANG, C.C. (1987). Binding of the A_1-selective adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine to rat brain membranes. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **335**, 59–63.

BURNSTOCK, G. (1978). A basis for distinguishing two types of purinergic receptor. In *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach*, ed. Straub, R.W. & Bolis, L., pp. 107–118. New York: Raven Press.

DALY, J.W. & JACOBSON, K.A. (1989). Molecular probes for adenosine receptors. In *Adenosine Receptors in the Nervous System*, ed. Ribeiro, J.A., pp. 41–52. London: Taylor & Francis.

DALY, J.W., UKENA, D. & JACOBSON, K.A. (1987). Analogues of adenosine, theophylline, and caffeine: selective interactions with A_1 and A_2 adenosine receptors. In *Topics and Perspectives in Adenosine Research*, ed. Gerlach, E. & Becker, B.F., pp. 23–36. Berlin: Springer-Verlag.

DUNWIDDIE, T.V. & FREDHOLM, B.B. (1984). Adenosine receptors mediating inhibitory electrophysiological responses in rat hippocampus are different from receptors mediating cyclic AMP accumulation. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **326**, 294–301.

FREDHOLM, B.B. & LINDGREN, E. (1987). Effects of N-ethylmaleimide and forskolin on noradrenaline release from rat hippocampal slices. Evidence that prejunctional adenosine and α-receptors are linked to N-proteins but not to adenylate cyclase. *Acta Physiol. Scand.*, **130**, 95–105.

GINSBORG, B.L. & HIRST, G.D.S. (1972). The effect of adenosine on the release of the transmitter from the phrenic nerve of the rat. *J. Physiol.*, **224**, 629–645.

KENAKIN, T.P. (1987). *Pharmacologic Analysis of Drug-Receptor Interaction*. New York: Raven Press.

LOHSE, M.J., KLOTZ, K.-N., LINDENBORN-FOTINOS, J., REDDINGTON, M., SCHWABE, U. & OLSSON, R.A. (1987). 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) – a selective high affinity antagonist radioligand for A_1 adenosine receptors. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **336**, 204–210.

LONDOS, C., COOPER, D.M.F. & WOLFF, J. (1980). Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 2551–2554.

MARTINSON, E.A., JOHNSON R.A. & WELLS, J.N. (1987). Potent adenosine receptor antagonists that are selective for the A_1 receptor subtype. *Molec. Pharmacol.*, **31**, 247–252.

PATON, W.D.M. & WAUD, D.R. (1967). The margin of safety of neuromuscular transmission. *J. Physiol.*, **191**, 59–90.

RIBEIRO, J.A. & SEBASTIÃO, A.M. (1985). On the type of receptor involved in the inhibitory action of adenosine at the neuromuscular junction. *Br. J. Pharmacol.*, **84**, 911–918.

RIBEIRO, J.A. & SEBASTIÃO, A.M. (1986). Adenosine receptors and calcium: basis for proposing a third (A_3) adenosine receptor. *Prog. Neurobiol.*, **26**, 179–209.

RIBEIRO, J.A. & SEBASTIÃO, A.M. (1989). Adenosine receptors mediating inhibition of peripheral and central neurons. *Symposium on Purine Nucleosides and Nucleotides in Cell Signalling: Targets for New Drugs*, Bethesda, U.S.A., abstract S30.

RIBEIRO, J.A. & WALKER, J. (1975). The effects of adenosine triphosphate and adenosine diphosphate on transmission at the rat and frog neuromuscular junctions. *Br. J. Pharmacol.*, **54**, 213–218.

SCHUBERT, P. & MAGER, R. (1989). Functional correlates of synaptic and extrasynaptic adenosine receptors in the hippocampus. In *Adenosine Receptors in the Nervous System*, ed. Ribeiro, J.A., pp. 159–167. London: Taylor & Francis.

SEBASTIÃO, A.M. & RIBEIRO, J.A. (1988). On the adenosine receptor and adenosine inactivation at the rat diaphragm neuromuscular junction. *Br. J. Pharmacol.*, **94**, 109–120.

SEBASTIÃO, A.M. & RIBEIRO, J.A. (1989). 1,3,8- and 1,3,7-substituted xanthines: relative potency as adenosine receptor antagonists at the frog neuromuscular junction. *Br. J. Pharmacol.*, **96**, 211–219.

SEBASTIÃO, A.M. & RIBEIRO, J.A. (1990a). Interactions between adenosine and phorbol esters or lithium at the frog neuromuscular junction. *Br. J. Pharmacol.*, **100**, 55–62.

SEBASTIÃO, A.M. & RIBEIRO, J.A. (1990b). Xanthine affinities for the adenosine receptor mediating inhibition of transmission at the rat hippocampus. *Br. J. Pharmacol.*, **99**, 141P.

SEBASTIÃO, A.M., RIBEIRO, J.A. & STONE, T.W. (1989). Antagonism of the adenosine receptor at the phrenic nerve-diaphragm and hippocampus of the rat by a xanthine amine congener. *Br. J. Pharmacol.*, **96**, 265P.

SINGH, Y.N., DRYDEN, W.F. & CHEN, H. (1986). The inhibitory effects of some adenosine analogues on transmitter release at the mammalian neuromuscular junction. *Can. J. Physiol. Pharmacol.*, **64**, 1446–1450.

UKENA, D., JACOBSON, K.A., PADGETT, W.L., AYALA, C., SHAMIM, M.T., KIRK, K.L., OLSSON, R.O. & DALY, J.W. (1986a). Species differences in structure-activity relationships of adenosine agonists and xanthine antagonists at rat brain A_1 adenosine receptors. *FEBS Lett.*, **209**, 122–128.

UKENA, D., DALY, J.W., KIRK, K.L. & JACOBSON, K.A. (1986b). Functionalized congeners of 1,3-dipropyl-8-phenylxanthine: potent antagonists for adenosine receptors that modulate membrane adenylate cyclase in pheochromocytoma cells, platelets and fat cells. *Life Sci.*, **38**, 797–807.

VAN CALKER, D., MULLER, M. & HAMPRECHT, B. (1979). Adenosine regulates via two different types of receptors the accumulation of cyclic AMP in cultured brain cells. *J. Neurochem.*, **33**, 999–1005.

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$GABA_A$ receptor-mediated stimulation of non-adrenergic non-cholinergic neurones in the dog ileocolonic junction

Guy E. Boeckxstaens, Paul A. Pelckmans, Marc Rampart, Isabelle F. Ruytjens, [1]Tony J. Verbeuren, Arnold G. Herman & [2]Yvan M. Van Maercke

Divisions of Gastroenterology and Pharmacology, Faculty of Medicine, University of Antwerp (UIA), B-2610 Antwerpen-Wilrijk, Belgium

1 The inhibitory effects of γ-aminobutyric acid (GABA), the $GABA_A$ receptor agonist homotaurine and the $GABA_B$ receptor agonist (±)-baclofen were investigated on circular muscle strips of the dog terminal ileum and ileocolonic junction.

2 In the presence of atropine, GABA and homotaurine induced concentration-dependent relaxations, similar to the non-adrenergic non-cholinergic (NANC)-mediated relaxations evoked by electrical stimulation or by acetylcholine. The ileocolonic junction was more sensitive to GABA and homotaurine than the ileum. (±)-Baclofen had no effect. Cross desensitization only occurred between GABA and homotaurine.

3 The $GABA_A$ receptor antagonist bicuculline shifted the concentration-response curves to GABA and homotaurine to the right. The maximal relaxation to GABA remained unaffected.

4 GABA-induced relaxations were not inhibited by timolol, guanethidine, domperidone, hexamethonium and desensitization to ATP, but were abolished by tetrodotoxin.

5 Bicuculline, and pretreatment with GABA or (±)-baclofen had no effect on the NANC-evoked relaxations to electrical stimulation and acetylcholine.

6 In conclusion, GABA stimulates $GABA_A$ receptors located on inhibitory NANC neurones in the dog ileocolonic junction. Our results suggest that it is unlikely that GABA is the final inhibitory NANC neurotransmitter.

Introduction

Since the proposal that γ-aminobutyric acid (GABA) may be a neurotransmitter in the vertebrate peripheral nervous system (Jessen *et al.*, 1979), evidence has accumulated demonstrating the presence of GABA receptors in the gastrointestinal tract (Krantis *et al.*, 1980; Giotti *et al.*, 1983; 1985; Ong & Kerr, 1983; Maggi *et al.*, 1984). By use of selective agonists and antagonists (Bowery *et al.*, 1981; Giotti *et al.*, 1983), two subclasses of GABA receptors can be distinguished: $GABA_A$ receptors, stimulated by GABA, homotaurine (3-amino-1-propane sulphonic acid) or muscimol and inhibited by bicuculline (competitively) and picrotoxin (non-competitively). $GABA_B$ receptors are stimulated by GABA and (−)-baclofen, and are blocked by phaclofen (Bowery *et al.*, 1981; Hill & Bowery, 1981; Dutar *et al.*, 1988).

Several lines of evidence suggest that GABA affects the motility of intestinal preparations mainly by altering the release of acetylcholine from intrinsic cholinergic neurones; stimulation of $GABA_A$ receptors may increase acetylcholine output (Krantis *et al.*, 1980; Giotti *et al.*, 1983; Ong & Kerr, 1983) whereas $GABA_B$ receptor stimulation may inhibit acetylcholine release (Ong & Kerr, 1983; Giotti *et al.*, 1983; 1985). In addition, evidence is available to suggest that GABA stimulates inhibitory intramural non-adrenergic non-cholinergic (NANC) neurones in the rat duodenum (Maggi *et al.*, 1984), and in the guinea-pig and rabbit distal colon (Krantis *et al.*, 1980; Giotti *et al.*, 1985, Tonini *et al.*, 1989).

Recently, we demonstrated that electrical stimulation and nicotinic receptor stimulation by acetylcholine or 1,1-dimethyl-4-phenylpiperazinium (DMPP) activates NANC neurones in the dog ileocolonic junction resulting in the release of an as yet unknown, inhibitory neurotransmitter (Pelckmans *et al.*, 1989). Several substances have been proposed as putative NANC inhibitory neurotransmitters, including GABA (Burnstock, 1986).

Since preliminary experiments provided evidence for GABA-induced relaxations in the dog ileocolonic junction (Boeckxstaens *et al.*, 1988), the present study was designed to investigate further the effects of GABA in this preparation and to characterize the receptors involved. Some of the results have been presented to the British Pharmacological Society (Boeckxstaens *et al.*, 1990c).

Methods

Tissue preparation

Mongrel dogs of either sex (body weight 10–30 kg) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.) and a laparotomy was performed. The ileum and the colon were resected 10 cm above and 3 cm below the ileocolonic junction respectively. After the resected specimen was cleaned and rinsed, the mucosa was removed from the ileum and ileocolonic junction by means of sharp dissection. Circular muscle strips were cut, approximately 1.5 cm long and 0.3 cm wide and mounted in organ baths (25 ml) (Pelckmans *et al.*, 1989) filled with modified Krebs-Ringer solution (mM: NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, CaEDTA 0.026 and glucose 11.1). The solution was maintained at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2.

Isometric tension recording

One end of each muscle strip was connected to a metal rod while the other end was attached to a strain gauge transducer (Statham UC_2) for continuous recording of the isometric tension. After the muscle strips were brought to the optimal point of their length-tension relationship (Vanhoutte &

[1] Present address: Institut de Recherches Servier, 11, rue des Moulineaux, 92150 Suresnes, France.
[2] Author for correspondence.

Leusen, 1969), they were washed three times and then allowed to equilibrate for at least 45 min before experimentation.

Experimental protocols

All experiments were performed on muscle strips maximally contracted with noradrenaline (Pelckmans *et al.*, 1990) (30 μM; 2.0 ± 0.3 g in the ileum and 2.8 ± 0.4 g in the ileocolonic junction, mean ± s.e.mean; n = at least 6) and in the presence of 0.3 μM atropine. Concentration-response curves to GABA, homotaurine and (±)-baclofen were constructed in a non-cumulative manner: only one concentration of agonist was administered during each noradrenaline-induced contraction and was left in contact with the tissue for 5 min. After each noradrenaline-induced contraction, the muscle strips were washed at least three times with an interval of 5 min, resulting in a cycle time of at least 20 min. This protocol was selected to avoid tachyphylaxis.

To investigate cross-desensitization, the preparations were incubated with the respective agonists (0.3 mM) 10 min before the administration of noradrenaline. Again, only one agonist was administered on the same noradrenaline-induced contraction. In these experiments, nitroglycerin (1 μM) was used as nonselective smooth muscle relaxant. In addition, the effect of various antagonists on the relaxation evoked by a submaximally-effective concentration of (GABA (0.1 mM) was assessed after a 15 min preincubation period.

In another series of experiments, the effects of bicuculline, tetrodotoxin (TTX) and GABA desensitization were studied on relaxations to electrical stimulation (2–16 Hz, 1–2 ms), acetylcholine (30 μM), GABA (0.1 mM) and nitroglycerin (1 μM). These stimuli were applied during the same noradrenaline-induced contraction. The muscle strips were washed at least three times with an interval of 5 min between each contraction. Electrical impulses (rectangular waves, 2–16 Hz, 1–2 ms, 200 mA, 9 V) were delivered by a Grass stimulator and a direct current (d.c.) amplifier for 10 s (Verbeuren *et al.*, 1983; Pelckmans *et al.*, 1989).

Drugs used

The following drugs were used: acetylcholine chloride, adenosine 5'-triphosphate (ATP), γ-aminobutyric acid (GABA), hexamethonium bromide, homotaurine (3-amino-1-propane sulphonic acid) (Sigma Chemical Co., St. Louis, MO., USA), atropine sulphate (Federa, Brussels, Belgium), (±)-baclofen, guanethidine monosulphate (Ciba-Geigy, Switzerland), domperidone (Janssen Pharmaceutica, Beerse, Belgium), nitroglycerin (Merck, Darmstadt, F.R.G.), noradrenaline hydrogentartrate (Fluka AG, Buchs SG, Switzerland), tetrodotoxin (Janssen Chimica, Beerse, Belgium) and timolol maleate (Merck Sharpe and Dohme Research Lab., Rahway, N.J., USA).

All drugs were administered as aqueous solutions except baclofen and bicuculline which were dissolved in 0.1 M citric acid. Ascorbic acid (0.6 mM) was added to the stock solution of noradrenaline. These were prepared on the day of experimentation and administered in volumes not exceeding 0.5% of the bath volume. The stock solution of tetrodotoxin (1 mM, in sodium citrate, pH: 4.8) was stored at −20°C.

Presentation of results and statistical analysis

The relaxations are expressed as percentage decrease of the noradrenaline-induced contraction or as a percentage of the relaxation of the respective agonist in control solution. The results are shown as mean ± s.e.mean for the number of dogs indicated.

The negative logarithm of the concentration of agonist (pD_2 or $-\log ED_{50}$) that produced a half-maximal response was calculated by linear regression analysis (Tallarida & Murray, 1981).

For statistical analysis, a two tailed Student's t test for paired or unpaired observations was used. P values of less than 0.05 were considered to be significant.

Results

Effect of GABA, homotaurine and (±)-baclofen

During a noradrenaline (30 μM)-induced contraction and in the presence of 0.3 μM atropine, both GABA and the $GABA_A$ receptor agonist homotaurine (10 μM–1 mM) produced concentration-dependent relaxations in the dog terminal ileum and ileocolonic junction (Figure 1). The ileocolonic junction was more sensitive to GABA and homotaurine than the terminal ileum, as demonstrated by the pD_2 ($-\log ED_{50}$) values (Table 1). The potency of GABA in relaxing the ileocolonic junction was greater than that of homotaurine, whereas in the ileum, no statistically significant differences could be demonstrated (Figure 1, Table 1). In six experiments, the $GABA_B$ receptor agonist (±)-baclofen (0.6 mM) failed to evoke relaxations in the terminal ileum or ileocolonic junction.

Effect of bicuculline on GABA- and homotaurine-induced relaxations

In both the terminal ileum and ileocolonic junction, the $GABA_A$ receptor antagonist bicuculline (3–10 μM) shifted the concentration-response curves to GABA and homotaurine to the right (Figure 2). In the ileocolonic junction, bicuculline had no effect on the maximal relaxation evoked by 1 mM GABA. Bicuculline had no effect on the spontaneous activity of the preparations.

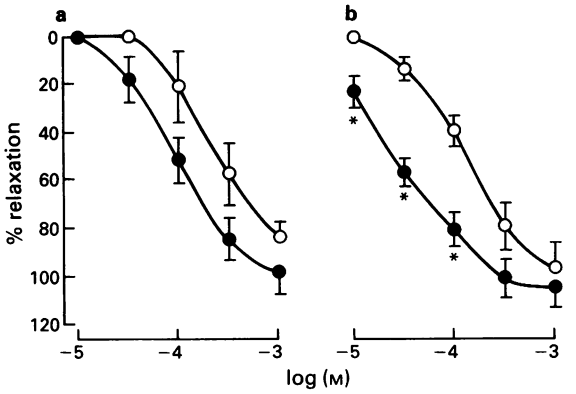

Figure 1 Concentration-response curves for the relaxant effect of GABA (●) and homotaurine (○) on circular muscle strip preparations of the dog terminal ileum (a) and ileocolonic junction (b). The experiments were performed during a noradrenaline (30 μM)-induced contraction and in the presence of 0.3 μM atropine. The results are shown as mean for 6 experiments and expressed as the percentage decrease of the noradrenaline-induced contraction; vertical bars show s.e.mean. *$P < 0.05$, significantly different from homotaurine; Student's t test for unpaired observations.

Table 1 pD_2 ($-\log ED_{50}$) values of GABA and homotaurine in the canine ileum and the ileocolonic junction

Agonist	*Ileum*	*Ileocolonic junction*
GABA	4.04 ± 0.15†	4.59 ± 0.11*
Homotaurine	3.68 ± 0.10†	3.94 ± 0.06

The results are shown as mean ± s.e.mean for at least 5 experiments.
* $P < 0.05$, GABA versus homotaurine in the ileocolonic junction.
† $P < 0.05$, ileum versus ileocolonic junction; Student's t test for unpaired observations.

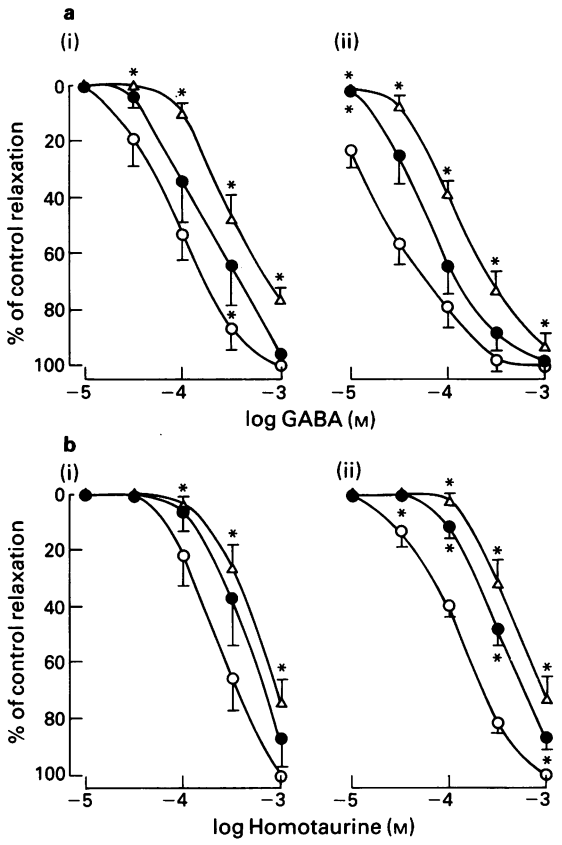

Figure 2 Effect of 3 μM (●) and 10 μM (△) bicuculline on the concentration-response curve to (a) GABA and (b) homotaurine (control, ○) in the canine terminal ileum (i) and ileocolinic junction (ii). The experiments were performed during a noradrenaline (30 μM)-induced contraction and in the presence of 0.3 μM atropine. The results are shown as mean for 6 experiments and are expressed as a percentage of the relaxation to 1 mM GABA (a) or homotaurine (b) in control solution; vertical bars show s.e.mean. These relaxations (expressed as percentage decrease of the noradrenaline-induced contraction) were 98 ± 9% and 84 ± 6% respectively in the ileum, and 101 ± 6% and 97 ± 10% respectively in the ileocolonic junction. *$P < 0.05$, significantly different from control, Student's t test for paired observations.

Effect of desensitization to GABA, homotaurine and (±)-baclofen on relaxations to GABA, homotaurine and nitroglycerin

The relaxations to GABA (0.3 mM in the ileum and 0.1 mM in the ileocolinic junction, $n = 5$–7) and homotaurine (0.3 mM, $n = 5$–7) were abolished after preincubation with 0.3 mM GABA or homotaurine. However, the relaxations to nitroglycerin remained unchanged. Incubation with 0.3 mM baclofen had no effect ($n = 6$) on the spontaneous activity of the preparations or on any of the relaxations studied (Table 2). Desensitization was readily reversed upon removal of the agonist.

Effect of various substances on GABA-induced relaxations

Timolol (3–10 μM), guanethidine (3 μM), domperidone (10 μM), hexamethonium (0.1–0.5 mM) and desensitization to ATP (achieved by successive administrations of 30 μM ATP) did not affect the relaxations to GABA (0.1 mM) in the terminal ileum and in the ileocolonic junction (Table 3).

Effect of GABA desensitization, bicuculline and TTX on relaxations to electrical stimulation, acetylcholine, GABA and nitroglycerin

GABA (0.1 mM)-induced relaxations are qualitatively similar to the NANC relaxations evoked by electrical stimulation (2–16 Hz, 1–2 ms) and acetylcholine (30 μM), as shown for the ileocolonic junction in Figure 3. Desensitization to GABA abolished the GABA-induced relaxations, whereas those to electrical stimulation and acetylcholine remained unaffected. Similarly, bicuculline (10 μM) only reduced the relaxations to GABA. Preincubation with 0.3 mM (±)-baclofen had no effect on any of the relaxations studied. TTX (0.3 μM) abolished the relaxations to electrical stimulation, acetylcholine and GABA, but not those to nitroglycerin (1 μM) (Figure 3).

Table 3 Effect of non-GABA antagonists on GABA (0.1 mM)-induced relaxations in the canine ileum and ileocolonic junction

Antagonist	*Ileum* (% of control)	*Ileocolonic junction* (% of control)
Timolol (10 μM)	99 ± 7	96 ± 5
Guanethidine (3 μM)	105 ± 3	107 ± 5
Domperidone (10 μM)	99 ± 5	103 ± 8
Hexamethonium (0.5 mM)	104 ± 3	105 ± 6
ATP desensitization	98 ± 7	99 ± 6

The experiments were performed during a noradrenaline (30 μM)-induced contraction and in the presence of 0.3 μM atropine. Results are shown as mean ± s.e.mean for 5–7 experiments and expressed as a percentage of the control values. These control relaxations (expressed as percentage decrease of the noradrenaline-induced contraction) are 63 ± 8% in the ileum and 76 ± 6% in the ileocolonic junction.

Table 2 Effect of pretreatment with GABA (0.3 mM), homotaurine or (±)-baclofen on GABA-, homotaurine-, and nitroglycerin-induced relaxations

	GABA pretreatment (% of control)	*Homotaurine pretreatment* (% of control)	*(±)-Baclofen pretreatment* (% of control)
Ileum			
GABA (0.3 mM)	0*	0*	104 ± 3
Homotaurine (0.3 mM)	0*	0*	93 ± 9
Nitroglycerin (1 μM)	99 ± 3	97 ± 5	93 ± 5
Ileocolonic junction			
GABA (0.1 mM)	0*	0*	98 ± 3
Homotaurine (0.3 mM)	0*	0*	109 ± 8
Nitroglycerin (1 μM)	98 ± 8	101 ± 6	97 ± 6

The experiments were performed during a noradrenaline (30 μM)-induced contraction and in the presence of 0.3 μM atropine. Results are shown as mean ± s.e.mean for 5–7 experiments and are expressed as a percentage of the control values. These control relaxations (expressed as percentage decrease of the noradrenaline-induced contraction) are in the ileum: GABA 67 ± 7%, homotaurine 51 ± 5%, nitroglycerin 64 ± 5%; in the ileocolonic junction: GABA 56 ± 4%, homotaurine 46 ± 5%, nitroglycerin 62 ± 8%.
* $P < 0.05$, significantly different from control, Student's t test for paired observations.

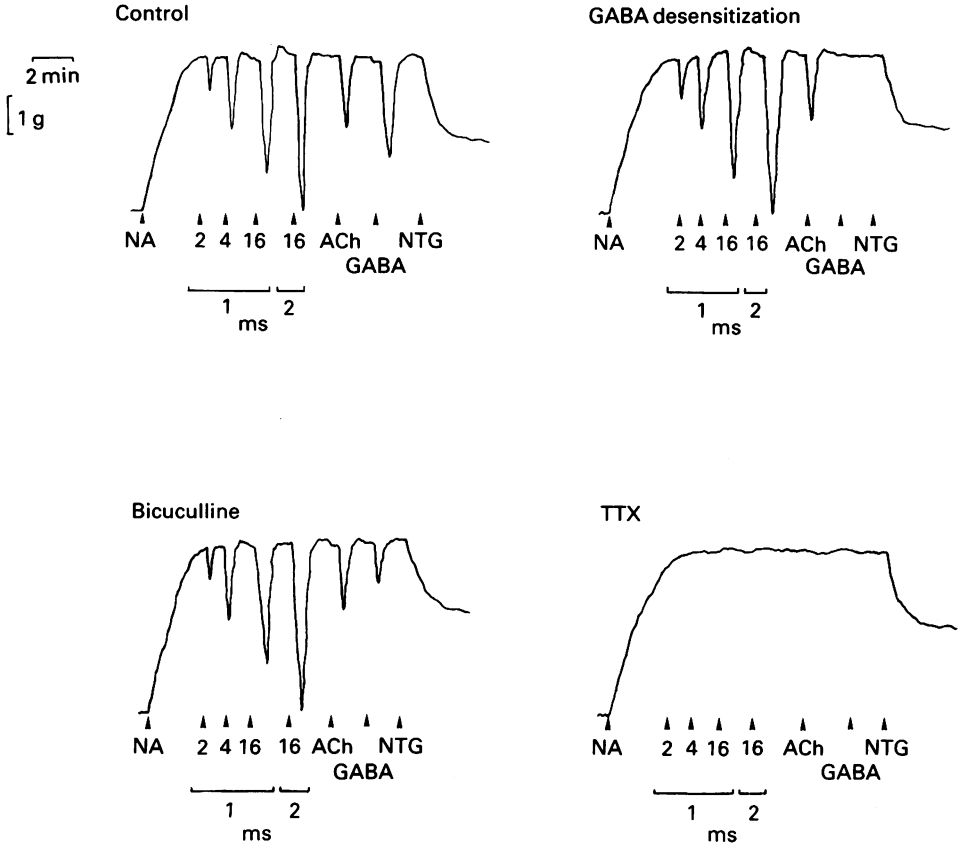

Figure 3 Typical example of the relaxant effects of electrical stimulation (2–16 Hz, 1–2 ms), acetylcholine (ACh, 30 μM), GABA (0.1 mM) and nitroglycerin (NTG, 1 μM) on circular muscle strips of the dog ileocolonic junction. GABA desensitization abolished and bicuculline (10 μM) reduced the relaxation to GABA, but had no effect on the relaxations to electrical stimulation and acetylcholine. Tetrodotoxin (TTX) abolished all relaxations, except those to nitroglycerin. The experiments were performed during a noradrenaline (NA, 30 μM)-induced contraction and in the presence of 0.3 μM atropine. Similar results were obtained in five additional experiments in both the terminal ileum and ileocolonic junction.

Discussion

The present results demonstrate that the effects of GABA on the canine terminal ileum and ileocolonic junction are due to activation of $GABA_A$ receptors; GABA-induced relaxations were antagonized by bicuculline and mimicked by the $GABA_A$ agonist homotaurine, but not by the $GABA_B$ agonist (±)-baclofen. In addition, the concentration-response curves for GABA and homotaurine were parallel, reached the same maximum and were subject to cross desensitization between GABA and homotaurine. Preincubation with (±)-baclofen had no effect on the GABA- and homotaurine-induced relaxations. The relaxations to GABA were abolished by TTX, but resistant to muscarinic and adrenoceptor antagonists, suggesting that GABA stimulates $GABA_A$ receptors on NANC inhibitory neurones.

These findings are in agreement with the observations in the guinea-pig and rabbit distal colon (Krantis *et al.*, 1980; Giotti *et al.*, 1985; Tonini *et al.*, 1989) and in the rat duodenum (Maggi *et al.*, 1984): GABA activates $GABA_A$ receptors resulting in NANC-induced relaxations. In contrast, stimulation of the $GABA_A$ receptors in the guinea-pig ileum (Krantis *et al.*, 1980; Kaplita *et al.*, 1982; Giotti *et al.*, 1983) and in the cat colon (Taniyama *et al.*, 1987) triggered cholinergic post-ganglionic neurones which resulted in a contractile response.

Inhibitory responses to GABA have also been attributed to $GABA_B$ receptor activation (Kaplita *et al.*, 1982; Ong & Kerr, 1983; Giotti *et al.*, 1983; 1985; Tonini *et al.*, 1989). In our experiments however, the $GABA_B$ agonist (±)-baclofen had no effect on the spontaneous activity of the preparations and did not induce relaxations when the muscle tension was raised with noradrenaline. It should be emphasised however that in the guinea-pig and rabbit intestine, $GABA_B$-mediated relaxation results from a reduction of resting and electrically-stimulated cholinergic nerve activity (Kaplita *et al.*, 1982; Giotti *et al.*, 1983; 1985; Ong & Kerr, 1983; Tonini *et al.*, 1989). Since our experiments were performed in the presence of atropine and since neither atropine nor TTX reduces the tone or spontaneous activity of the dog terminal ileum and ileocolonic junction, the failure to detect $GABA_B$ receptor-mediated effects can be explained by the absence of cholinergic tone in our preparations.

In previous experiments, we demonstrated the presence of an inhibitory NANC innervation in the canine terminal ileum and ileocolonic junction; acetylcholine and the nicotinic receptor agonist 1,1-dimethyl-phenylpiperazinium induced relaxations via the stimulation of nicotinic receptors located on inhibitory NANC neurones (Pelckmans *et al.*, 1989; Boeckxstaens *et al.*, 1990b). These neurones could also be activated by electrical stimulation resulting in the release of an unknown inhibitory neurotransmitter, which is not vasoactive intestinal polypeptide, an opioid (Pelckmans *et al.*, 1989), 5-hydroxytryptamine (Boeckxstaens *et al.*, 1990a), or ATP (Boeckxstaens *et al.*, 1990b). The NANC-induced relaxations in response to electrical stimulation and acetylcholine could also be mimicked by GABA but were not affected by bicuculline and desensitization to GABA or baclofen. These experiments, therefore, provide evidence against the involvement of GABA as the final NANC neurotransmitter in dog terminal ileum and ileocolonic junction. However, the finding that TTX abolishes the relaxation to GABA suggests a neuronal involvement. GABA may, therefore, stimulate NANC inhibitory neurones to release an as yet unidentified neurotransmitter. Since ATP desensitization had no effect on GABA-evoked relaxations, it is unlikely that this neurotransmitter is ATP, contrasting with observations in the rat duodenum (Maggi *et al.*, 1984; Manzini *et al.*, 1985).

In conclusion, our results indicate that GABA produces a transient relaxation of the dog terminal ileum and ileocolonic junction through a $GABA_A$ receptor-mediated activation of inhibitory NANC neurones. This finding adds further evidence for the presence of a NANC inhibitory innervation in this region of the canine gut. In addition, we provide evidence against GABA as the final NANC neurotransmitter. The nature of the latter remains to be determined.

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References

BOECKXSTAENS, G.E., PELCKMANS, P.A., RAMPART, M., BOGERS, J.J., VERBEUREN, T.J., HERMAN, A.G. & VAN MAERCKE, Y.M. (1990a). Pharmacological characterization of 5-hydroxytryptamine receptors in the canine terminal ileum and ileocolonic junction. *J. Pharmacol. Exp. Ther.*, (in press).

BOECKXSTAENS, G.E., PELCKMANS, P.A., RAMPART, M., VERBEUREN, T.J., HERMAN, A.G. & VAN MAERCKE, Y.M. (1990b). Evidence against ATP being the inhibitory transmitter released by non-adrenergic non-cholinergic nerves in the canine ileocolonic junction. *J. Pharmacol. Exp. Ther.*, (in press).

BOECKXSTAENS, G.E., PELCKMANS, P.A., RUYTJENS, I.F., RAMPART, M., HERMAN, A.G. & VAN MAERCKE, Y.M. (1990c). $GABA_A$-receptor mediated non-adrenergic non-cholinergic relaxations in the canine ileocolonic junction. *Br. J. Pharmacol.*, **99**, 52P.

BOECKXSTAENS, G.E., PELCKMANS, P.A., VAN MAERCKE, Y.M., HERMAN, A.G. & VERBEUREN, T.J. (1988). Neural origin of ATP- and GABA-induced non-adrenergic non-cholinergic relaxations in the canine ileocolonic junction. *Hepato-Gastroenterol.*, **35**, 186–187.

BOWERY, N.G., DOBLE, A., HILL, D.R., HUDSON, A.L., SHAW, J.S., TURNBULL, M.J. & WARRINGTON, R. (1981). Bicuculline-insensitive GABA receptors on peripheral autonomic nerve terminals. *Eur. J. Pharmacol.*, **71**, 53–70.

BURNSTOCK, G. (1986). The non-adrenergic non-cholinergic nervous system. *Arch. Int. Pharmacodyn.*, **280** (Suppl.), 1–15.

DUTAR, P. & NICOLL, R.A. (1988). A physiological role for $GABA_B$ receptors in the central nervous system. *Nature*, **332**, 156–158.

GIOTTI, A., LUZZI, S., MAGGI, C.A., SPAGNESI, S. & ZILLETTI, L. (1985). Modulatory activity of $GABA_B$ receptors on cholinergic tone in guinea-pig distal colon. *Br. J. Pharmacol.*, **84**, 883–895.

GIOTTI, A., LUZZI, S., SPAGNESI, S. & ZILLETTI, L. (1983). $GABA_A$ and $GABA_B$ receptor-mediated effects in guinea-pig ileum. *Br. J. Pharmacol.*, **78**, 469–478.

HILL, D.R. & BOWERY, N.G. (1981). ^{3}H-baclofen and ^{3}H-GABA bind to bicuculline-insensitive $GABA_B$ sites in rat brain. *Nature*, **190**, 149–152.

JESSEN, K.R., MIRSKY, R., DENNISON, M.E. & BURNSTOCK, G. (1979). GABA may be a neurotransmitter in the vertebrate peripheral nervous system. *Nature*, **281**, 71–74.

KAPLITA, P.V., WATERS, D.H. & TRIGGLE, D.J. (1982). γ-Aminobutyric acid action on guinea-pig ileal myenteric plexus. *Eur. J. Pharmacol.*, **79**, 43–51.

KRANTIS, A., COSTA, M., FURNESS, J.B. & ORBACH, J. (1980). γ-Aminobutyric acid stimulates intrinsic inhibitory and excitatory nerves in the guinea-pig intestine. *Eur. J. Pharmacol.*, **67**, 461–468.

MAGGI, C.A., MANZINI, S. & MELI, A. (1984). Evidence that $GABA_A$ receptors mediate relaxation of rat duodenum by activating intramural nonadrenergic-noncholinergic neurons. *J. Auton. Pharmacol.*, **4**, 77–85.

MANZINI, S., MAGGI, C.A. & MELI, A. (1985). Further evidence for involvement of adenosine-5'-triphosphate in non-adrenergic non-cholinergic relaxation of the isolated rat duodenum. *Eur. J. Pharmacol.*, **113**, 399–408.

ONG, J. & KERR, D.I.B. (1983). $GABA_A$- and $GABA_B$-receptor mediated modification of intestinal motility. *Eur. J. Pharmacol.*, **86**, 9–17.

PELCKMANS, P.A., BOECKXSTAENS, G.E., VAN MAERCKE, Y.M., HERMAN, A.G. & VERBEUREN, T.J. (1989). Acetylcholine is an indirect inhibitory transmitter in the canine ileocolinic junction. *Eur. J. Pharmacol.*, **170**, 235–242.

PELCKMANS, P.A., VAN MAERCKE, Y.M., DE MAEYER, M.H., HERMAN, A.G. & VERBEUREN, T.J. (1990). Cholinergic and adrenergic contractile properties of the canine ileocolonic junction. *J. Pharmacol. Exp. Ther.*, (in press).

TALLARIDA, R.J. & MURRAY, P.B. (1981). *Manual of Pharmacological Calculations*. New York: Springer Verlag.

TANIYAMA, K., SAITO, N., MIKI, Y. & TANAKA, C. (1987). Enteric γ-aminobutyric acid-containing neurons and the relevance to motility of the cat colon. *Gastroenterology*, **93**, 519–525.

TONINI, M., CREMA, A., FRIGO, G.M., RIZI, C.A., MANZO, L., CANDURA, S.M. & ONORI, L. (1989). An *in vitro* study of the relationship between GABA receptor function and propulsive motility in the distal colon of the rabbit. *Br. J. Pharmacol.*, **98**, 1109–1118.

VANHOUTTE, P.M. & LEUSEN, I. (1969). The reactivity of isolated venous preparations to electrical stimulation. *Pflügers Arch.*, **306**, 341–353.

VERBEUREN, T.J., JORDAENS, F.H. & HERMAN, A.G. (1983). Accumulation and release of (^{3}H)-5-hydroxytryptamine in saphenous veins and cerebral arteries of the dog. *J. Pharmacol. Exp. Ther.*, **226**, 579–588.

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Protein kinase C-mediated contractile responses of arteries from diabetic rats

W. Abebe & [1]K.M. MacLeod

Division of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, B.C., Canada V6T 1W5

1 The role of protein kinase C (PKC) in mediating enhanced contractile responses of aortae and mesenteric arteries from male rats with 12–14 week streptozotocin-induced diabetes to noradrenaline (NA) was investigated using the PKC activator, phorbol 12,13-dibutyrate (PDB), and the PKC inhibitor, staurosporine.

2 Maximum contractile responses of aortae and mesenteric arteries from diabetic rats to NA were significantly enhanced compared with responses of arteries from age-matched control animals. The maximum NA responses were increased by 59.6 ± 7.9% in aortae and by 54.9 ± 7.4% in mesenteric arteries from diabetic animals, compared to their respective controls.

3 Pretreatment of aortae and mesenteric arteries from both control and diabetic animals with staurosporine (5×10^{-8} M) caused marked inhibition of contractile responses to a maximum concentration of NA (10^{-5} M in aortae; 3×10^{-5} M in mesenteric arteries). In the presence of staurosporine, no difference was observed in the magnitude of contractile responses of arteries from control and diabetic rats to NA.

4 Maximum contractile responses of mesenteric arteries from diabetic rats to PDB were significantly increased (by 45.0 ± 4.9%) compared to responses of arteries from control animals. In contrast, no significant difference was found in the magnitude of contractile responses of aortae from control and diabetic rats to PDB.

5 Staurosporine (5×10^{-8} M) caused marked attenuation of contractile responses of arteries from control and diabetic rats to a maximum concentration of PDB (3×10^{-6} M). In the presence of staurosporine, the difference in magnitude of contractile responses of mesenteric arteries from control and diabetic rats to PDB was abolished.

6 Contractile responses of aortae and mesenteric arteries from control and diabetic rats to PDB were reduced in the absence of extracellular Ca^{2+}, and in the presence of the Ca^{2+} channel blockers, nifedipine (3×10^{-6} M) or verapamil (3×10^{-6} M). Under these conditions, no difference was found in the magnitude of contractile responses of mesenteric arteries from control and diabetic rats to PDB.

7 These data suggest that enhanced contractile responses of aortae and mesenteric arteries from streptozotocin-induced diabetic rats to NA may result, at least in part, from increased activation of PKC. In addition, increased activation of PKC-mediated processes, which are dependent on the presence of extracellular Ca^{2+}, may further contribute to the enhanced contractile responses of diabetic mesenteric arteries to NA.

Introduction

It is now well known that vascular deterioration is one of the complicating features of diabetes mellitus (Garcia *et al.*, 1974; Christlieb *et al.*, 1976). It has been proposed that this is in part due to altered reactivity of vascular smooth muscle to neurotransmitters and circulating hormones (Weidmann *et al.*, 1979). In an attempt to investigate this hypothesis, the responsiveness of vascular preparations from animals with chemically-induced diabetes to various vasoactive agents has been extensively studied (Brody & Dixon, 1964; Ramanadhan & Tenner, 1984; MacLeod & McNeill, 1985; Agrawal *et al.*, 1987; White & Carrier, 1988). Although the results of these investigations appear to be controversial, previous studies from our laboratory have demonstrated that aortae and mesenteric arteries from male rats with streptozotocin (STZ)-induced diabetes of 12 weeks duration are more responsive to the contractile effects of noradrenaline (NA) than are the corresponding arteries from age-matched control rats (MacLeod, 1985; Harris & MacLeod, 1988). The increased responsiveness of the diabetic vessels to NA did not appear to result from a generalized increase in the contractility of the tissues (MacLeod, 1985), or to a decrease in the release of endothelium-derived relaxing factor (Harris & MacLeod, 1988), but was a consequence of stimulation of α_1-adrenoceptors (Abebe *et al.*, 1990). It could be prevented from occurring, or reversed once established, by treatment of the diabetic animals with insulin, indicating that it is a result of the diabetic state (MacLeod, 1985).

Contractions of arteries in response to α_1-adrenoceptor stimulation are known to be associated with increased metabolism of sarcolemmal phosphoinositides (for review see Heagerty & Ollerenshaw, 1987). The hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C results in the generation of the second messengers, inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 has been shown to be involved in the release of Ca^{2+} from intracellular stores while DAG activates protein kinase C (PKC), resulting in protein phosphorylation and the generation of physiological responses. It has been suggested that the activation of PKC is associated with the tonic component (Campbell *et al.*, 1985; DeFeo & Morgan, 1985; Chatterjee & Tejada, 1986; Heagerty & Ollerenshaw, 1987), while IP_3 mediates the initial phasic component of the contractile responses of vascular smooth muscle to NA (Suematsu *et al.*, 1984; Hashimoto *et al.*, 1986). The tumour promoting phorbol esters have been shown to mimic the actions of DAG in activating PKC and causing contraction of vascular tissues (Castagna *et al.*, 1982; Danthaluri & Deth, 1984; Nishizuka, 1984; Rasmussen *et al.*, 1984; Gleason & Flaim, 1986; Miller *et al.*, 1986; Chiu *et al.*, 1987; Singer & Baker, 1987).

In view of the observed increases in contractile responsiveness of arteries from diabetic rats to NA (MacLeod, 1985; Harris & MacLeod, 1988), it is possible that the phosphoinositide messenger system is augmented in these tissues. The present study was undertaken to investigate whether

[1]Author for correspondence.

PKC-mediated contractile responses are altered in aortae and mesenteric arteries from male rats with STZ-induced diabetes of 12–14 weeks duration. In this study, we used the phorbol ester, phorbol 12,13-dibutyrate (PDB) as a PKC activator (Castagna *et al.*, 1982), and the microbial alkaloid, staurosporine as a PKC inhibitor (Tamaoki *et al.*, 1986) to probe the status of PKC-mediated contractile mechanisms.

Methods

Induction of diabetes

Male Wistar rats weighing 190–220 g received a single injection of STZ (60 mg kg^{-1}) into the lateral tail vein. Age-matched control rats were injected with the citrate buffer vehicle (pH 4.5) used to dissolve the STZ. All injections were given to the animals under light ether anaesthesia. Three days after injection, rats were monitored for the development of glucosuria using Lilly Tes-tape. The control and diabetic rats were caged separately and housed in a similar environment. Both groups of animals were given the same diet (Purina Rat Chow) and water *ad libitum*.

Tissue preparation and contraction studies

Twelve to fourteen weeks after injection, diabetic and age-matched control rats were weighed and killed by stunning followed by decapitation. Blood was collected at the same time for serum glucose assay. The thoracic aorta and superior mesenteric artery were removed from each rat and placed in Krebs solution of composition (mM): NaCl 113, KCl 4.7, $NaHCO_3$ 25.0, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2 and dextrose 11.5. After removal of adhering fat and connective tissues, ring preparations of aorta and mesenteric artery were prepared and placed individually in isolated tissue baths, containing 20 ml Krebs solutions maintained at 37°C and aerated with 95% O_2–5% CO_2, for isometric tension studies as described previously (MacLeod, 1985). Care was taken in these experiments not to damage the endothelium lining the vessels; the function of the endothelium was occasionally checked by monitoring responses to acetylcholine. The muscle preparations were equilibrated for 90 min under a resting tension of 2 g for aorta and 1 g for mesenteric artery. These resting tensions were found in preliminary experiments to be optimum for both control and diabetic arteries. During the equilibration period, the bathing solution was changed every 20–30 min. All experiments were carried out in the presence of 0.1 μM desipramine, 1 μM hydrocortisone, and 1 μM timolol to block neuronal and extraneuronal uptake, and β-adrenoceptors, respectively.

Following the equilibration period, cumulative concentration-response curves to NA and PDB were obtained in arterial rings from control and diabetic rats, in Krebs solution containing 2.5 mM Ca^{2+}. For determination of the involvement of PKC in NA- and PDB-induced contractions, responses to a single maximum concentration of NA (10^{-5} M in aortae or 3×10^{-5} M in mesenteric arteries) or PDB (3×10^{-6} M) were obtained in arteries pretreated with staurosporine (5×10^{-8} M) for 25 min. Responses of arteries to 8×10^{-2} M KCl were also obtained in the presence of staurosporine (5×10^{-8} M). In preliminary concentration-response experiments, 8×10^{-2} M KCl was found to produce maximum contractile responses in arteries from both control and diabetic rats. Responses to KCl were measured in the presence of 10^{-7} M phentolamine to block the effects of any neurally released NA. Control responses to NA, PDB and KCl were obtained 60 min before incubation of the tissues with staurosporine. The specificity of the action of PDB was further established by treating tissues with the biologically inactive phorbol ester, 4α-phorbol (10^{-8}–10^{-5} M for 30 min) (Castagna *et al.*, 1982). To assess the relative importance of extracellular Ca^{2+} in PKC-mediated contractile responses in control and diabetic preparations, concentration-response curves to PDB were also obtained in Ca^{2+}-free Krebs solution containing 1 mM ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid (EGTA) following a 30 min equilibration period in this solution. Calcium chloride was replaced by NaCl to maintain osmolarity. The effect of Ca^{2+} entry blockade on contractile responses to PDB was assessed by adding verapamil or nifedipine (3×10^{-6} M) to the tissue bath 15 min before a single maximum concentration (3×10^{-6} M) of PDB. In preliminary experiments, this concentration of each of the blockers was found to produce the maximum inhibitory effect on PDB-induced contractions. Control responses to PDB were obtained 60 min before addition of the blockers.

At the end of each experiment, tissues were blotted dry, and their lengths and weights determined in order to calculate their cross-sectional areas, using the formula:

Cross-sectional area (mm^2) = weight (mg) (length (mm) × density (mg mm^{-3})$^{-1}$, where the density of vascular smooth muscle was assumed to be 1.05 mg mm^{-3} (Wyse, 1980). Contractile responses of each preparation to agonists were calculated as the increase in tension (g) per mm^2 in response to each concentration of agonist. Concentration-response curves were analysed by non-linear regression analysis using all the data points for determination of pD_2 ($-\log ED_{50}$) values.

Blood glucose determination

Serum glucose levels were monitored by the glucose oxidation method, employing the Boehringer periodochrome glucose kit.

Statistical analysis

All values are expressed as mean ± s.e.mean. Statistical significance was evaluated by one-way analysis of variance (ANOVA) and considered to be significantly different if $P < 0.05$.

Drugs

Streptozotocin, (−)-noradrenaline hydrochloride, phorbol 12,13-dibutyrate, 4α-phorbol, verapamil and nifedipine were all obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Staurosporine was purchased from Calbiochem Corporation (La Jolla, CA, U.S.A.). A stock solution of noradrenaline was prepared daily in deionized water containing 1 mg ml^{-1} ascorbic acid. Phorbol 12,13-dibutyrate, 4α-phorbol and staurosporine were dissolved in dimethylsulphoxide, and verapamil and nifedipine in ethanol. Stock solutions of all drugs were diluted with deionized water to appropriate concentrations. The total volume of drug added to the tissue bath never exceeded 0.4 ml. The final dimethylsulphoxide or ethanol concentration in the bathing medium was without effect on contractile responses. Experiments involving PDB, 4α-phorbol, verapamil, nifedipine and staurosporine were performed in tissue baths protected from light.

Results

General characteristics of diabetic rats

Twelve to fourteen weeks after injection, the diabetic rats had significantly decreased body weights and elevated serum glucose levels compared to age-matched control animals (Table 1). Arterial preparations from diabetic rats were smaller in cross-sectional areas than those from control animals. The diabetic rats also manifested other symptoms of the disease including glucosuria, diarrhoea, polyuria, polydipsia and cataracts.

Table 1 Characteristics of diabetic and control rats

	n	*Body weight* (g)	*Serum glucose* (mg dl^{-1})	*Cross-sectional area* (mm^2) *Aortae*	*Mesenteric arteries*
Control	23	521 ± 6	94.7 ± 3.9	1.43 ± 0.04	0.39 ± 0.02
Diabetic	23	341 ± 8*	337.0 ± 13.8*	1.00 ± 0.03*	0.30 ± 0.02*

Values are mean ± s.e.mean.
* $P < 0.05$ with respect to corresponding control values.

Contractile responses to NA and PDB

As previously found (MacLeod, 1985; Harris & MacLeod, 1988), contractile responses to NA were significantly greater in aortae and mesenteric arteries from diabetic rats compared to control arteries (Figure 1). In aortic preparations, the maximum response to NA was increased from 2.12 ± 0.18 g mm^{-2} (mean ± s.e.mean, $n = 12$) in control vessels to 3.21 ± 0.38 g mm^{-2} ($n = 12$) in diabetic vessels. The maximum increase in tension in response to NA in diabetic mesenteric arteries was 5.28 ± 0.53 g mm^{-2} ($n = 10$) which was significantly greater than the corresponding value of 3.60 ± 0.45 g mm^{-2} ($n = 10$) obtained in control preparations. However, diabetes did not alter the sensitivities (as reflected by pD_2 values) of these arteries to NA (pD_2 values in diabetic aortae = 6.65 ± 0.14, mean ± s.e.mean; pD_2 value in control aortae = 6.88 ± 0.06; pD_2 value in diabetic mesenteric arteries = 6.19 ± 0.11; pD_2 value in control mesenteric arteries = 6.40 ± 0.11).

Phorbol 12,13-dibutyrate caused relatively slowly developing, concentration-dependent contractions of arterial rings. The magnitude of the maximum contractions and sensitivities of aortae from control and diabetic rats to PDB were similar (Figure 2; Table 2). In contrast to the aortae, maximum contractile responses of mesenteric arteries to PDB were enhanced in diabetic vessels compared to control tissues in the presence of 2.5 mM Ca^{2+} (Figure 2; Table 2). However, as with the aortae, sensitivities of the mesenteric preparations from the two groups of animals to PDB did not differ from each other (Table 2). Arteries from both control and diabetic rats did not contract in response to the inactive phorbol ester, 4α-phorbol (10^{-8}–10^{-5} M, $n = 3$) (data not shown).

Effects of staurosporine

The effect of the PKC inhibitor, staurospórine, on maximum contractile responses of arteries to NA (10^{-5} M in aortae or 3×10^{-5} M in mesenteric arteries) was investigated (Figure 3). In the absence of staurosporine, NA induced greater maximum contractile responses in both aortae and mesenteric arteries from diabetic rats than in control preparations. Pretreatment of arteries from both groups of animals with staurosporine (5×10^{-8} M) caused marked inhibition of the maximum contractile responses to NA. Staurosporine

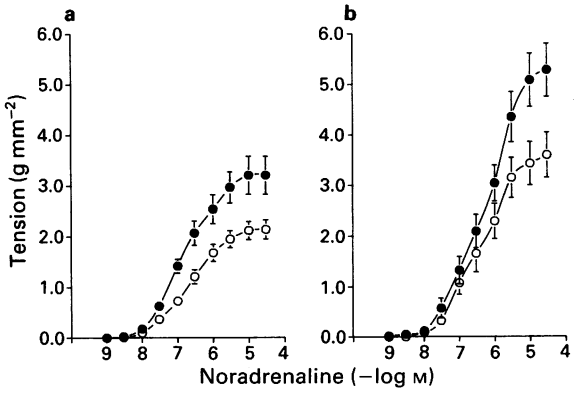

Figure 1 Concentration-response curves for noradrenaline-induced contractions of (a) aortae and (b) mesenteric arteries from control (○) and diabetic (●) rats. Each point represents the mean and vertical lines indicate s.e.mean of 10–12 observations.

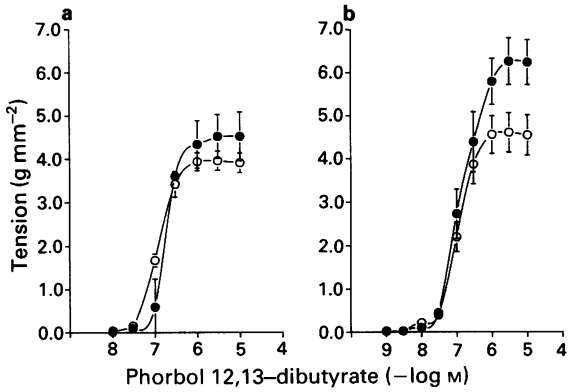

Figure 2 Concentration-response curves for phorbol 12,13-dibutyrate-induced contractions of (a) aortae and (b) mesenteric arteries from control (○) and diabetic (●) rats in Krebs solution containing 2.5 mM Ca^{2+}. Each point represents the mean and vertical lines show s.e.mean of 9–10 observations.

Table 2 Phorbol 12,13-dibutyrate pD_2 values and maximum contractile responses in the presence and absence of Ca^{2+} in aortae and mesenteric arteries from diabetic and control rats

	pD_2 values Control	Diabetic	*Contractile response* (g mm^{-2}) Control	Diabetic
Aorta				
2.5 mM Ca^{2+}	6.90 ± 0.06	6.73 ± 0.04	3.96 ± 0.23	4.50 ± 0.59
0 Ca^{2+}	6.03 ± 0.07	6.05 ± 0.08	1.24 ± 0.81	1.15 ± 0.22
Mesenteric artery				
2.5 mM Ca^{2+}	6.91 ± 0.09	6.77 ± 0.12	4.61 ± 0.46	6.26 ± 0.58*
0 Ca^{2+}	6.09 ± 0.20	5.83 ± 0.09	1.45 ± 0.11	1.30 ± 0.14

Values are mean ± s.e.mean of 6–10 observations.
* $P < 0.05$ with respect to corresponding control value.

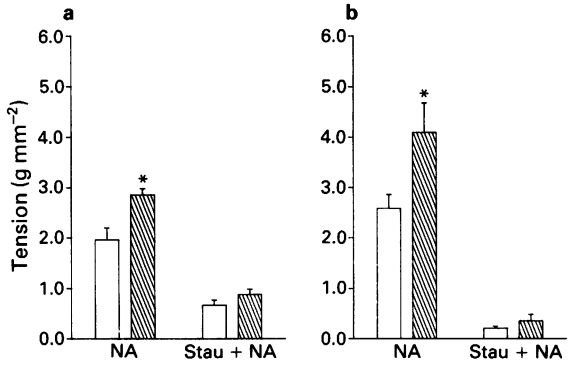

Figure 3 Effects of staurosporine (Stau) on noradrenaline-induced contractions of (a) aortae and (b) mesenteric arteries from control (open columns) and diabetic (hatched columns) rats. Tissues were incubated with 5×10^{-8} M staurosporine for 25 min before the addition of noradrenaline (10^{-5} M in aortae or 3×10^{-5} M in mesenteric arteries). Each column represents the mean and bars show s.e.mean of 8–9 observations. * $P < 0.05$ with respect to corresponding control values.

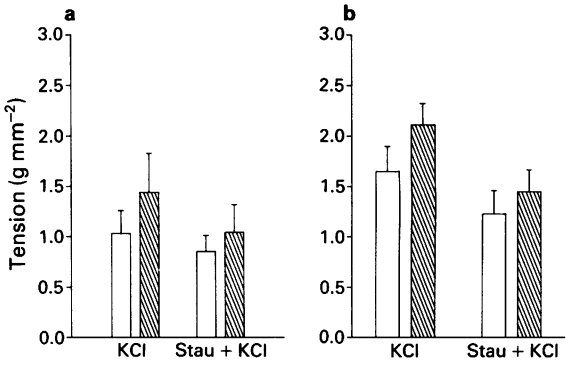

Figure 5 Effects of staurosporine (Stau) on KCl-induced contractions of (a) aortae and (b) mesenteric arteries from control (open columns) and diabetic (hatched columns) rats. Tissues were incubated with 5×10^{-8} M staurosporine for 25 min before the addition of 8×10^{-2} M KCl. Each point represents the mean and bars show s.e.mean of 5–10 observations.

appeared to exert a greater inhibitory effect on responses of mesenteric arteries. In the presence of staurosporine, the difference in contractile responses observed between control and diabetic arteries to NA was abolished.

Responses of arteries to PDB were also measured after pretreatment with staurosporine (Figure 4). In the absence of staurosporine, contractile responses of mesenteric arteries, but not aortae, from diabetic rats to 3×10^{-6} M PDB were significantly greater than control. Staurosporine (5×10^{-8} M) also produced marked inhibition of contractions of both arteries from control and diabetic rats to PDB, with greater antagonism of responses of mesenteric arteries. The inhibitory effect of staurosporine abolished the difference in contractile responses to PDB found between control and diabetic mesenteric arteries.

Contractions of arteries induced by KCl were also measured in the presence of staurosporine in order to assess the specificity of the action of the inhibitor in antagonizing PKC-mediated responses to NA and PDB (Figure 5). Consistent with our previous results (MacLeod, 1985), the magnitudes of contractions of untreated arteries from control and diabetic rats to KCl were similar. In contrast to its action against NA and PDB, staurosporine (5×10^{-8} M) caused relatively less inhibition of contractions induced by KCl (8×10^{-2} M) in both aortae and mesenteric arteries from control and diabetic animals. In the presence of staurosporine, no differences were observed in contractile responses of arteries from control and diabetic rats to KCl.

Effects of extracellular Ca^{2+} removal on PDB responses

Phorbol 12,13-dibutyrate produced concentration-dependent contractions of aortae and mesenteric arteries incubated in Ca^{2+}-free solution containing 1 mM EGTA. However, the magnitude of these responses was markedly reduced compared to the responses observed in the presence of 2.5 mM Ca^{2+} (Figure 6; Table 2). In Ca^{2+}-free solution, no differences were observed in the magnitude of contractile responses or sensitivities of control and diabetic aortae to PDB. Similarly, contractile responses and sensitivities of diabetic mesenteric arteries to PDB in Ca^{2+}-free solution did not differ from those in control vessels (Figure 6; Table 2).

Effects of Ca^{2+} channel blockers on PDB responses

The effects of the Ca^{2+} channel blockers, verapamil and nifedipine, on PDB responses were evaluated in Krebs solution containing 2.5 mM Ca^{2+} (Figure 7). Contractile responses of mesenteric arteries, but not aortae, from diabetic rats to 3×10^{-6} M PDB were significantly enhanced compared with those from controls in the absence of the Ca^{2+} channel blockers. Pretreatment of arteries with 3×10^{-6} M verapamil or nifedipine resulted in inhibition of responses of both arteries from diabetic and control rats to 3×10^{-6} M PDB, although these effects were greater in diabetic mesenteric preparations. The inhibitory effects of the Ca^{2+} channel blockers

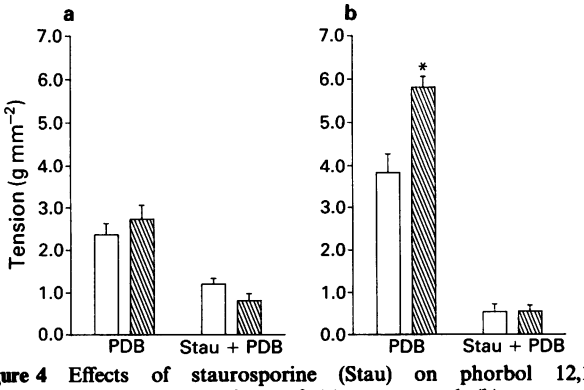

Figure 4 Effects of staurosporine (Stau) on phorbol 12,13-dibutyrate-induced contractions of (a) aortae and (b) mesenteric arteries from control (open columns) and diabetic (hatched columns) rats. Tissues were incubated with 5×10^{-8} M staurosporine for 25 min before the addition of 3×10^{-6} M phorbol 12,13-dibutyrate. Each column represents the mean and bars show s.e.mean of 5–10 observations. * $P < 0.05$ with respect to corresponding control values.

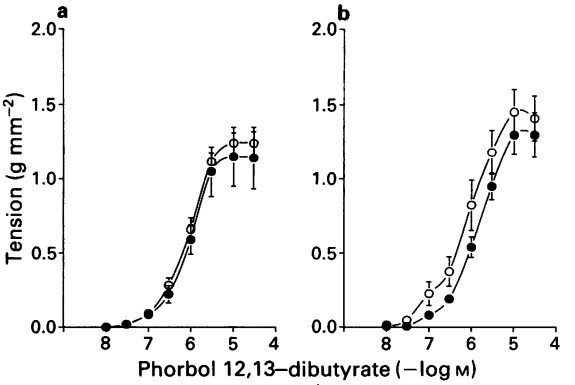

Figure 6 Concentration-response curves for phorbol 12,13-dibutyrate-induced contractions of (a) aortae and (b) mesenteric arteries from control (○) and diabetic (●) rats in Ca^{2+}-free solution containing 1 mM EGTA. Each point represents the mean and vertical lines show s.e.mean of 6 observations.

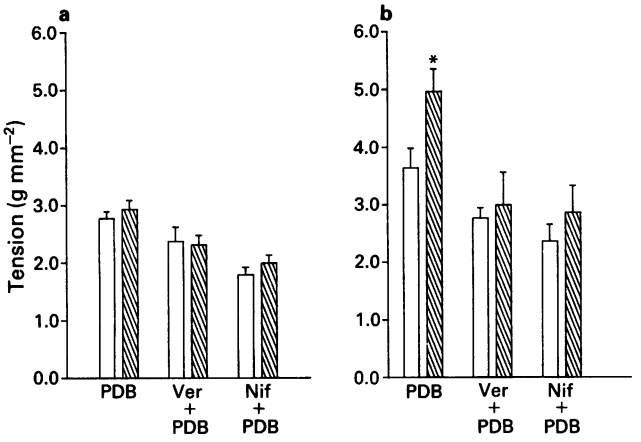

Figure 7 Effects of verapamil (Ver) and nifedipine (Nif) on phorbol 12,13-dibutyrate-induced contractions of (a) aortae and (b) mesenteric arteries from control (open columns) and diabetic (hatched columns) rats. Tissues were incubated with 3×10^{-6} M of the blockers for 15 min before the addition of 3×10^{-6} M phorbol 12,13-dibutyrate (PDB). Each column represents the mean and bars show s.e.mean of 8–16 observations. * $P < 0.05$ with respect to corresponding control values.

abolished the difference between control and diabetic mesenteric arteries in contractile responses to PDB.

Discussion

The results of the present study confirm previous findings from this laboratory that aortae and mesenteric arteries from rats with STZ-induced diabetes are more responsive to the contractile effects of NA than are the corresponding arteries from age-matched control rats (MacLeod, 1985; Harris & MacLeod, 1988). This observation is also consistent with the results of other investigators who used intact animals as well as isolated tissues from various experimental diabetic models (Brody & Dixon, 1964; Agrawal *et al.*, 1987; White & Carrier, 1988). However, the mechanism underlying this observation is not well understood. The current study has examined the possibility that diabetes induces changes in contractile mechanisms resulting from activation of PKC.

Our finding that contractile responses of arteries to NA were inhibited by the putative PKC inhibitor, staurosporine (Tamaoki *et al.*, 1986) suggests that at least part of the NA response of these arteries is mediated via activation of PKC. This observation is consistent with the view that PKC activation is involved in the tonic contractile responses of vascular smooth muscle to agents like NA which stimulate the phosphoinositide messenger system (Campbell *et al.*, 1985; DeFeo & Morgan, 1985; Chatterjee & Tejada, 1986; Heagerty & Ollerenshaw, 1987). Recently, it was suggested that staurosporine may block contractile responses of arteries to agonists, including those causing PKC activation, by a PKC-independent mechanism, possibly involving inhibition of myosin light chain kinase (Ruegg & Burgess, 1989). If this were the case, staurosporine would be expected to block KCl-induced contractions, which are believed to be associated with activation of myosin light chain kinase, and occur independently of PKC activation (Nakaki *et al.*, 1985; Turla & Webb, 1990). However, the concentration of staurosporine used in the present investigation caused much greater inhibition of contractile responses of arteries to NA and the PKC activator, PDB, than to KCl. This suggests that the inhibitor was relatively more selective in inhibiting PKC-mediated responses. Therefore, it seems likely that the contractile responses of arteries to NA measured in the present study are mediated, at least partly, by PKC activation. The fact that the enhanced contractile responses of arteries from diabetic rats to NA were abolished in the presence of staurosporine suggests that at least part of the increased responsiveness is the result of enhanced activation of PKC-mediated mechanisms in these tissues. However, these data do not demonstrate whether this results from increased activation of PKC by increased production of DAG, and/or from increased responsiveness of contractile processes to activation of PKC.

Phorbol 12,13-dibutyrate was used in the present investigation to probe further the status of PKC-mediated contractile mechanisms because of its greater water solubility than the commonly used analogue, 12-O-tetradecanoyl-phorbol 13-acetate (TPA). Contraction of vascular smooth muscle by active phorbol esters such as PDB or TPA, in the absence and presence of extracellular Ca^{2+}, is a well documented phenomenon (Danthaluri & Deth, 1984; Kojima *et al.*, 1984; Rasmussen *et al.*, 1984; Gleason & Flaim, 1986; Miller *et al.*, 1986; Chiu *et al.*, 1987; Singer & Baker, 1987; Swamura *et al.*, 1987). The assumption that the contractions induced by these agents are mediated by PKC is supported by a variety of evidence including the lack of contractile effect in response to inactive phorbol analogues, blockade of the action of phorbol esters by PKC inhibitors and a lack of reported non-specific actions of phorbol esters (Miller *et al.*, 1986; Singer & Baker, 1987). Moreover, there are studies showing that the phorbol ester receptor and PKC are the same protein (Kikkawa *et al.*, 1983; Niedel *et al.*, 1983). In the present study, we have shown that the biologically inactive phorbol ester, 4α-phorbol, (Castagna *et al.*, 1982) did not cause contractions of arteries. In addition, contractile responses of arteries from both control and diabetic rats to PDB were inhibited by staurosporine. Staurosporine also abolished the difference in contractile responses observed between diabetic and control mesenteric arteries to PDB. These data suggest that the contractile responses of arteries to PDB observed in the present investigation are also mediated by PKC activation, and that the increased responsiveness of mesenteric arteries from diabetic rats to this agent is the result of enhanced activation of PKC-mediated mechanisms in these tissues.

A part of the contractile response of both arteries to PDB was dependent on the presence of extracellular Ca^{2+}, since Ca^{2+}-free solution containing EGTA or the Ca^{2+} channel blockers verapamil or nifedipine caused a reduction in the magnitude of the PDB contractions. The observation that the extent of reduction of the PDB response by verapamil or nifedipine was not as great as that observed in Ca^{2+}-free, EGTA solution is in agreement with previous findings that a substantial component of the PDB response is resistant to Ca^{2+} channel blockers (Sybertz *et al.*, 1986; Chiu *et al.*, 1987). Similar Ca^{2+}-dependencies of contractile responses to PDB and TPA have been observed by a number of other investigators in rat aortae and mesenteric arteries as well as in other types of blood vessels (Rasmussen *et al.*, 1984; Baraban, 1985; Gleason & Flaim, 1986; Chiu *et al.*, 1987; Singer & Baker, 1987; Turla & Webb, 1987). Phorbol 12,13-dibutyrate has also been shown to stimulate the influx of [$^{45}Ca^{2+}$] in rabbit and rat aortae (Gleason & Flaim, 1986; Chiu *et al.*, 1987). These data suggest that activation of PKC with phorbol esters leads to opening of Ca^{2+} channels allowing the influx of extracellular Ca^{2+} into smooth muscle cells. The results of the present investigation demonstrate that the enhanced responsiveness of mesenteric arteries from diabetic rats to PDB was dependent on the availability or entry of extracellular Ca^{2+}, since in the absence of Ca^{2+} or in the presence of verapamil or nifidepine, the magnitudes of the PDB-induced contractions were similar in control and diabetic mesenteric arteries. Further evidence supporting this observation was obtained from experiments showing that upon the re-addition of Ca^{2+} to Ca^{2+}-free medium, diabetic mesenteric arteries pretreated with PDB contracted more than control arteries (data not shown). These observations suggest that PDB-mediated activation of PKC in mesenteric arteries from STZ-diabetic rats may result in increased influx of Ca^{2+} through cell membrane Ca^{2+} channels, thereby leading to increased tension development. In this

regard, it is possible that the increased Ca^{2+} influx in response to PDB is due to increased activation of Ca^{2+} channels by PKC-dependent phosphorylation (Berridge *et al.*, 1987; Heagerty & Ollerenshaw, 1987; Fish *et al.*, 1988) and/or an increased number of Ca^{2+} channels available for activation by PKC, in the diabetic vessels. However, other explanations which include (a) that the Ca^{2+} sensitivity of PKC may be increased in the presence of PDB to a greater extent in the diabetic vessels, and/or (b) augmentation of other Ca^{2+}-dependent cellular events occurs subsequent to PKC activation in diabetes, cannot be excluded.

It is not clear why diabetes induced enhanced contractile responses of mesenteric arteries to direct activation of PKC by PDB in the presence of extracellular Ca^{2+} without affecting the responses in aortae. One possible explanation for this variation in tissue responsiveness is that different types of PKC may be present in the two types of blood vessels, with differential responsiveness to diabetes. Alternatively, the number and/or nature of Ca^{2+} channels activated by PKC may be affected by diabetes in a different manner in the two kinds of vascular tissues, and/or other events occurring subsequent to PKC activation may be influenced differently by diabetes in the two types of vessels. These possibilities appear to be partly supported by the results of other investigators who have shown that differences in PKC activity exist in different vascular beds (Wagner *et al.*, 1987; Silver *et al.*, 1988).

Our data indicate that contractile responses to NA mediated via PKC activation are enhanced, although those resulting from direct activation of PKC are unaltered, in aortae from diabetic rats. This suggests that the increased contractions observed in aortae from diabetic rats in response to NA may result, at least in part, from increased activation of PKC by enhanced production of DAG in these tissues. In support of this, recently we have found greater phosphoinositide turnover in diabetic aortae than in control preparations in response to NA (Abebe & MacLeod, unpublished observations). On the other hand, contractile responses to both direct activation of PKC, and to activation of PKC in response to NA, were enhanced in mesenteric arteries from diabetic rats. Enhancement of NA-induced phosphoinositide turnover was also observed in mesenteric arteries from diabetic rats (Abebe & MacLeod, unpublished observations). Therefore, both increased activation of PKC by enhanced production of DAG, and increased responsiveness of contractile mechanisms to PKC activation, may contribute to the increased contractile responses of diabetic mesenteric arteries to NA.

In conclusion, the results of the present study provide evidence that enhanced contractile responses of aortae and mesenteric arteries from STZ-induced diabetic rats to NA may result, at least in part, from increased activation of PKC. In addition, increased activation of PKC-mediated processes, which are dependent on the presence of extracellular Ca^{2+}, may further contribute to the enhanced contractile responses of diabetic mesenteric arteries to NA.

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References

ABEBE, W., HARRIS, K.H. & MACLEOD, K.M. (1990). Enhanced contractile responses of arteries from diabetic rats to α_1-adrenoceptor stimulation in the absence and presence of extracellular Ca^{2+}. *J. Cardiovasc. Pharmacol.* (in press).

AGRAWAL, D.K., BHIMJI, S. & MCNEILL, J.H. (1987). Effect of chronic experimental diabetes on vascular smooth muscle function in rabbit carotid artery. *J. Cardiovasc. Pharmacol.*, **9**, 584–593.

BARABAN, J.M., GOULD, R.J., PEROUTKA, S.J. & SNYDER, S.H. (1985). Phorbol ester effects on neurotransmission: interaction with neurotransmitters and calcium in smooth muscle. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 604–607.

BERRIDGE, M.J. (1987). Inositol trisphosphate and diacylglycerol: two interacting second messengers. *Ann. Rev. Biolchem.*, **56**, 159–193.

BRODY, M.J. & DIXON, R.L. (1964). Vascular reactivity in experimental diabetes mellitus. *Circ. Res.*, **14**, 494–501.

CAMPBELL, M.D., DETH, R.C., PAYNE, R.A. & HONEYMAN, T.W. (1985). Phosphoinositide hydrolysis is correlated with agonist-induced calcium flux and contraction in the rabbit aorta. *Eur. J. Pharmacol.*, **116**, 129–136.

CASTAGNA, M., TAKAZ, Y., KAIBUCHI, K., SANO, K., KIKKAWA, U. & NISHIZUKA, Y. (1982). Direct activation of calcium activated phospholipid dependent protein kinase by tumor promoting phorbol esters. *J. Biol. Chem.*, **257**, 7847–7851.

CHATTERJE, M. & TEJADA, M. (1986). Phorbol ester induced contraction in chemically-skinned vascular smooth muscle. *Am. J. Physiol.*, **251**, C356–C361.

CHIU, A.T., BOZARTH, J.M., FORSYTHE, M.S. & TIMMERMANS, P.B.M.W.M. (1987). Ca^{2+} utilization in the contraction of rat aorta to stimulation of protein kinase C by phorbol dibutyrate. *J. Pharmacol. Exp. Ther.*, **242**, 934–939.

CHRISTLIEB, A.R., JANKA, H.V. & SOLANO, A. (1976). Vascular reactivity to angiotensin II and to norepinephrine in diabetic subjects. *Diabetes*, **25**, 268–274.

DANTHULURI, N.R. & DETH, R.C. (1984). Phorbol ester-induced contraction of arterial smooth muscle and inhibition of α-adrenergic response. *Biochem. Biophys. Res. Commun.*, **125**, 1103–1109.

DEFEO, T.T. & MORGAN, K.G. (1985). Calcium-force relationships as detected with acquorin in two different vascular smooth muscles of the ferret. *J. Physiol.*, **369**, 269–282.

FISH, R.D., SPERTI, G., COLUCCI, W.S. & CLAPHAM, D.E. (1988). Phorbol ester increases the dihydropyridine-sensitive calcium conductance in a vascular smooth muscle cell line. *Circ. Res.*, **62**, 1049–1054.

GARCIA, M.J., MCNAMARA, P.M., GORDON, T. & KINNELL, W.B. (1974). Morbidity and mortality in diabetics in the Framingham population: sixteen-year follow-up study. *Diabetes*, **23**, 104–111.

GLEASON, M.M. & FLAIM, S.F. (1986). Phorbol ester contracts rabbit thoracic aorta by increasing intracellular Ca^{2+} and by activating calcium influx. *Biochem. Biophys. Res. Commun.*, **138**, 1362–1369.

HARRIS, K.H. & MACLEOD, K.M. (1988). Influence of the endothelium on contractile responses of arteries from diabetic rats. *Eur. J. Pharmacol.*, **153**, 55–64.

HASHIMOTO, T., MIRATA, M., ITOH, T., KANMURA, Y. & KURIYAMA, H. (1986). Inositol 1,4,5-trisphosphate activates pharmacomechanical coupling in smooth muscle of the rabbit mesenteric artery. *J. Physiol.*, **370**, 605–618.

HEAGERTY, A.M. & OLLERENSHAW, J.D. (1987). The phosphoinositide signalling system and hypertension. *J. Hypertension*, **5**, 515–524.

KIKKAWA, U., TAKAI, Y., TANAKA, Y., MYAKE, R. & NISHIZUKA, Y. (1983). Protein kinase C as a possible receptor protein of tumor-promoting phorbol esters. *J. Biol. Chem.*, **258**, 11442–11445.

KOJIMA, I., KOJIMA, K., KREUTTER, D. & RASMUSSEN, H. (1984). The temporal integration of the aldosterone secretory response to angiotensin occurs via two intracellular pathways. *J. Biol. Chem.*, **259**, 14448–14457.

MACLEOD, K.M. (1985). The effect of insulin treatment on changes in vascular reactivity in chronic, experimental diabetes. *Diabetes*, **34**, 1160–1167.

MACLEOD, K.M. & MCNEILL, J.H. (1985). The influence of chronic experimental diabetes on contractile responses of rat isolated blood vessels. *Can. J. Physiol. Pharmacol.*, **63**, 52–57.

MILLER, J.R., HAWKINS, D.J. & WELLS, J.N. (1986). Phorbol diesters alter the contractile responses of porcine coronary artery. *J. Pharmacol. Exp. Ther.*, **239**, 38–42.

NAKAKI, J., ROTH, B.L., CHUANG, D.M. & COSTA, E. (1985). Phasic and tonic components of 5-HT_2 receptor-mediated rat aorta contraction: participation of Ca^{2+} channels and phospholipase C. *J. Pharmacol. Exp. Ther.*, **234**, 442–446.

NIEDEL, J.E., KUNN, L.J. & VANDENBARK, G.R. (1983). Phorbol diester receptor copurifies with protein kinase C. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 36–40.

NISHIZUKA, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature*, **308**, 693–698.

RAMANADHAN, S. & TENNER, T.E. (1984). Alterations in aortic and tail artery reactivity to agonists after STZ treatment. *Can. J.*

Physiol. Pharmacol., **62**, 418–423.

RASMUSSEN, H., FORDER, J., KOJIMA, I. & SCRIABINE, A. (1984). TPA-induced contraction of isolated rabbit vascular smooth muscle. *Biochem. Biophys. Res. Commun.*, **122**, 776–784.

RUEGG, U.T. & BURGESS, G.M. (1989). Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinase C. *Trends Pharmacol. Sci.*, **10**, 218–220.

SILVER, P.J., LEPORE, R.E., CUMISKEY, W.R., KIEFER, D. & HARRIS, A.L. (1988). Protein kinase C activity and reactivity to phorbol ester in vascular smooth muscle from spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto rats (WKY). *Biochem. Biophys. Res. Commun.*, **84**, 272–277.

SINGER, H.A. & BAKER, K.M. (1987). Calcium dependence of phorbol-12,13-dibutyrate-induced force and myosin light chain phosphorylation in arterial smooth muscle. *J. Pharmacol. Exp. Ther.*, **243**, 814–820.

SUEMATSU, E., HIRATA, M., HASHIMOTO, T. & KURIYAMA, H. (1984). Inositol 1,4,5-trisphosphate releases Ca^{2+} from intracellular storage sites in skinned single cells of porcine coronary artery. *Biochem. Biophys. Res. Commun.*, **120**, 481–485.

SWAMURA, M., KOBAYASHI, Y., NARA, Y., HATTORI, K. & YOMORI, Y. (1987). Effect of extracellular calcium on vascular contraction induced by phorbol ester. *Biochem. Biophys. Res. Commun.*, **145**, 492–501.

SYBERTZ, E.J., DESIDERIO, D.M., TETZLOFF, G. & CHIU, P.J.S. (1986). Phorbol dibutyrate contractions in rabbit aorta: calcium dependence and sensitivity to nitrovasodilators and 8-Br-cyclic GMP. *J. Pharmacol. Exp. Ther.*, **239**, 78–83.

TAMAOKI, T., NOMOTO, H., TAKAMASHI, I., KATO, Y., MORIMOTO, M. & TOMITO, F. (1986). Staurosporine, a potent inhibitor of phospholipid/Ca^{2+} dependent protein kinase. *Biochem. Biophys. Res. Commun.*, **135**, 397–402.

TURLA, M.B. & WEBB, C. (1987). Enhanced vascular reactivity to protein kinase C activators in genetically hypertensive rats. *Hypertension*, (Suppl. III), III-151–III-154.

TURLA, M.B. & WEBB, C. (1990). Augmented phosphoinositide metabolism in aortas from genetically hypertensive rats. *Am. J. Physiol.*, **258**, H173–H178.

WAGNER, B., SCHACHTELE, C. & MARME, D. (1987). Phorbol 12,13-dibutyrate-induced contraction of isolated rabbit vascular smooth muscle. *Eur. J. Pharmacol.*, **140**, 227–232.

WEIDMANN, P., BERETTA-PICOLI, C., KEUSCH, G., GLUCK, Z., MUJAGIC, M., GRIMM, M., MEIER, A. & ZEIGLER, W.H. (1979). Sodium-volume factor, cardiovascular reactivity and hypertensive mechanism of diuretic therapy in mild hypertension associated with diabetes mellitus. *Am. J. Med.*, **67**, 779–784.

WHITE, R.E. & CARRIER, G.O. (1988). Enhanced vascular α-adrenergic neuroeffector system in diabetes: importance of calcium. *Am. J. Physiol.*, **255**, H1036–H1042.

WYSE, D.G. (1980). On 'the normalization' of active developed force of isolated helical strips of muscular and elastic arteries for variation in wall thickness. *J. Pharmacol. Methods*, **4**, 313–326.

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Inhibition of nicotine-induced relaxation of the bovine retractor penis muscle by compounds known to have ganglion-blocking properties

Sakari Alaranta, [1]Erik Klinge, Tuomo Pätsi & *Nils O. Sjöstrand

Division of Pharmacology and Toxicology, Department of Pharmacy, University of Helsinki, Kirkkokatu 20, SF-00170 Helsinki, Finland and * Department of Physiology, Karolinska Institute, S-104 01 Stockholm, Sweden

1 The relative potency in blocking the nicotine-induced relaxation of the bovine retractor penis muscle (BRP) was estimated for 12 drugs known to have ganglion-blocking properties.

2 The order of potency of the drugs studied was mecamylamine > chlorisondamine > pentolinium > propantheline > (+)-tubocurarine > hexamethonium > emepronium > tetraethylammonium > glycopyrrolate > decamethonium > butylscopolamine > scopolamine.

3 The results conform well to those obtained with other pharmacological methods used for the estimation of ganglion-blocking activity.

4 It is concluded that blockade of the nicotinic relaxation of the BRP can be used as an alternative method for quantitative assessment of ganglion-blocking activity.

5 Advantages of this technique are that it discriminates well between antinicotinic and antimuscarinic activity and that it satisfies most or all ethical and economical demands.

6 It is also possible that this method has certain value in predicting whether a drug has enough ganglion-blocking activity to be likely to cause impotence.

Introduction

It has been shown that strips of the bovine retractor penis muscle (BRP) are concentration-dependently relaxed by low concentrations of nicotine and other nicotinic agonists including acetylcholine, and that these relaxations are totally blocked by low concentrations of mecamylamine and lidocaine and by 50–90% by tetrodotoxin (Klinge *et al.*, 1988). It is further known that in the stroma of the BRP there are sparsely scattered small ganglia and single nerve cell bodies (Alaranta *et al.*, 1989). On the basis of these results and earlier comparative studies (Klinge & Sjöstrand, 1977) the authors have presented two hypotheses. Firstly, the relaxant effect of nicotine on the BRP is, in part or principally, mediated via receptors located on the above-mentioned nerve cell bodies. Secondly, nicotinic activation of these neurones can be used as a model for further pharmacological and possibly also physiological characterization of the postganglionic part of mammalian erectile nerves.

This paper describes how the relaxation of the BRP induced by a small standard dose of nicotine is affected by actual ganglion-blocking drugs as well as by antimuscarinic and neuromuscular blocking drugs known to have ganglion-blocking properties. One purpose of the study was to find out whether it is possible to use the nicotinic relaxation of the BRP for quantitative assessment of the ganglion-blocking activity of a given drug. Another aim was to clarify whether there is any correlation between the results of the present study and the knowledge of the impotence causing effect of the drugs used for the treatment of ambulatory patients. Finally, it was of interest to see whether the results throw any new light on the proposed mechanism of action of nicotine in the BRP.

Methods

Muscle strips

Retractor penis muscles were obtained from bulls of different breeds weighing 250–500 kg. Samples were dissected 10–30 cm distal to the points where the paired muscle bundles pass the anal orifice. Within 10–25 min of slaughter the samples were put in 2–5°C Tyrode solution, the composition of which was the same as that described earlier (Klinge & Sjöstrand, 1974) with the exception that the amount of $MgCl_2$ was reduced to 50 mg l^{-1} (0.525 mM). Strips, 15–25 mm in length and 2–3 mm wide, were cut at 2–5°C. It was of importance that the strips were as even and homogeneous as possible. During the cutting procedure touching the muscles with fingers was avoided. The strips were then mounted in 20 ml organ baths containing Tyrode solution at 35°C aerated with 95% O_2 and 5% CO_2. For fresh preparations an equilibration time of 2–3 h was required until their responsiveness to nicotine was fully developed, whereas about 4 h were required for strips mounted on the day after slaughter. An initial resting isometric tension of 4–5 g was applied. During the equilibration period washes were performed at about 60 min intervals. Changes in tension were recorded by means of Grass FT 03 force displacement transducers coupled to a Grass model 7D polygraph.

Estimation of ganglion-blocking activity

A high enough tone for studying the nicotinic relaxation, usually 6–10 g, was generated by adding 5-hydroxytryptamine (5-HT) to the organ bath. The concentration of 5-HT needed ranged between 0.2 and 6 μM. In addition to biological variation, this was due to the phenomena that some strips developed tone spontaneously and most strips increased their sensitivity to 5-HT during the first 2 or 3 h of the experiment, whereafter their responsiveness to this contractile agent remained stable (cf. Klinge *et al.*, 1988; Alaranta *et al.*, 1989). These facts had to be taken into consideration when the same level of tone was secured in successive contractions. For adjustment of the tone 5-HT was added cumulatively (Figure 1).

For the nicotine-induced relaxation a standard dose, i.e. 30 μM, was used. The magnitude of the nicotinic relaxation was not influenced by the concentrations of 5-HT used, whereas the recontraction occurring after the relaxation was more rapid in the presence of higher concentrations. Washing was performed 2 min after application of nicotine; 60–80 min later the tone was again raised and application of nicotine was

[1] Author for correspondence.

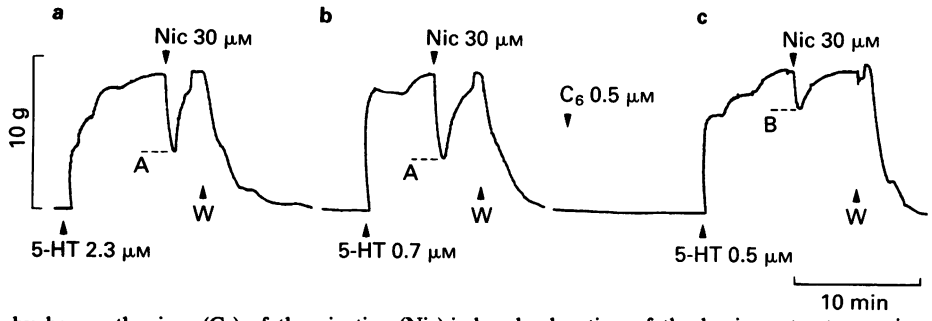

Figure 1 Inhibition by hexamethonium (C_6) of the nicotine (Nic)-induced relaxation of the bovine retractor penis muscle. (a and b) Two successive controls. (c) Effect of C_6. (A) Indicates the magnitude of the relaxation in the controls, and (B) indicates its magnitude in the presence of C_6. The concentrations of 5-hydroxytryptamine (5-HT) are final cumulative concentrations needed to generate the same level of tone. In this particular strip the increase in sensitivity to 5-HT was quite clear. W = wash.

repeated (Figure 1). The effect of a blocking drug was studied only if the relaxations caused by nicotine in two consecutive controls were equal in size. In the third experiment the blocking drug was added 12–15 min before the application of nicotine. The use of a strip for additional experiments is not recommended, because the washout of a blocking drug is complete only in the case of highly water soluble compounds.

The blocking activity of a certain concentration of a drug was expressed as % reduction in the relaxation of the muscle strip, according to the following equation:

$$\frac{A - B}{A} \times 100,$$

where A is the size of the control relaxation in mm, and B the size of the relaxation in the presence of the blocking drug (Figure 1). In order to construct a regression line the activity of 4 dose levels from the assumed linear part of the concentration-effect curve was studied except for (+)-tubocurarine, hexamethonium and decamethonium for which 5 dose levels were used. The activity of each dose level was studied in at least 5 strips obtained from 4 to 6 bulls. In Table 1 two examples of the procedure are presented. The IC_{50} values were calculated from the regression lines. The parallelism of the regression lines was tested by using the analysis of covariance (SAS, 1985). $P < 0.05$ was considered to be statistically significant.

Drugs

The following drugs were generously donated: butylscopolamine Br (C.H. Boehringer, Ingelheim am Rhein, F.R.G.), chlorisondamine Cl (Ciba-Geigy AG, Basle, Switzerland), emepronium Br and (+)-tubocurarine Cl (both from Orion Pharmaceutica, Espoo, Finland), glycopyrrolate Br (H. Lundbeck & Co., Copenhagen, Denmark) and propantheline Br (Star, Tampere, Finland). The following drugs were purchased: decamethonium Br, pentolinium bitartrate and tetraethylammonium Cl (all from Sigma Chemical Company, St. Louis, MO, U.S.A.), hexamethonium Br and 5-hydroxytryptamine creatinine sulphate (both from Fluka AG, Buchs SG, Switzerland), mecamylamine HCl (Merck Sharp & Dohme, Rahway, NJ, U.S.A.), nicotine bitartrate (BDH Chemicals, Poole, U.K.) and scopolamine HBr (C.H. Boehringer, Ingelheim am Rhein, F.R.G.).

Results

The molar potency-ratio of the drugs studied in inhibiting the nicotine-induced relaxation of the BRP is shown in Table 2. Of the actual ganglion-blocking agents mecamylamine was about 5.4, chlorisondamine about 3.7 and pentolinium about 2.8 times more potent than hexamethonium, whereas tetraethylammonium was about 8.3 times less potent. Of the antimuscarinic drugs used for the treatment of ambulatory patients propantheline was the most potent, being about 3.5 times more potent than emepronium, about 32 times more potent than glycopyrrolate, about 140 times more potent than butylscopolamine, and about 400 times more potent than scopolamine. The weak effect of scopolamine is noteworthy. Of the neuromuscular blocking drugs, (+)-tubocurarine was about 43 times more potent than decamethonium.

The regression lines obtained for the inhibitory activity of the 5 actual ganglion-blocking drugs as well as for the 2 neuromuscular blocking drugs are presented in Figure 2. The regression lines representing the inhibitory activity of the 5 antimuscarinic drugs are illustrated in Figure 3. In the latter figure the line denoting the activity of hexamethonium is

Table 2 Inhibition of nicotine-induced relaxation of the bovine retractor penis muscle by various drugs known to have ganglion-blocking properties

Drug	IC_{50} − log [M]	*Molar potency-ratio* (Hexamethonium = 1.00)
Mecamylamine	7.123	5.43
Chlorisondamine	6.960	3.72
Pentolinium	6.838	2.80
Propantheline	6.689	1.99
(+)-Tubocurarine	6.579	1.54
Hexamethonium	6.391	1.00
Emepronium	6.146	0.57
Tetraethylammonium	5.478	0.12
Glycopyrrolate	5.188	0.063
Decamethonium	4.948	0.036
Butylscopolamine	4.535	0.014
Scopolamine	4.061	0.005

Table 1 Construction of regression lines for calculation of IC_{50} values for the inhibition by pentolinium and hexamethonium of the nicotine-induced relaxation of the bovine retractor penis muscle

Pentolinium					
−log [M]	7.283	6.982	6.681	6.380	
% inhibition					
(mean ± s.e., $n = 4$)	23.4 ± 1.6	42.0 ± 6.3	59.0 ± 5.4	77.2 ± 7.1	
Equation of regression line: $y = 59.3x + 455.3$; $r = 0.9999$					
Hexamethonium					
− log [M]	6.704	6.550	6.403	6.253	6.102
% inhibition					
(mean ± s.e., $n = 6$)	23.6 ± 4.5	29.4 ± 9.5	49.6 ± 11.2	63.2 ± 7.8	78.6 ± 4.6
Equation of regression line: $y = 95.7x + 661.7$; $r = 0.9897$					

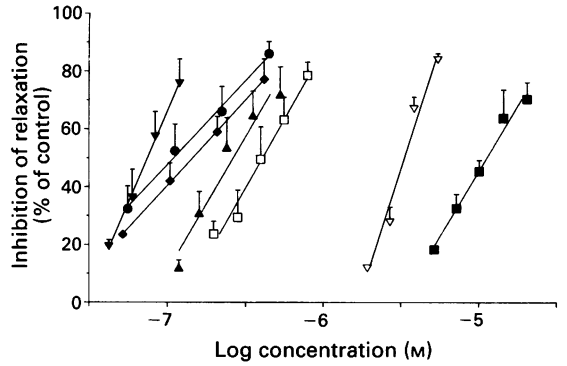

Figure 2 The regression lines, i.e. the linear parts of the concentration-effect curves for the inhibition by mecamylamine (▼), chlorisondamine (●), pentolinium (◆), (+)-tubocurarine (▲), hexamethonium (□), tetraethylammonium (▽) and decamethonium (■) of the nicotine-induced relaxation of the bovine retractor penis muscle. Each point represents the arithmetical mean; vertical lines show s.e.mean.

included as a reference. Four drugs yielded a regression line the slope of which was significantly different from that of hexamethonium, i.e. pentolinium ($P < 0.0049$; d.f. = 4), chlorisondamine ($P < 0.0061$; d.f. = 4), tetraethylammonium ($P < 0.012$; d.f. = 4) and mecamylamine ($P < 0.037$; d.f. = 4).

Discussion

The relative ganglion-blocking activity of various drugs measured with the bovine isolated retractor penis muscle (Table 2) conforms well to that obtained with other pharmacological methods. This is illustrated in the following where all comparisons have been made by using the molar IC_{50} values. For example, in blocking contractions of the cat nictitating membrane evoked by preganglionic sympathetic stimulation, mecamylamine has been shown by Corne & Edge (1958) to be about as potent as hexamethonium, the potency-ratio being 1.01, whereas with the same method Spinks *et al.* (1958) and Bowman & Webb (1972) found this potency-ratio to be 1.13 and 17 respectively. The discrepancy between the activities was probably in part due to differencies in the site of injection of the drugs, i.e. intra-arterial injection increased the activity of mecamylamine (Bowman & Webb, 1972). The activities may also have been influenced by differencies in the stimulation parameters. In the present study the potency-ratio of mecamylamine to hexamethonium was 5.4. It is possible that the thickness of the strips of the BRP was a factor that increased the relative potency of lipophilic compounds.

In a study in which the preganglionically stimulated nictitating membrane was used, chlorisondamine was found to be

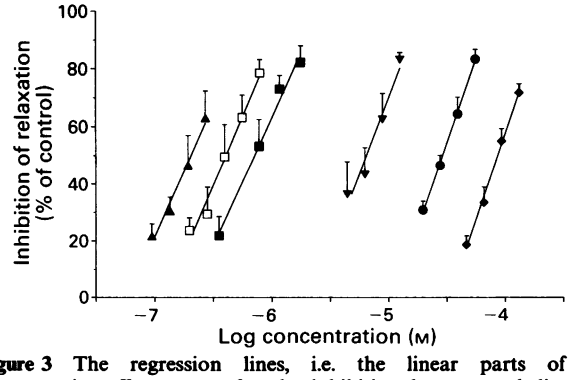

Figure 3 The regression lines, i.e. the linear parts of the concentration-effect curves for the inhibition by propantheline (▲), hexamethonium (□), emepronium (■), glycopyrrolate (▼), butylscopolamine (●) and scopolamine (◆) of the nicotine-induced relaxation of the bovine retractor penis muscle. Each point represents the arithmetical mean; vertical lines show s.e.mean. None of the slopes was significantly different from that of hexamethonium.

1.6 times more potent than pentolinium (Plummer *et al.*, 1955), while it was shown to be 1.3 times more potent in the present study. In the former study chlorisondamine was 7.6 times more potent than hexamethonium, whereas it was about 3.7 times more potent in our study. When the same nictitating membrane technique was employed the potency-ratios of pentolinium to hexamethonium were found to be 5, 4.8 and 4.7 by Mason & Wien (1955), Plummer *et al.* (1955) and Corne & Edge (1958), respectively, whereas this potency ratio in blocking the peristaltic reflex in the guinea-pig isolated ileum was found to be 1.5 (Mason & Wien, 1955). In the present study the potency-ratio of pentolinium to hexamethonium was 2.8.

In the present study, the only actual ganglion-blocking drug that exhibited a lower potency than hexamethonium was tetraethylammonium, the potency-ratio being 0.12. The potency-ratios of these 2 drugs in blocking the contractions of the nictitating membrane obtained by various authors differ considerably from each other, the average value being 0.19 (Table 3). When estimated with methods other than the nictitating membrane technique, the ganglion-blocking potency-ratio of these drugs also displays considerable differences, the average value being 0.15 (Table 4). The method which recorded the depression of postganglionic action potentials evoked by preganglionic sympathetic stimulation (Bainbridge & Brown, 1960) is an electrophysiological rather than a pharmacological method. It still yielded a potency-ratio which is in agreement with the ratios obtained with the ordinary nictitating membrane technique (Table 3).

With regard to the ganglion-blocking activity of the neuromuscular blocking drugs, it has been estimated that the potency ratio of (+)-tubocurarine to hexamethonium in blocking the contractions of the guinea-pig isolated ileum induced by a small dose of nicotine is about 0.68 (Feldberg, 1951). In our study this ratio was about 1.54. In the study of Feldberg (1951) the potency ratio of (+)-tubocurarine to tetraethylammonium was about 7.2, while it was about 12.8 in the present study. (+)-Tubocurarine has been shown to be

Table 3 Molar ganglion-blocking potency ratios of tetraethylammonium to hexamethonium obtained by measuring the inhibition of the contraction of the cat nictitating membrane evoked by preganglionic sympathetic stimulation

Potency-ratio	*Authors*
0.028	Paton & Zaimis (1951)
0.12	Lape & Hoppe (1956)
0.29	Di Gregorio & Dipalma (1967)
0.34	Spinks *et al.* (1958)
0.19 (mean)	

Table 4 Molar ganglion-blocking potency-ratios of tetraethylammonium to hexamethonium obtained with methods other than the commonly used nictitating membrane technique

Potency ratio	*Method*
0.079	Inhibition of the peristaltic reflex in rabbit isolated ileum (Paton & Zaimis, 1951)
0.094	Inhibition of contraction of guinea-pig isolated ileum induced by a small dose of nicotine (Feldberg, 1951)
0.19	Induction of mydriasis in mouse by blocking the ciliary ganglion (Edge, 1953)
0.226	Depression of postganglionic action potentials evoked by preganglionic sympathetic stimulation (Bainbridge & Brown, 1960)
0.15 (mean)	

about 5 times more potent than hexamethonium in blocking the contractions of the preganglionically stimulated cat nictitating membrane (Bowman & Webb, 1972). The same authors found the potency ratio of (+)-tubocurarine to mecamylamine to be about 0.29, which is the same as that obtained in the present study, i.e. 0.28. In blocking the contractions of the cat nictitating membrane, the potency ratio of (+)-tubocurarine to decamethonium has been estimated to be 118 (Hoppe *et al.*, 1955), whereas it was found to be 43 in the present study.

Thus, the ganglion-blocking activity of various drugs, as estimated with the most commonly used method, i.e. the preganglionically stimulated cat nictitating membrane, is in most cases in quite good agreement with the results of the present study. Further, if the fact that the activities obtained with different methods often exhibit considerable discrepancies is taken into consideration, it is feasible that the nicotinic relaxation of the BRP could be used as a screening method for the assessment of the ganglion-blocking activity of a given drug. Although there is at present no clinical need to develop new ganglion-blocking drugs it may sometimes be important to exclude a too high ganglion-blocking activity of drugs developed for other purposes. A distinct advantage of the present method is that it discriminates well between antinicotinic and antimuscarinic activity. Additional advantages are that it satisfies most or all ethical and economical demands. Furthermore, it is likely that reliable results can be obtained with less than 4, i.e. 3 dose levels. One disadvantage is that it will probably be difficult to record postganglionic action potentials after preganglionic stimulation.

There is no ideal method for the assessment of ganglion-blocking activity, and even within the same species the results which are relevant to a certain type of ganglion may not be directly applicable to another type (Bevan, 1968; Gyermek, 1980). Against this background it may be difficult to assume that the results of the present study could bear a relationship to the impotence causing effect of the drugs studied. It is generally accepted that actual ganglion-blocking drugs do cause impotence (e.g. Volle & Koelle, 1970; Erina, 1980), although we have found no study ranking their propensity to bring about this side-effect. It is, however, of more interest that, of the anticholinergics included in the present study, propantheline exhibited the highest ganglion-blocking activity, and the propensity of this drug to cause impotence is well known (Manners, 1966; Goldstein & Krane, 1983; Bagley, 1988). Also methantheline, which has a slightly weaker ganglion-blocking activity, is well known to cause impotence (Schwartz & Robinson, 1952; Innes & Nickerson, 1965). We have found no accounts of an impotence causing effect of emepronium, but this should not, until the question has been adequately settled, exclude the possibility that emepronium could slightly reduce the strength of erection. Finally, there are no convincing reports that clinical doses of butylscopolamine or scopolamine cause impotence. It therefore seems that with the present method it is, to a certain extent, possible to predict whether a drug has enough ganglion-blocking activity to be likely to cause impotence.

The differences between the slopes of the regression lines are probably, in part, due to differences in the mechanism of action of the drugs. The existence of such differences between the mechanism of action of ganglion-blocking drugs is a well known fact (Brown, 1980; Skok, 1986; Rang & Dale, 1987). On the other hand, the ganglion-blocking potency-ratios obtained in the present study indirectly support the hypothesis that, in the BRP small doses of nicotine exert their action by activating the type of nicotinic receptors primarily encountered in autonomic ganglia.

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References

ALARANTA, S., UUSITALO, H., KLINGE, E., PALKAMA, A. & SJÖSTRAND, N.O. (1989). Histochemical demonstration of nerve cell bodies in the retractor penis muscle and the penile artery of the bull. *Neuroscience*, **32**, 823–827.

BAGLEY, J.L. (1988). When medications impair men's sexuality. *Am. Druggist*, **198**, 69–72.

BAINBRIDGE, J.G. & BROWN, D.M. (1960). Ganglion-blocking properties of atropine-like drugs. *Br. J. Pharmacol. Chemother.*, **15**, 147–151.

BEVAN, J.A. (1968). Ganglionic blocking agents. In *Selected Pharmacological Testing Methods*. ed. Burger, A. pp. 235–254. New York: Marcel Dekker.

BOWMAN, W.C. & WEBB, S.N. (1972). Neuromuscular blocking and ganglion blocking activities of some acetylcholine antagonists in the cat. *J. Pharm. Pharmacol.*, **24**, 762–772.

BROWN, D.A. (1980). Locus and mechanism of action of ganglion-blocking agents. In *Pharmacology of Ganglionic Transmission*. ed. Kharkevich, D.A. pp. 185–235. Berlin-Heidelberg-New York: Springer Verlag.

CORNE, S.J. & EDGE, N.D. (1958). Pharmacological properties of pempidine (1:2:2:6:6-pentamethylpiperidine), a new ganglion-blocking compound. *Br. J. Pharmacol. Chemother.*, **13**, 339–349.

DI GREGORIO, G.J. & DIPALMA, J.R. (1967). Some pharmacological actions of 2-phenylquinoline methiodide. *Br. J. Pharmacol. Chemother.*, **30**, 531–540.

EDGE, N.D. (1953). Mydriasis in the mouse: a quantitative method of estimating parasympathetic ganglion block. *Br. J. Pharmacol. Chemother.*, **8**, 10–14.

ERINA, E.V. (1980). Ganglion-blocking agents in internal medicine. In *Pharmacology of Ganglionic Transmission*. ed. Kharkevich, D.A. pp. 417–438. Berlin-Heidelberg-New York: Springer Verlag.

FELDBERG, W. (1951). Effects of ganglion-blocking substances on the small intestine. *J. Physiol.*, **113**, 483–505.

GOLDSTEIN, I. & KRANE, R.J. (1983). Drug-induced sexual dysfunction. *World J. Urol.*, **7**, 239–243.

GYERMEK, L. (1980). Methods for the examination of ganglion-blocking activity. In *Pharmacology of Ganglionic Transmission*. ed. Kharkevich, D.A. pp. 63–121. Berlin-Heidelberg-New York: Springer Verlag.

HOPPE, J.O., FUNNEL, J.E. & LAPE, H. (1955). The effects of structural variation in the quaternary nitrogen centers of benzquinonium chloride upon neuromuscular blocking activity. *J. Pharmacol. Exp. Ther.*, **115**, 106–119.

INNES, I.R. & NICKERSON, M. (1965). Drugs inhibiting the action of acetylcholine on structures innervated by postganglionic parasympathetic nerves. In *The Pharmacological Basis of Therapeutics*. ed. Goodman, L.S. & Gilman, A. pp. 521–545. New York: Macmillan Publishing Company.

KLINGE, E. & SJÖSTRAND, N.O. (1974). Contraction and relaxation of the retractor penis muscle and the penile artery of the bull. *Acta Physiol. Scand.*, **93**, Suppl. 420, 1–88.

KLINGE, E. & SJÖSTRAND, N.O. (1977). Comparative study of some isolated mammalian smooth muscle effectors of penile erection. *Acta Physiol. Scand.*, **100**, 354–367.

KLINGE, E., ALARANTA, S. & SJÖSTRAND, N.O. (1988). Pharmacological analysis of nicotinic relaxation of the bovine retractor penis muscle. *J. Pharmacol. Exp. Ther.*, **245**, 280–286.

LAPE, H.E. & HOPPE, J.O. (1956). The use of serial carotid occlusion, nictitating membrane and cross-circulation techniques in the investigation of the central hypotensive activity of ganglionic blocking agents. *J. Pharmacol. Exp. Ther.*, **116**, 453–461.

MANNERS, D. (1966). Probanthine. *Med. J. Australia*, **2**, 436.

MASON, D.F.J. & WIEN, R. (1955). The actions of heterocyclic bisquaternary compounds, especially of a pyrrolidinium series. *Br. J. Pharmacol. Chemother.*, **10**, 124–132.

PATON, W.D.M. & ZAIMIS, E.J. (1951). Paralysis of autonomic ganglia by methonium salts. *Br. J. Pharmacol. Chemother.*, **6**, 155–168.

PLUMMER, A.J., TRAPOLD, J.H., SCHNEIDER, J.A., MAXWELL, R.A. & EARL, A.E. (1955). Ganglionic blockade by a new bisquaternary series, including chlorisondamine dimethochloride. *J. Pharmacol. Exp. Ther.*, **115**, 172–184.

RANG, H.P. & DALE, M.M. (1987). Ganglion blocking drugs. In *Pharmacology*. ed. Rang, H.P. & Dale, M.M. pp. 125–129. Edinburgh-London-Melbourne-New York: Churchill Livingstone.

SAS user's guide (1985). *Statistics*, Version 5 edition, Cary, North Carolina: SAS Institute Inc.

SCHWARTZ, N.J. & ROBINSON, B.D. (1952). Impotence due to methantheline bromide. *N.Y. State J. Med.*, **52**, 1530.

SKOK, V.I., (1986). Ganglion blockers. In *Mechanisms of Drug Action.* Vol. 1, ed. Woodruff, G.N., pp. 97–129. Houndmills: Macmillan Press.

SPINKS, A., YOUNG, E.H.P., FARRINGTON, J.A. & DUNLOP, D. (1958). The pharmacological actions of pempidine and its ethyl homologue. *Br. J. Pharmacol. Chemother.*, **13**, 501–520.

VOLLE, R.L. & KOELLE, G.B. (1970). Ganglion stimulating and blocking agents. In *The Pharmacological Basis of Therapeutics.* ed. Goodman, L.S. & Gilman, A. pp. 585–600. New York: Macmillan Publishing Company.

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Distinct antinociceptive actions mediated by different opioid receptors in the region of lamina I and laminae III–V of the dorsal horn of the rat

P.J. Hope, [1]S.M. Fleetwood-Walker & *R. Mitchell

Department of Preclinical Veterinary Sciences, University of Edinburgh, Summerhall, Edinburgh EH9 1QH and *M.R.C. Brain Metabolism Unit, 1 George Square, Edinburgh EH8 9JZ

1 In view of the presence of μ, δ and κ opioid receptors in the spinal dorsal horn and their apparent involvement in behavioural analgesia, the present experiments addressed the action of selective agonists ionophoresed in the vicinity of rat dorsal horn neurones which were located either in lamina I or in laminae III–V.

2 In laminae III–V, κ agonists (U50488H and dynorphin A) caused a selective inhibition of the nociceptive responses of multireceptive cells, whilst μ and δ agonists ([D-Ala$_2$,MePhe$_4$,Gly-ol]enkephalin and [D-Pen$_2$,D-Pen$_5$]enkephalin respectively) failed to alter either the spontaneous activity or the responses to noxious and innocuous cutaneous stimuli and to D,L-homocysteic acid or glutamate. Nocispecific neurones were encountered too rarely in laminae III–V to study their properties.

3 In lamina I, agonists had no effects on either nocispecific or multireceptive neurones. In contrast, the μ agonist [D-Ala$_2$,MePhe$_4$,Gly-ol]enkephalin consistently inhibited nociceptive responses of both multireceptive and nocispecific lamina I cells. The δ agonist [D-Pen$_2$,D-Pen$_5$]enkephalin consistently caused selective inhibition of the nociceptive responses of multireceptive cells but had a mixed profile of action on nocispecific cells.

4 These results suggest that μ, δ and κ opioid receptors mediate different antinociceptive actions in both laminae III–V and lamina I. The study reveals a distinct physiological role for δ receptors in modulating nociceptive inputs to lamina I neurones. In contrast to μ and κ receptor actions, δ receptors heterogeneously influence subpopulations of neurones.

Introduction

Clinical observations indicate that systemically administered morphine and other opiates selectively attenuate sensations evoked by noxious, but not innocuous stimuli (Behar *et al.*, 1979; Wang *et al.*, 1979). Opiates can act at both spinal and supraspinal sites to reduce neuronal responsiveness to nociceptive stimuli (see Duggan & North, 1984, for review). Intrathecal administration of opiates or opioid peptides can elicit behavioural analgesia in man and animals (Wang *et al.*, 1979; Onofrio & Yaksh, 1983; Yaksh, 1983; Schmauss & Yaksh, 1984). Behavioural studies have suggested the efficacy of intrathecally administered opioids at both μ- (Schmauss & Yaksh, 1984) and δ-sites (Hill *et al.*, 1987). Systemic and intrathecal administration of κ-agonists have been reported as antinociceptive, particularly in visceral and mechanical nociceptive tests (Hayes *et al.*, 1983; Schmauss & Yaksh, 1984). Other groups have observed thermal antinociception with intrathecal dynorphins and U50488H although there appears to be little margin between antinociception and motor dysfunction and paralysis (see Fleetwood-Walker, 1988; Millan, 1990, for reviews). The presence of these receptor subtypes and endogenous opioid peptides within the dorsal horn of the spinal cord, especially the superficial laminae (Hökfelt *et al.*, 1977; Botticelli *et al.*, 1981; Morris & Herz, 1987; Mansour *et al.*, 1988) suggests a complex role for opioids in influencing the spinal processing of nociceptive information at the single neurone level. Although the CNS of the rat contains relatively low levels of κ receptors (Mansour *et al.*, 1988) their presence in dorsal horn, especially the superficial zone is now well-established (Morris & Herz, 1987).

In electrophysiological studies, single neurones of the dorsal horn in lamina I, lamina II (the substantia gelatinosa) or in the deeper dorsal horn (laminae III–V) exhibit somewhat different functional characteristics (Menétrey *et al.*, 1977; Cervero & Iggo, 1980; McMahon & Wall, 1983; Schouenborg & Sjolund, 1983) including a range of responses to noxious and/or innocuous cutaneous stimuli. The effects of microionophoretically-applied opioids (including morphine) on single dorsal horn neurones have been investigated by a number of groups (Calvillo *et al.*, 1974; Dostrovsky & Pomeranz, 1976; Zieglgansberger & Bayerl, 1976; Duggan *et al.*, 1977; Belcher & Ryall, 1978; Davies & Dray, 1978; Piercey *et al.*, 1980). In general, either depression or excitation of multireceptive neurones in the deeper dorsal horn was reported. These effects were rarely reversible by naloxone. In contrast, more consistent antinociceptive and naloxone-reversible effects were observed with deeper dorsal horn neurones when opiates were administered into the substantia gelatinosa (lamina II) (Duggan *et al.*, 1977; Sastry & Goh, 1983). These results indicated that the effects of agonists at different opioid receptors may be rather discretely mediated within distinct laminae of the dorsal horn.

A recent study from our laboratory supported this hypothesis. Fleetwood-Walker *et al.* (1988) reported that when administered near to spinocervical tract (SCT) neurones in laminae III–V of cat spinal cord, only κ agonists exerted a selective antinociceptive action whilst μ and δ agonists were ineffective. In contrast, μ, but not δ or κ agonists ionophoretically applied in the region of the substantia gelatinosa had a selective antinociceptive effect on SCT neurones. In these experiments there was little evidence for any significant antinociceptive role of δ agonists at either of these two loci. However, results of intrathecal studies showed that δ, as well as μ and κ, agonists have distinct analgesic actions (Onofrio & Yaksh, 1983; Hill *et al.*, 1987). Whilst the effects of receptor-selective opioid agonists administered in lamina II and laminae III–V were investigated in detail by Fleetwood-Walker *et al.* (1988), effects on cells in lamina I remained to be studied. Furthermore, the majority of neurones tested in previous studies were multireceptive. The present study investigated the actions of μ, δ and κ agonists on nocispecific and multireceptive neurones in lamina I and compared these effects with actions on multireceptive laminae III–V cells, in the rat. Preliminary reports of this work have

[1] Author for correspondence.

appeared in abstract form (Fleetwood-Walker et al., 1986; Hope et al., 1987).

Methods

Experiments were carried out on rats (270–330 g) anaesthetized with intravenous α-chloralose (35 mg kg^{-1}) and urethane (700 mg kg^{-1}), after induction with halothane. Supplementary doses of anaesthetic were given as required. Core temperature was maintained at 37–38°C and carotid blood pressure was monitored throughout the experiment. If blood presure fell below 90 mmHg, the experiment was terminated. Humidified oxygen (0.1 l min^{-1}) was passed through one arm of a patent Y-shaped cannula to enrich the inspired air of spontaneously respiring rats. A craniotomy was performed to allow sterotaxic placement of electrodes at supraspinal sites and a laminectomy (thoracic 12–lumbar 4) allowed access for electrophysiological recordings from dorsal horn neurones.

Electrophysiological methods

Extracellular recordings were made via the central barrel (4 M NaCl, pH 4.0–4.5) of a 7 barrelled glass microelectrode. Electrode tip sizes were 4.0–4.5 μm and d.c. resistances were 5–8 MΩ. The band-width of the recording amplifier was 1 Hz–7 kHz.

One side barrel contained 1 M NaCl (pH 4.0–4.5) for automatic current balancing and current controls (Neurophore Ionophoresis System, Medical Systems Corporation). Another side barrel contained Pontamine Sky Blue dye (2% in 0.5 M sodium acetate for marking recording sites by ejection for 50 μA min). Other barrels contained various opioid receptor-selective agonists or antagonists (Corbett et al., 1984; Fleetwood-Walker et al., 1988): 2.5 mM dynorphin A_{1-13} (DYNO), 5 mM [D-Ala_2,$MePhe_4$,Gly-ol]enkephalin (DAMGO), 5 mM [D-Pen_2,D-Pen_5]enkephalin (DPDPE), 100 mM naloxone hydrochloride, 30 mM ICI 174864 (N,N-diallyl-Tyr-Aib-Aib-Phe-Leu) (all in aqueous solution at pH 4.0–4.5), 5 mM U50488H (*trans*-3,4-dichloro-N-methyl-N-(2-pyrrolidinyl)cyclohexyl-benzene acetamide, U50) in 100 Mm NaCl at pH 4.0–4.5 and 100 mM D,L-homocysteic acid (DLH) and 100 mM sodium glutamate in aqueous solution at pH 8.0–8.5. Synthetic peptides were obtained from Sigma or Cambridge Research Biochemicals, except for ICI 174864 which was a gift from ICI Pharmaceuticals plc.

All drugs were applied with a cathodal current except DLH and glutamate (anodal). Retention currents of 10–15 nA were applied to minimize drug leakage between tests.

The location of each neurone was examined histologically, having been marked by Pontamine Sky Blue dye ejection, at the end of each test. Many neurones located in lamina I were also identified as belonging to the spinomesencephalic tract (SMT) by use of standard antidromic criteria (Lipski, 1981). Concentric bipolar electrodes (Rhodes Medical SNE-100) were stereotaxically placed in the region of the contralateral parabrachial nucleus (posterior 9.5–10.0 mm, ventral 6.5–7.0 mm and lateral 0.5–1.0 mm, with respect to bregma (Hylden et al., 1986; Paxinos & Watson, 1986). The positions of all stimulating electrodes were marked by iron deposition from the electrode (30–60 s, 30 μA d.c.) and verified histologically, following ferryocyanide staining.

Test protocols

Action protocols of the recorded neurones were of uniform size and shape and could be clearly discriminated from other field potentials. All dorsal horn neurones had cutaneous excitatory receptive fields on the ipsilateral hindlimb. The field properties were carefully examined by use of both noxious and innocuous cutaneous stimuli before and after testing with drugs. Neurones were selected that could be classified as either multireceptive or nocispecific. Multireceptive neurones responded to both innocuous stimuli (light brushing, gentle squeeze and/or tapping) and noxious stimuli (pinch and/or radiant heat, 46–49°C). In contrast, nocispecific neurones responded only to noxious stimuli and gave no response to any innocuous stimuli tested. Having thus classified the receptive field of the neurone, controlled cutaneous stimuli were applied (usually for 10 s periods) to adjacent cutaneous areas, within the receptive field. The innocuous stimulus used was a motorized rotating brush. Noxious stimuli were provided by a calibrated pinch (serrated forceps with a graduated controlled displacement) or a thermocouple-controlled radiant heat lamp (giving a surface temperature ramp between 30–49°C). Responses to these stimuli and DLH- or glutamate-evoked activity (in response to ionophoresis at 5–60 nA for 10–15 s) were regularly repeated over 3 or 4 min cycles. These responses were always submaximal and approximately matched in terms of neuronal firing rates. Duplicate or triplicate controls varied by less than 15%. Neuronal firing was recorded on FM tape and firing rates (400 ms bins) were plotted on-line by computer (Cromemco System III), together with analogue signals from the stimulators and the ionophoresis unit. Responses to pinch, heat, brush, DLH or glutamate and epochs of spontaneous activity were integrated over appropriate time scales and normalized against control values meaned from the duplicate or triplicate pre-drug control responses. These normalized values were expressed graphically (see Figure 1, for example) to facilitate comparisons of drug effects on different classes of evoked activity. All drugs were continuously ionophoresed for 1 min before and for the duration of the test cycle. Ionophoretic ejection was started at currents of 5 nA and was increased between each subsequent cycle of tests by 5, 10 or 20 nA intervals until a distinct action of an opioid agonist was observed. Ionophoresis of agonists was then either terminated and testing continued until responses recovered to control levels or was maintained for testing of reversal by an opioid antagonist.

Results

Characteristics of cells

The excitatory receptive fields of all neurones tested were carefully mapped and were always located on the ipsilateral hind paw or limb. Inhibitory or contralateral receptive fields were rarely seen and were not tested. Twenty-seven neurones were subsequently located in the region of lamina I. Histological examination of transverse sections of the spinal cord revealed 27 Pontamine Sky Blue dye spots located immediately dorsal to the substantia gelatinose (lamina II) demonstrating the loci of the lamina I neurones. Six dye spots were located ventral to the substantia gelatinosa, revealing the loci of neurones in the deeper dorsal horn laminae III–V.

Excitatory neuronal responses were evoked from 12 out of 27 neurones located in lamina I and 6 out of 6 neurones in laminae III–V, by both noxious and innocuous cutaneous stimuli. These multireceptive lamina I neurones, including 4 SMT neurones, all responded to an unequivocally noxious pinch and 4 out of 6 cells tested also responded to noxious radiant heat (44–48°C). In all 6 deeper dorsal horn cells tested and 7 out of 12 lamina I cells the initial vigorous response evoked by innocuous stimulation could be subsequently maintained throughout the experiment. However, 5 out of 12 lamina I cells demonstrated an adaptation to testing with the first two or three innocuous brush stimuli applied. Thereafter repeated brush-evoked responses were consistently maintained at a lower level without alteration of the stimulus, for the duration of the testing period. This characteristic adaptation to non-nociceptive stimuli was seen both in SMT neurones and in lamina I neurones unidentified in terms of their ascending projections. However, neither the nociceptive responses of any lamina I neurones nor any responses of neurones located in laminae III–V demonstrated this feature of adaptation to controlled cutaneous stimuli. In such cases,

Table 1 Opioid effects on lamina I neurones

	Selective antinociceptive effect		*No effect*		*Non-selective effect*	
	multireceptive	nocispecific	multireceptive	nocispecific	multireceptive	nocispecific
DAMGO	9/9	9/9	0/9	0/9	0/9	0/9
DPDPE	8/8	5/15	0/8	3/15	0/8	7/15
U50488H or DYNO	0/5	0/4	4/5	4/4	1/5	0/4

DAMGO = [D-Ala$_2$,MePhe$_4$,Gly-ol]enkephalin; DPDPE = [D-Pen$_2$,D-Pen$_5$]enkephalin; DYNO = dynorphin A_{1-13}; U50488H = *trans*-3,4-dichloro-N-methyl-N-(2-pyrrolidinyl)cyclohexyl-benzene acetamide.

stimuli could be repeatedly evoked at 3 min intervals for over 2 h.

A variety of innocuous stimuli (including brushing, gentle squeeze or tap) completely failed to evoke any response from 15 out of 27 lamina I neurones, including 10 SMT neurones. These neurones, however, consistently responded to noxious cutaneous stimuli and were thus classified as nocispecific.

Both nocispecific and multireceptive neurones in lamina I showed a characteristic lack or minimal level (< 2 Hz) of spontaneous background activity. These neuronal characteristics were seen in both SMT and other lamina I neurones, unidentified in terms of their ascending projections. In contrast, neurones in the deeper dorsal horn consistently demonstrated background activity ranging from 2 to 10 Hz.

Effects of opioids applied in the vicinity of multireceptive neurones located in lamina I

Twelve multireceptive neurones in lamina I (of which 4 were SMT cells) were tested. In all cases both the μ (DAMGO) and the δ (DPDPE) agonists had profound selective antinociceptive effects, inhibiting both mechanical and thermal nociceptive responses whilst responses to innocuous and DLH- or glutamate-evoked activity were not altered from control levels (Table 1 and Figure 1). In the 4 cases where recovery from DAMGO (20 nA) was investigated, the substantial inhibition of nociceptive responses had recovered to near control values (98 ± 5% for pinch) within 5 ± 2 min (means ± s.e.mean, $n = 4$). Corresponding recovery from 15–20 nA DPDPE was similar, with pinch responses being restored to 93 ± 5% of controls in 7 ± 2 min ($n = 4$). The action of DAMGO was reversed by naloxone (20–60 nA) but not by a selective δ-antagonist, ICI 174864 (60–75 nA). In contrast, ICI 174864 (20–50 nA) consistently reversed typical DPDPE-mediated effects, as shown in Figure 2. In the 4 cases of DAMGO action in which reversal was investigated, the substantial inhibition of nociceptive responses caused by 15–20 nA DAMGO was reversed to 96 ± 12% of controls (pinch) by 4 ± 2 min of naloxone at 20–60 nA. Intravenous naloxone was not tested. The δ antagonist, ICI 178864 was ineffective, with DAMGO-suppressed pinch responses remaining at 26 ± 4% of controls despite ionophoresis of ICI 174864 at 60–75 nA for 4 ± 1 min ($n = 3$). In contrast, the substantial inhibition of noxious pinch responses caused by 15–20 nA DPDPE was reversed to 84 ± 6% of control responses by 20–50 nA of ICI 174864 for 5 ± 1 min ($n = 4$). In no case was there any effect of antagonists alone. In contrast to the selective antinociceptive effects evoked by ionophoresis of μ and δ agonists at low ejection currents (5–25 nA) on both SMT and unidentified revealed Pontamine Sky Blue dye spots located immediately dorsal to the substantia gelatinosa (lamina II). Six dye spots were located ventral to the substantia gelatinosa, revealing the loci of neurones in the deeper dorsal horn laminae III–V.

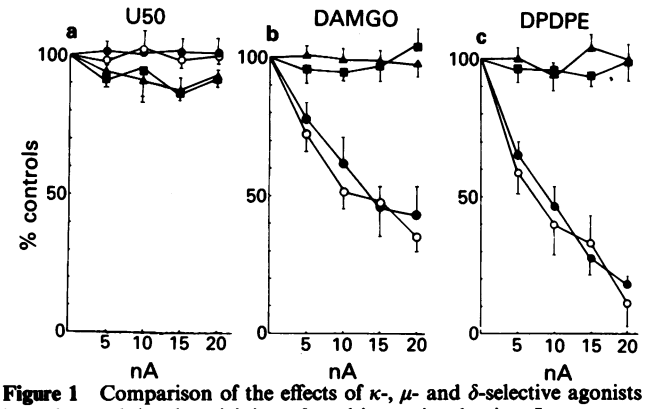

Figure 1 Comparison of the effects of κ-, μ- and δ-selective agonists ionophoresed in the vicinity of multireceptive lamina I neurones. Action potentials were counted over a set period of stimulation (or 10 s duration of spontaneous activity) and expressed as a percentage of responses evoked during the control period. Duplicate or triplicate responses evoked during the control period (before drug ionophoresis) were averaged and the mean considered as 100%. Neuronal responses in the presence of drugs are shown as mean values from samples of neurones (n indicated) with s.e.mean shown by vertical bars. Responses were evoked by noxious heat (●), noxious pinch (○), innocuous brush (▲) and D,L-homocysteic acid (DLH) (■). The presence of both μ [D-Ala$_2$,MePhe$_4$,Gly-ol]enkephalin (DAMGO) (b) and δ [D-Pen$_2$,D-Pen$_5$]enkephalin (DPDPE) (c) agonists caused a profound selective inhibition of the nociceptive responses ($n = 9$ and 8 respectively), whilst innocuous and DLH-evoked responses were unaffected. In contrast the κ-agonist, U50488H (U50) (a) had no effect on the evoked activity of these neurones ($n = 4$).

Excitatory neuronal responses were evoked from 12 of the neurones located in lamina I and 6 of the neurones in laminae III–V, by both noxious and innocuous cutaneous stimuli. These multireceptive lamina I neurones, including 4 SMT neurones, all responded to an unequivocally noxious pinch and 4 out of 6 cells tested also responded to noxious radiant heat (44–48°C). In all 6 deeper dorsal horn cells tested and 7 out of 12 lamina I cells the initial vigorous response evoked by innocuous stimulation could be subsequently maintained throughout the experiment. However, 5 out of 12 lamina I cells demonstrated a gradual adaptation to testing with the first two or three innocuous brush stimuli applied. Thereafter repeated brush-evoked responses were consistently maintained at a lower level without alteration of the stimulus, for the duration of the experiment. This characteristic adaptation to non-nociceptive stimuli was seen both in SMT neurones and in lamina I neurones unidentified in terms of their ascending projections. However, neither the nociceptive responses of any lamina I neurones nor any responses of neurones located in laminae III–V demonstrated this feature of adaptation to controlled cutaneous stimuli. In such cases, stimuli could be repeatedly evoked at 3 min intervals for over 2 h.

A variety of innocuous stimuli (including brushing, gentle squeeze or tap) completely failed to evoke any response from 15 out of 27 lamina I neurones, including 10 SMT neurones. These neurones, however, consistently responded to noxious cutaneous stimuli and were thus classifed as nocispecific.

Both nocispecific and multireceptive neurones in lamina I showed a characteristic lack or minimal level (< 2 Hz) of spontaneous background activity. These neuronal characteristics were seen in both SMT and other cells, κ agonists (U50488H or DYNO) had no effect at either the same (5–25 nA) or much higher (up to 100 nA) ejection currents, in 4 out of 5 neurones tested (Figure 1). In a single example, DYNO inhibited all evoked activity at ejection currents around 60 nA. In 8 neurones, multiple testing of different agonists was carried out, including 3 where μ, δ and κ agonists were all studied. All

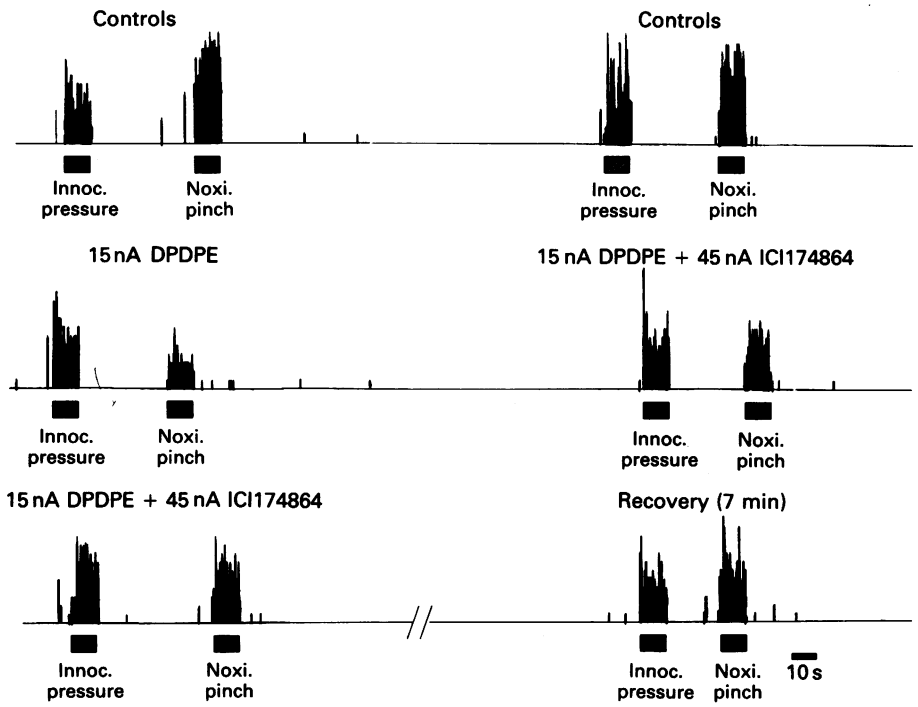

Figure 2 The typical effects of δ-receptor drugs on a multireceptive lamina I neurone. A continuous record is shown as ongoing firing frequency which is plotted as the number of action potentials per 400 ms bin against time. The three panels of firing frequency record follow on directly from each other in descending order. Initial excitatory control responses are shown to innocuous brush, and noxious pinch. A selective antinociceptive effect of [D-Pen$_2$,D-Pen$_5$]enkephalin (DPDPE) developed rapidly (ejection began 20 s after termination of the second control pinch stimulus) and this inhibition of the nociceptive response was prevented by the additional ionophoresis of the δ-antagonist, ICI 174864 (ejection began again 20 s after the preceding pinch stimulus).

such results were consistent with the overall conclusion that μ and/or δ agonists would suppress nociceptive inputs to any individual neurone, whereas κ agonists were ineffective.

Effects of opioids applied in the vicinity of nocispecific neurones located in lamina I

A total of 15 cells in lamina I were classified as nocispecific, responding only to noxious cutaneous stimuli and ionophoresed DLH, including 10 neurones antidromically identified as SMT cells. In contrast to the consistent selective antinociceptive effects on multireceptive lamina I neurones described above, the δ agonist DPDPE had 3 different actions on nocispecific cells (Table 1 and Figure 3). DPDPE selectively inhibited the nociceptive responses of 5 out of 15 neurones, whilst the DLH-evoked responses were unaffected, with ejection currents of up to 25 nA (Figure 3a). In one neurone of this type for which recovery was closely examined, the substantial inhibition of nociceptive responses due to DPDPE was reversed to more than 90% of control levels 5 min after stopping ejection of DPDPE. With the same test protocol both the nociceptive and DLH-evoked responses were inhibited to a similar degree in 7 out of 15 cells (Table 1 and Figure 3c). In the 3 of these neurones for which recovery was examined, the DPDPE-induced suppression of evoked responses had recovered to near control values [for example 102 ± 12% (DLH) and 87 ± 9% (pinch)] by 6 ± 1 min after stopping ejection of DPDPE. Both of these actions of DPDPE were reversed by ionophoresis of the δ antagonist, ICI 174868 (20–70 nA). In all 3 of the selectively affected neurones that were tested with the δ antagonist (including 2 SMT cells), the substantial inhibitions of nociceptive responses caused by 10–15 nA DPDPE (Figure 3a) were readily reversed by ICI 174864. Pinch responses, for example, were restored to 92 ± 9% of controls by 20–60 nA ICI 174864 applied for 4 ± 1 min (n = 3). In all 4 of the non-selectively affected neurones that were tested with the δ antagonist (including 2 SMT cells), the effects of DPDPE were also readily reversed by ICI 174864. For example, the suppressed responses to DLH and to noxious pinch were reversed to 88 ± 12% and 85 ± 3% of controls by 30–70 nA ICI 174864 applied for 5 ± 1 min (n = 4). No effect of ICI 174864 alone was ever apparent. DPDPE failed to alter the responses of the remaining 3 neurones at either similar (5–20 nA) (Figure 3b) or in 2 of these cases, even at much higher ejection currents (up to 100 nA) (not shown). The κ agonists U50488H and DYNO failed to cause any change in responses tested (5–15 nA) (Figure 4) or even at up to 40 nA (not shown). However, in all 9 cells tested, including 5 SMT cells, the selective μ agonist, DAMGO inhib-

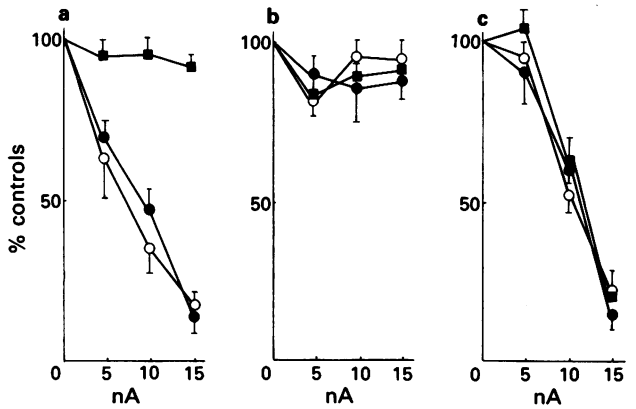

Figure 3 The different effects of the δ-agonist [D-Pen$_2$,D-Pen$_5$]enkephalin (DPDPE) ionophoresed in the vicinity of nocispecific lamina I neurones. Responses were integrated and expressed as a percentage of control levels. Mean values from samples of neurones (n indicated) are shown; s.e.mean shown by vertical bar. Responses were evoked by noxious heat (●), noxious pinch (○) and D,L-homocysteic acid (DLH) (■). In (a) are shown cases (n = 5) in which DPDPE inhibited only the nociceptive responses (DLH-evoked activity was unaffected). In some cells (n = 3) DPDPE had no effect on levels of any evoked activity (b). However, the predominant action of DPDPE is shown in (c) (in a total of 7 out of 15 neurones tested) in which all types of evoked activity (including DLH) were inhibited to a similar degree.

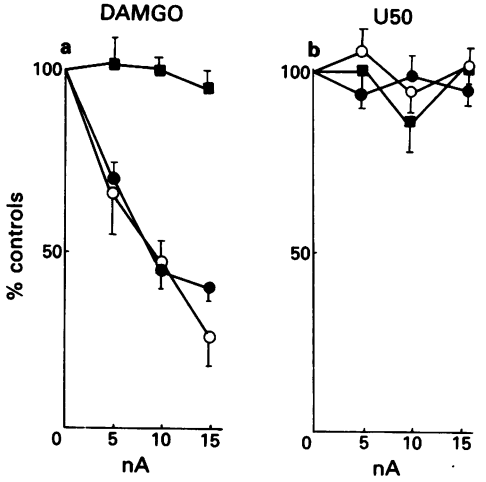

Figure 4 The effects of μ- and κ-selective agonists ionophoresed in the vicinity of nocispecific lamina I neurones. Responses were integrated and expressed as a percentage of control levels. Mean values from samples of neurones (n indicated) are shown; s.e.mean shown by vertical bars. Responses were evoked by noxious heat (●), noxious pinch (○) and (DLH) (■). (a) The μ agonist, [D-Ala$_2$,MePhe$_4$,Gly-ol]enkephalin (DAMGO), inhibited only nociceptive responses, leaving DLH-evoked activity unaffected (n = 9). (b) In contrast the κ agonist, U50488H (U50), failed to alter the levels of any of the evoked activity, in all neurones tested (n = 3).

ited only nociceptive responses (Figure 4), leaving DLH- or glutamate-evoked responses quite unaffected (5–25 nA).

Effects of opioids applied in the vicinity of multireceptive neurones located in laminae III–V

On six multireceptive neurones located in the deeper dorsal horn (laminae III–V) neither the μ (DAMGO) nor the δ (DPDPE) agonists caused selective antinociceptive effects (Figure 5), such as were apparent for lamina I neurones. However, the κ agonists (DYNO or U50488H) typically caused a selective antinociceptive effect on the pinch response of all neurones tested, as shown in Figure 5. On 2 neurones

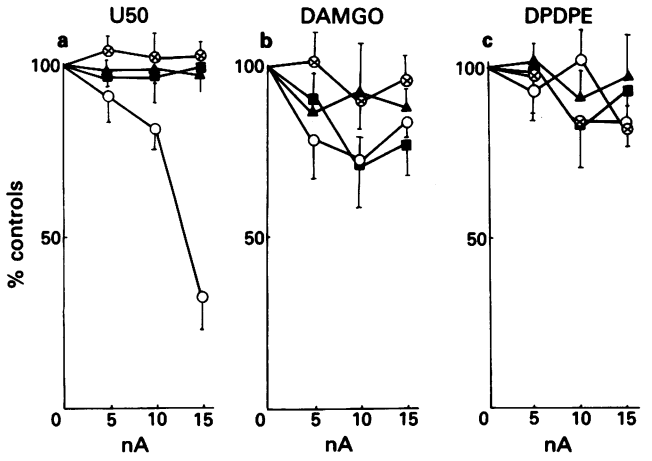

Figure 5 The effects of κ-, μ- and δ-selective agonists ionophoresed in the vicinity of multireceptive laminae III–V neurones. Responses were integrated and expressed as a percentage of control levels. Mean values from samples of neurones (n indicated) are shown; vertical bars show s.e.mean. Responses were evoked by innocuous brush (▲), noxious pinch (○), and D,L-homocysteic acid (DLH) (■). The levels of spontaneous background activity (⊗) were also calculated. (a) The κ agonist, U50488H (U50), consistently caused selective inhibition of nociceptive responses (leaving DLH-, innocuous and spontaneous activity unaffected) in all neurones tested (n = 4). In contrast, (b and c) neither the μ-agonist [D-Ala$_2$,MePhe$_4$,Gly-ol]enkephalin (DAMGO) nor the δ-agonist [D-Pen$_2$,D-Pen$_5$]enkephalin (DPDPE), altered evoked or spontaneous levels of activity (n = 3 and 5 respectively).

tested, U50488H also selectively inhibited the nociceptive heat response. Three of the 4 neurones tested with U50488H were examined for recovery. The substantial inhibition of pinch responses caused by 15 nA U50488H had recovered to 83 ± 10% of control values in 12 ± 3 min after stopping ionophoresis of the drug. No further studies were carried out on these neurones, but when examining the same phenomenon in cat laminae III–V neurones, reversal by naloxone and inactivity of [des-Tyr]$_1$-dynorphin A$_{1-13}$ were seen (Fleetwood-Walker *et al.*, 1988). Ionophoresis of either naloxone or ICI 174864 alone had no apparent effect on the evoked responses tested in lamina I or laminae III–V. No clear differences in the profile of drug actions could be discerned between antidromically-identified SMT neurones and unidentified nocispecific neurones, with examples of SMT cells showing each of the profiles of DPDPE action and the typical responses to μ and κ agonists. The administration of antagonists by other routes was not tested.

Discussion

A variety of antinociceptive actions are mediated by μ, δ and κ opioid agonists. As in the cat (Fleetwood-Walker *et al.*, 1988), the antinociceptive action of κ agonists was exerted here on both the thermal and mechanical nociceptive responses of laminae III–V neurones to an apparently similar degree. Systemic or intrathecal administration of selective κ agonists are most effective against visceral and mechanical nociception (Hayes *et al.*, 1983; Schmauss & Yaksh, 1984) but there is little margin between doses causing antinociception and motor dysfunction or paralysis (see Millan, 1990 for review). In the present study, the range over which opioids could act was considerably restricted by virtue of the experimental design, although some diffusion of ionophoresed drugs must occur. The excitatory effects observed by Knox & Dickenson (1987) may have involved actions of intrathecally administered κ agonists at various sites remote to the dorsal horn neurone being recorded. In our studies, the sites mediating such excitation may have been beyond the reach of ionophoresed κ agonists.

Selective inhibition of nociceptive responses, without affecting innocuous, DLH-evoked or spontaneous activity, indicates that inhibitory influences may be exerted specifically on the nociceptive pathway, rather than generally on any polysynaptic input or directly on the neurones being tested, either in laminae III–V and lamina I. For example, κ agonists may inhibit nociceptive transmission to the deeper dorsal horn neurone being tested, via a closely antecedent interneurone, rather than by action on the nociceptive afferent fibres which predominantly terminate in the superficial dorsal horn (Cervero & Iggo, 1980). In the case of κ agonists, these proposed interneurones are unlikely to be located as far dorsal as the region of the substantia gelatinosa, because Fleetwood-Walker *et al.* (1988) reported that ionophoresis of κ agonists there failed to influence neurones located in laminae III–V. Similarly, the quite different profiles of drug action when applied in superficial laminae rather than laminae III–V suggest that drugs did not readily diffuse between the two. The majority (but not all) of the κ receptors are present in superficial laminae (Slater & Patel, 1983; Morris & Herz, 1987) as are the endogenous κ agonists (Botticelli *et al.*, 1981). At first sight there appears to be a mismatch between structure and function. However, the superficial dynorphin-related peptides may have other roles than the modulation of acute cutaneous nociceptive inputs surveyed in the present study.

In contrast to the lack of action in the vicinity of deeper dorsal horn neurones, μ and δ receptor selective agonists often had profound influences on both nocispecific and multireceptive lamina I neurones. Autoradiographic studies have revealed μ opioid binding sites correspondingly concentrated in the superficial dorsal horn (laminae I and II) (Atweh & Kuhar, 1977; Traynor *et al.*, 1982; Mack *et al.*, 1984; Dash-

wood et al., 1985; Morris & Herz, 1987) and μ agonists can exert an antinociceptive influence on deeper dorsal horn neurones, via the substantia gelatinosa, in the cat (Fleetwood-Walker et al., 1988). It is possible that the actions of μ agonists ionophoresed in the vicinity of lamina I neurones in this study may exert their influence either via lamina I or via the adjacent substantia gelatinosa. Diffusion over such short distances is very likely. Whether such actions are mediated by effects on primary afferent terminals (Lamotte et al., 1976; Fields et al., 1980) or interneurones of the substantia gelatinosa (Yoshimura & North, 1983), is not clear. The non-selective effects described may be due to actions either on antecedent interneurones or afferent terminals, in both the non-nociceptive pathways, or directly on the neurone being tested.

A recent autoradiographic study by Morris & Herz (1987) found δ binding sites restricted to lamina I of the dorsal horn of the rat. This evidence is in accordance with our findings that δ agonists failed to influence somatosensory inputs to deeper dorsal horn neurones (laminae III–V) when either applied nearby these cells (in the rat, this study; in the cat, Fleetwood-Walker et al., 1988) or in the substantia gelatinosa dorsal to the cells being recorded (Fleetwood-Walker et al., 1988). The use of the selective δ antagonist, ICI 174864, confirmed the involvement of δ receptors in the modulation of somatosensory inputs to both multireceptive and nocispecific neurones in lamina I. Although the terminal excitability of primary afferent Aδ- and C-fibres is reduced by opiate derivatives active at both μ and δ sites (Carstens et al., 1979; Hentall & Fields, 1983) there must be some differential action of μ and δ agonists to account for the different effects caused by DAMGO and DPDPE on nocispecific lamina I neurones. Whilst DAMGO consistently inhibited only nociceptive responses, leaving non-nociceptive activity unaffected in multireceptive and nocispecific cells, DPDPE also regularly caused inhibition of both noxious and DLH-evoked responses to nocispecific cells. It therefore seems that there is not an ubiquitous inhibitory mechanism mediated by δ-receptors. The variety of both selective and non-selective actions of δ agonists indicates a complex and perhaps important role for δ-receptor sites, restricted to lamina I (Morris & Herz, 1987) in the modulation of nociceptive transmission through the superficial dorsal horn and on to supraspinal sites.

References

ATWEH, S.A. & KUHAR, M.J. (1977). Autoradiographic localisation of opiate receptors in rat brain. I. Spinal cord and lower medulla. *Brain Res.*, **124**, 53–67.

BEHAR, M., DAVIDSON, J.T., MAGORA, F. & OLSHWANG, D. (1979). Epidural morphine in treatment of pain. *Lancet*, **i**, 527–529.

BELCHER, G. & RYALL, R.W. (1978). Differential excitatory and inhibitory effects of opiates on non-nociceptive and nociceptive neurones in the spinal cord of the cat. *Brain Res.*, **141**, 303–314.

BOTTICELLI, L.J., COX, B.M. & GOLDSTEIN, A. (1981). Immunoreactive dynorphin in mammalian spinal cord and dorsal root ganglia. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 7783–7786.

CALVILLO, O., HENRY, J.L. & NEUMAN, R.S. (1974). Effects of morphine and naloxone on dorsal horn neurones in the cat. *Can. J. Physiol. Pharmacol.*, **52**, 1207–1211.

CARSTENS, E., TULLOCH, I., ZIEGLGANSBERGER, W. & ZIMMERMAN, M. (1979). Presynaptic excitability changes induced by morphine in single cutaneous afferent A and C-fibers. *Pflugers Arch. Gen. Physiol.*, **379**, 142–147.

CERVERO, F. & IGGO, A. (1980). The substantia gelatinosa of the spinal cord. A critical review. *Brain*, **103**, 717–772.

CORBETT, A.D., GILLAN, M.G.C., KOSTERLITZ, H.W., McKNIGHT, A.T., PATERSON, S.J. & ROBSON, L.E. (1984). Selectivity of opioid peptide analogues as agonists and antagonists at the δ receptor. *Br. J. Pharmacol.*, **83**, 271–279.

DASHWOOD, M.R., FLEETWOOD-WALKER, S.M., GILBEY, M.P., MITCHELL, R. & SPYER, K.M. (1985). Distribution of α-adrenergic, muscarinic and opiate receptors within lumber spinal cord of cat. *J. Physiol.*, **362**, 42P.

DAVIES, J. & DRAY, A. (1978). Pharmacological and electrophysiological studies of morphine and enkephalin on rat supraspinal neurones and cat spinal neurones. *Br. J. Pharmacol.*, **63**, 87–96.

DOSTROVSKY, J.O. & POMERANZ, B. (1976). Interaction of ionophoretically applied morphine with responses of interneurons in cat spinal cord. *Exp. Neurol.*, **52**, 325–338.

DUGGAN, A.W., HALL, J.G. & HEADLEY, P.M. (1977). Suppression of transmission of nociceptive impulses by morphine: selective effects of morphine administered in the region of the substantia gelatinosa. *Br. J. Pharmacol.*, **61**, 65–76.

DUGGAN, A.W. & NORTH, R.A. (1984). Electrophysiology of opioids. *Pharmacol. Rev.*, **35**, 219–282.

FIELDS, H.L., EMSON, P.C., LEIGH, B.K., GILBERT, R.F.T. & IVERSEN, L.L. (1980). Multiple opiate receptor sites on primary afferent fibres. *Nature*, **284**, 351–353.

FLEETWOOD-WALKER, S.M., HOPE, P.J. & MITCHELL, R. (1986). Delta opioids exert antinociceptive effects in lamina I, but not laminae III-V of rat dorsal horn. *J. Physiol.*, **382**, 152P.

FLEETWOOD-WALKER, S.M., HOPE, P.J., MITCHELL, R., EL-YASSIR, N. & MOLONY, V. (1988). The influence of opioid receptor subtypes on the processing of nociceptive inputs to the spinal cord of the cat. *Brain Res.*, **451**, 213–226.

HAYES, A.G., HILL, R.G., SKINGLE, M. & TYERS, M.B. (1983). Further investigations into the properties of agonists for the κ opiate receptor. *Br. J. Pharmacol.*, **80**, 47P.

HENTALL, I.D. & FIELDS, H.L. (1983). Actions of opiates, substance P and serotonin on the excitability of primary afferent terminals and observations on interneuronal activity in the neonatal rat's dorsal horn in vitro. *Neurosci.*, **9**, 521–528.

HILL, R.G., HUGHES, J., LEIGHTON, G. & RODRIGUEZ, R.E. (1987). Behavioural effects of [D-Pen^2, D-Pen^5]-enkephalin and ICI 174,864 after intrathecal injection in the rat. *J. Physiol.*, **382**, 154P.

HÖKFELT, T., LUNDBERG, J.M., TERENIUS, L., ELDE, R.P. & NILSSON, G. (1977). Immunohistochemical analysis of peptide pathways possibly related to pain and analgesia: enkephalin and substance P. *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 3081–3085.

HOPE, P.J., FLEETWOOD-WALKER, S.M. & MITCHELL, R. (1987). The antinociceptive actions of opioids on lamina I neurons of the dorsal horn. *Pain Suppl.*, **4**, S409.

HYLDEN, J.L.K., HAYASHI, H., DUBNER, R. & BENNETT, G.J. (1986). Physiology and morphology of the lamina I spinomescephalic projection. *J. Comp. Neurol.*, **247**, 505–515.

KNOX, R.J. & DICKENSON, A.H. (1987). Effects of selective and non-selective κ-opioid receptor agonists on cutaneous C-fibre-evoked responses of rat dorsal horn neurones. *Brain Res.*, **415**, 21–29.

LAMOTTE, C., PERT, C.B. & SNYDER, S.H. (1976). Opiate receptor binding in primate spinal cord: distribution and changes after dorsal root section. *Brain Res.*, **112**, 407–412.

LIPSKI, J. (1981). Antidromic activation of neurons as an analytical tool in the study of the central nervous system. *J. Neurosci. Methods*, **4**, 1–32.

MACK, K.J., KILLIAN, A. & WEYHENMEYER, J.A. (1984). Comparison of mu, delta and kappa opiate binding sites in rat brain and spinal cord. *Life Sci.*, **34**, 281–285.

McMAHON, S.B. & WALL, P.D. (1983). A system of rat spinal cord lamina I cells projecting through the contralateral dorsolateral funiculus. *J. Comp. Neurology*, **214**, 217–233.

MANSOUR, A., KHACHATURIAN, H., LEWIS, M.E., AKIL, H. & WATSON, S.J. (1988). Anatomy of CNS opioid receptors. *Trends Neurosci.*, **11**, 309–314.

MENÉTREY, C., GIESLER, G.J. & BESSON, J.-M. (1977). An analysis of response properties of spinal cord dorsal horn neurons to non-noxious and noxious stimuli in the spinal rat. *Exp. Brain Res.*, **27**, 15–33.

MILLAN, M.J. (1990). κ opioids and analgesia. *Trends Pharmacol. Sci.*, **11**, 70–76.

MORRIS, R.J. & HERZ, A. (1987). Distinct distribution of opioid receptor types in rat lumbar spinal cord. *Naunyn Schmiedebergs Arch. Pharmacol.*, **336**, 240–243.

ONOFRIO, B.M. & YAKSH, T.L. (1983). Intrathecal delta-receptor ligand produces analgesia in man. *Lancet*, **i**, 1386–1387.

PAXINOS, G. & WATSON, C. (1986). *The Rat Brain in Stereotaxic Coordinates*, 2nd Edition. New York: Academic Press.

PIERCEY, M.F., EINSPAHR, F.J., DOBRY, P.J.K., SCHROEDER, L.A. & HOLLISTER, R.P. (1980). Morphine does not antagonise the substance P mediated excitation of dorsal horn neurons. *Brain Res.*, **186**, 421–434.

SASTRY, B.R. & GOH, J.W. (1983). Actions of morphine and met-

enkephalinamide on nociceptor driven neurones in substantia gelatinosa and deeper dorsal horn. *Neuropharmacol.*, **22**, 119–122.

SCHMAUSS, C. & YAKSH, T.L. (1984). In vivo studies on spinal opiate receptor systems mediating antinociception. II. Pharmacological profiles suggesting a differential association of mu, delta and kappa receptors with visceral, chemical and cutaneous thermal stimuli in the rat. *J. Pharmacol. Exp. Ther.*, **228**, 1–12.

SCHOUENBOURG, J. & SJOLUND, B.H. (1983). Activity evoked by A- and C-afferent fibres in rat dorsal horn neurons and its relation to the flexion reflex. *J. Neurophysiol.*, **50**, 1108–1121.

SLATER, P. & PATEL, S. (1983). Autoradiographic localisation of opiate κ receptors in the rat spinal cord. *Eur. J. Pharmacol.*, **92**, 159–160.

TRAYNOR, J.R., KELLY, P.D. & RANCE, M.J. (1982). Multiple opiate binding sites in rat spinal cord. *Life Sci.*, **31**, 1377–1380.

WANG, J.K., NAUSS, L.A. & THOMAS, J.A. (1979). Pain relief by intrathecally applied morphine in humans. *Anaesthesiol.*, **50**, 149–151.

YAKSH, T.L. (1983). In vivo studies on spinal opiate receptor systems mediating antinociception. I. Mu and delta profiles in the primate. *Exp. Ther.*, **226**, 303–316.

YOSHIMURA, M. & NORTH, R.A. (1983). Substantia gelatinosa neurones in vitro hyperpolarised by enkephalin. *Nature*, **305**, 529–530.

ZIEGLGANSBERGER, W. & BAYERL, H. (1976). The mechanisms of inhibition of neuronal activity by opiates in the spinal cord of the cat. *Brain Res.*, **115**, 111–128.

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The anti-adrenergic effect of adenosine and its blockade by pertussus toxin: a comparative study in myocytes isolated from guinea-pig, rat and failing human hearts

[1]L.A. Brown, S.M. Humphrey & S.E. Harding

Department of Cardiac Medicine, National Heart & Lung Institute, Dovehouse St, London SW3 6LY

1 In intact ventricular preparations, adenosine has been shown to reduce the β-adrenoceptor-induced increase in contraction (the anti-adrenergic effect). In the present study we have investigated this effect of adenosine on isolated ventricular myocytes from failing human heart and normal guinea-pig and rat heart.

2 Adenosine in the absence of β-adrenoceptor-mediated stimulation had no effect on contraction in human and guinea-pig myocytes but produced a variable effect in rat myocytes.

3 8-Cyclopentyl 1,3-dipropylxanthine (CPX), a selective A_1-receptor antagonist, antagonised the anti-adrenergic effect of adenosine in guinea-pig myocytes.

4 The anti-adrenergic effect of adenosine was greater in guinea-pig than rat myocytes and even more pronounced in cells isolated from failing human heart.

5 Pertussis toxin-pretreatment at 35°C of guinea-pig and human myocytes abolished the anti-adrenergic effect of adenosine. Longer exposure to higher concentrations of pertussis toxin was required for complete abolition in human compared to guinea-pig cells.

6 These results support the suggestion that the adenosine receptors mediating the anti-adrenergic effect of adenosine are of the A_1 subtype and are coupled to the inhibitory guanine nucleotide binding protein, G_i/G_o.

7 Pertussis toxin pretreatment increased the sensitivity of guinea-pig myocytes to isoprenaline in the absence of adenosine; the EC_{50} value was decreased by a factor of 10. This suggests that G_i may exert a tonic inhibitory effect on the β-adrenoceptor/adenylate cyclase interaction in normal myocardium.

Introduction

The anti-adrenergic effect of adenosine in the myocardium was first demonstrated by Schrader and co-workers in 1977 on isolated perfused whole hearts of the guinea-pig. It has since been demonstrated in many preparations, including papillary muscles (Hopwood *et al.*, 1987) and isolated ventricular myocytes (Belardinelli & Isenberg, 1983), and is often referred to as an 'indirect' effect of adenosine (Bohm *et al.*, 1989b). Adenosine is thought to exert a negative inotropic effect by attenuation of the accumulation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) which follows catecholamine-induced activation of adenylate cyclase (Schrader *et al.*, 1977; Dobson, 1978). Catecholamine stimulation of adenylate cyclase via β-adrenoceptors is believed to involve a complex transduction-coupling process composed of agonist, receptor, a stimulatory guanine nucleotide protein (G_s) and adenylate cyclase (Gilman, 1987). Exactly how adenosine attenuates the adenylate cyclase activation by catecholamines in the heart is still unknown. Evidence for the linking of the adenosine receptor which is involved in the anti-adrenergic effect, to adenylate cyclase, possibly via the inhibitory guanine nucleotide binding protein (G_i), has been provided for the rat (Hazeki & Ui, 1981), the guinea-pig (Bohm *et al.*, 1986) and recently for diseased human heart (Bohm *et al.*, 1989a,b). The pathophysiological role of this effect of adenosine in the heart is not well established, but it is of particular interest in the failing human heart where levels of a 40 KDa pertussis toxin-sensitive substrate, presumably G_i/G_o, are raised (Feldman *et al.*, 1989). An increase in G_i/G_o could augment the anti-adrenergic effect of adenosine, resulting in a more pronounced decrease in β-adrenoceptor effects when adenosine levels are high. Adenosine levels in the pericardial space have been found to be raised in heart failure (Newman *et al.*, 1984), therefore, the increased effectiveness of adenosine could well contribute to the observed subsensitivity to β-agonists in these patients (Bristow *et al.*, 1982; Brown *et al.*, 1986; Bohm *et al.*, 1988).

Isolated myocytes are a useful model for the study of the mechanical performance of heart muscle as their contractile response may be observed free of neural and humoral influences. The presence of adenosine deaminase in conjunction with a deaminase resistant analogue is necessary when studying the effects of adenosine in whole muscle (Hopwood *et al.*, 1987). However, endogenous adenosine is not a problem when using isolated superfused myocytes. Previous work on the effects of adenosine in isolated cardiac myocytes has mainly involved cells isolated from rat (Hazeki & Ui, 1981; Martens *et al.*, 1987; Henrich *et al.*, 1987), guinea-pig (Belardinelli & Isenberg, 1983) and bovine (Belardinelli & Isenberg, 1983) hearts but not myocytes isolated from human heart tissue. This earlier work has characterized the adenosine receptor mediating the anti-adrenergic effect of adenosine as being of the A_1 subtype. Hazeki & Ui studied the effects of pertussis toxin on rat myocytes and determined that it blocked the adenosine inhibition of catecholamine-induced increase in cyclic AMP (Hazeki & Ui, 1981). Isolated myocytes are especially useful for studies involving pertussis toxin as they can be treated directly, unlike muscle preparations which must be isolated from animals previously injected with the toxin. Since it is impossible to treat patients with toxin the only studies to date involving human heart and pertussis toxin have been carried out on membrane preparations (Bohm *et al.*, 1989a).

In this present study we have investigated and characterised the anti-adrenergic effect of adenosine on myocytes isolated from failing human hearts and compared them with those isolated from normal guinea-pig and rat hearts.

Methods

Single isolated myocytes were prepared from the ventricle of adult guinea-pig, rat and human hearts by enzymatic dissociation as previously described (Harding *et al.*, 1988; 1990).

[1] Author for correspondence.

Guinea-pig myocytes were prepared by a modified perfusion method (Harding *et al.*, 1988). The hearts were mounted on a Langendorff apparatus and equilibrated with Krebs-Henseleit solution of the following composition (in mM): NaCl 119, KCl 4.2, $MgSO_4$ 0.94, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.5, bubbled with 95% O_2 + 5% CO_2, pH 7.4 containing 1 mM calcium for 5 min at 37°C. Low calcium medium (in mM): NaCl 120, KCl 5.4, $MgSO_4$ 5, pyruvate 5, glucose 20, taurine 20, HEPES 10, bubbled with 100% O_2, pH 6.95, containing 12–15 μM calcium was then perfused through for 5 min at 37°C. This was followed by a 2 min perfusion with the same low calcium solution with 200 μM calcium and 4 u ml^{-1} (Sigma) type XXIV protease added. The solution was then changed to one containing no added protease but 0.3 mg ml^{-1} (Worthington) collagenase and 0.6 mg ml^{-1} (Sigma) hyaluronidase for a further 10 min. The ventricles were then carefully removed from the cannula, chopped and incubated in the same enzyme solution for 2 × 5 min. At the end of each 5 min period the solution containing the dispersed cells was filtered through a 300 μm gauze and centrifuged at 40*g* for 1 to 2 min. The cells were washed twice by centrifugation in Krebs-Henseleit solution and resuspended in the same medium.

Rat myocytes were prepared by a perfusion method (Harding *et al.*, 1988) which is similar to that of the guinea-pig but does not use protease type XXIV.

Human ventricular tissue was obtained from explanted hearts at the time of heart transplantation, the patients (7 male, 2 female, age range 23–63 years) were in end-stage heart failure. Six had ischaemic heart disease, two congenital abnormalities and one primary pulmonary hypertension. Tissue from the left or right ventricle was transported in cold cardioplegic solution (Tomlins *et al.*, 1986), total transfer time to the laboratory was 1–2 h. Pieces of ventricle, chopped quickly into chunks of approximately 1 mm^3 by use of an array of razor blades, were incubated at 35°C in 25–50 ml of low calcium medium (free Ca^{2+} concentration of 1–2 μM) for 3 × 4 min. The chunks were then transferred to 10 ml of the above solution, with nitrilotriacetic acid omitted and 4 u ml^{-1} (Sigma) type XXIV protease and 30 μM calcium added, and incubated again at 35°C for 45 min. This incubation was repeated for a further 2 × 45 min with the protease omitted and 400 iu ml^{-1} (BCL) collagenase added. The dispersed cells were then strained and centrifuged in the same way as those prepared by the perfusion method. It has been shown that the characteristics of cells prepared by either the perfusion or the chunk method are similar (Harding *et al.*, 1988).

Isolated cells were superfused with Krebs-Hensleit solution on the stage of an inverted microscope, all experiments were carried out at 32°C following preincubation at 22°C or at 35°C with or without pertussis toxin. The myocytes were electrically stimulated (human 0.2 Hz; guinea-pig and rat 0.5 Hz) and the contraction of a single myocyte monitored with a video camera and length detection system as previously described (Harding *et al.*, 1988). Cells used in all experiments were chosen by selection criteria involving sarcomere length and response to electrical stimulation (Harding *et al.*, 1988). Concentration-response curves (CRC) to isoprenaline and adenosine (in the presence of isoprenaline) were performed in a cumulative fashion. Calcium concentration-response curves were also cumulative and were performed in a phosphate-free Krebs-Henseleit solution to prevent precipitation at high concentrations of calcium (up to 25 mM). 8-Cyclopentyl 1,3-dipropylxanthine (CPX) was used as a selective A_1-receptor antagonist (Collis *et al.* 1989). Cells were exposed to CPX 15 min before the adenosine concentration-response curve was carried out. Isolated myocytes were incubated in pertussis toxin (5 μg ml^{-1} for human and 1 μg ml^{-1} for guinea-pig diluted with low calcium solution) at 35°C.

The concentration to produce half-maximal change (EC_{50}) was calculated by use of an iterative curve fitting programme. The % reduction induced by adenosine in myocytes contracting stably to isoprenaline was derived from the following equation; ((mean isoprenaline response − mean basal response) − (adenosine + isoprenaline − mean basal response))/(mean isoprenaline − mean basal response) × 100. The mean basal response was the average of the contraction amplitude before and after isoprenaline exposure. The mean isoprenaline response was the average of the contraction amplitude, in the presence of isoprenaline, before and after adenosine (see Figures 3 and 5).

The anti-adrenergic effect of adenosine on the guinea-pig and rat myocytes was tested by use of a concentration of isoprenaline which produced approximately 75% of its maximum. EC_{50} values for isoprenaline in guinea-pig and rat ventricular myocytes were 2.32 ± 0.34 nM ($n = 7$) and 2.27 ± 0.24 nM ($n = 6$), respectively. A final concentration of 3 nM isoprenaline was used to determine the anti-adrenergic effect of adenosine in these myocytes. For the ventricular myocytes isolated from failing human hearts it was not always possible to produce a response to 3 nM isoprenaline due to the extent of β-adrenoceptor desensitization in the tissue (Harding *et al.*, 1989). The EC_{50} values for isoprenaline and the isoprenaline/calcium response ratio (the maximum response to isoprenaline relative to that produced by the maximally stimluating concentration of calcium) for ventricular myocytes isolated from failing human heart have been determined to be an indication of the severity of the heart failure (Harding *et al.*, 1989). Therefore when the anti-adrenergic effect was studied in the human myocytes, an isoprenaline concentration-response curve was carried out initially and the appropriate isoprenaline concentration for each cell used to test the effects of adenosine.

Statistical significance was assessed by Student's *t* test. Statistical values quoted throughout are mean ± s.e.mean and *n* values are for numbers of animals or explanted hearts, with results being pooled from 1–5 cells for each heart. For EC_{50} values, *t* tests were performed on the log transformed values.

Compounds used

Adenosine and (−)-isoprenaline hydrochloride were obtained from Sigma. 8-Cyclopentyl 1,3-dipropylxanthine (CPX) was obtained from Research Biochemicals Inc and was dissolved in Krebs-Henseleit solution pH 8.0. Pertussis toxin, derived from *Bordetella pertussis*, was obtained from Porton Products Ltd.

Results

Adenosine had no effect on the basal contraction amplitudes (% change in cell length) of the guinea-pig myocytes or cells isolated from failing human heart, the contraction amplitude remaining at 3.7% cell shortening ($n = 7$) and 2.2% cell shortening ($n = 4$) respectively during the addition of 10 μM adenosine. These same cells had previously responded to a maximally stimulating concentration of calcium. In rat myocytes the effect of 10 μM adenosine alone was variable, with 7 out of 14 cells ($n = 11$ animals) responding with a slight increase in contraction which was not readily reversible. The basal contraction amplitude was raised from 2.13 ± 0.4% cell shortening to 3.8 ± 0.56% cell shortening in the presence of 10 μM adenosine. With different cells the contraction amplitude was increased to 5.63 ± 1.4% cell shortening ($n = 3$) when a concentration of 20 μM adenosine was added.

Concentration-response curves (CRC) for isoprenaline in the presence of 10 μM adenosine were shifted to the right compared to control isoprenaline CRC in both guinea-pig (Figure 1) and human myocytes. In the guinea-pig, EC_{50} values were significantly increased from 2.02 ± 0.39 to 5.57 ± 0.73 nM isoprenaline in the presence of 10 μM adenosine ($P < 0.002$, $n = 7$). In myocytes isolated from failing human ventricle 10 μM adenosine increased the EC_{50} values for isoprenaline from 1.69 ± 0.31 to 21.5 ± 6.5 nM ($n = 2$). Adenosine (100 μM) did not produce any further significant shift of the curve to the

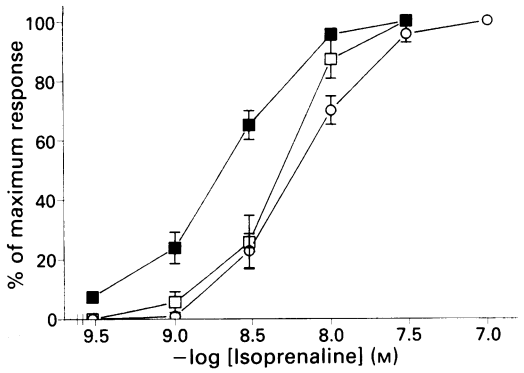

Figure 1 The effect of adenosine on the concentration-response curve to isoprenaline in isolated guinea-pig ventricular myocytes. (■) Control, $n = 14$; (□) plus 10 μM adenosine, $n = 7$; (○) plus 100 μM adenosine, $n = 7$. Responses are expressed as a % of the maximum response obtained to isoprenaline; vertical lines show s.e.mean.

right in guinea-pig or human myocytes. Hence, 10 μM adenosine was used in all further experiments.

CPX antagonized the negative inotropic effect of adenosine on guinea-pig myocytes which were contracting stably to 3 nM isoprenaline. At a final concentration of 3.5 nM, CPX caused a significant displacement of the adenosine CRC to the right (Figure 2). The EC_{50} value increased from 1.13 ± 0.18 μM to 10.40 ± 5.12 μM adenosine ($P < 0.001$, $n = 6$). CPX had no effects upon the basal or the isoprenaline-stimulated contractions of the guinea-pig myocytes in the absence of adenosine ($n = 6$, data not shown).

Figure 3 shows a typical trace of the anti-adrenergic effect of adenosine on a human ventricular myocyte. The same experimental protocol shown in this figure was used for all the cells when the negative inotropic effect of adenosine was being studied. A value for the % reduction induced by adenosine was derived from the mean isoprenaline response minus the adenosine response both of which had previously been corrected for basal contractions (see Methods). The attenuation produced by 10 μM adenosine in myocytes contracting stably to isoprenaline was greater in guinea-pig than in rat myocytes ($P < 0.01$) and even more pronounced in cells from failing human hearts ($P < 0.001$ cf rat, and $P < 0.01$ cf guinea-pig) (Figure 4).

Myocytes were incubated with pertussis toxin at 35°C to accelerate the reaction. Experiments were then carried out at 32°C. Comparisons were made with myocytes which had been kept at room temperature (22°C) during this period, as well as ones which had been incubated at 35°C in the absence of pertussis toxin. Preincubation at 35°C alone did not affect the anti-adrenergic effect of adenosine in guinea-pig or human myocytes ($n = 11$, $n = 5$ respectively, data not shown).

Incubation of guinea-pig myocytes with 1 μg ml^{-1} pertussis toxin at 35°C for a minimum of 2 h abolished the anti-adrenergic effect of 10 μM adenosine (Figure 5a and b). After pertussis toxin treatment, addition of adenosine did not reduce the contraction amplitude of isoprenaline-stimulated cells. Adenosine 10 μM alone had no effect on the basal contraction amplitudes of the guinea-pig myocytes following pertussis toxin pretreatment ($n = 5$, data not shown). Pretreatment of myocytes isolated from failing human heart with pertussis toxin, 5 μg ml^{-1} at 35°C for a minimum of 3 h, abolished the negative inotropic effect of 10 μM adenosine (Figure 5c and d). Initial experiments using a final concentration of 1 μg ml^{-1} pertussis toxin were unsuccessful in blocking

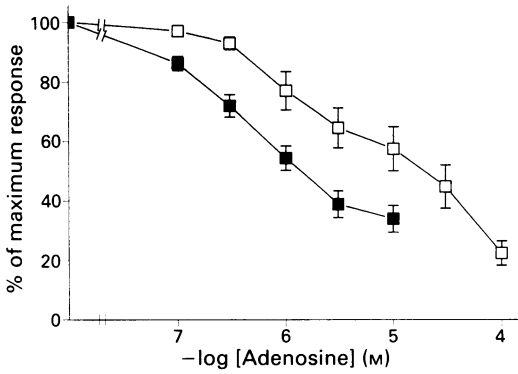

Figure 2 The effect of 8-cyclopentyl 1,3-dipropylxanthine (CPX) on the concentration-response curve to adenosine in guinea-pig isolated myocytes which were contracting stably to 3 nM isoprenaline. (■) Control, $n = 6$; (□) plus 3.5 nM CPX. $n = 6$. Responses are expressed as a % of the maximum response to 3 nM isoprenaline; vertical lines show s.e.mean.

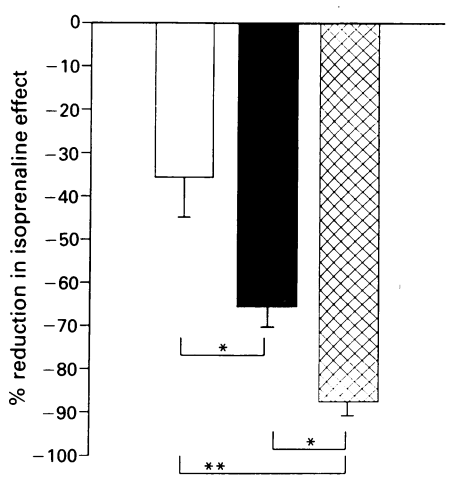

Figure 4 The attenuation produced by 10 μM adenosine in myocytes contracting stably to a given concentration of isoprenaline (see text). Open column, rat myocytes, $n = 7$; solid column, guinea-pig myocytes, $n = 13$; cross-hatched column, human myocytes $n = 9$. Responses are expressed as a % reduction in the mean isoprenaline response (see text); bars show s.e.mean. * $P < 0.01$, ** $P < 0.001$.

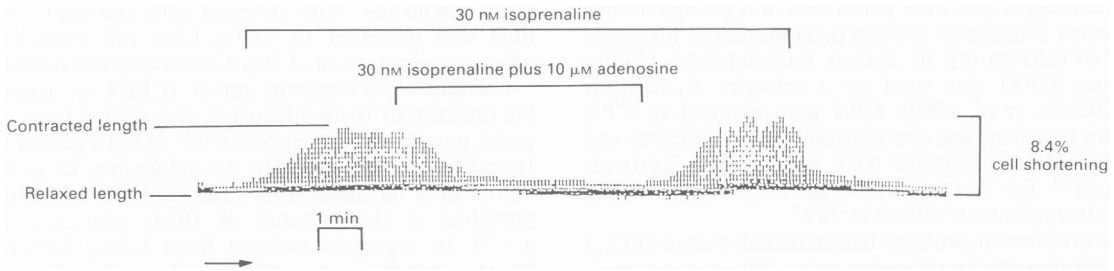

Figure 3 Typical trace recording of the contraction amplitude of a ventricular myocyte isolated from a failing human heart. The trace illustrates the anti-adrenergic effect of 10 μM adenosine on a cell contracting stably in 30 nM isoprenaline.

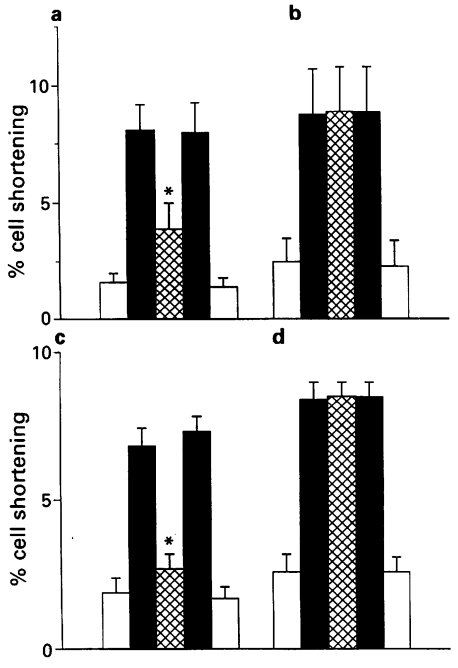

Figure 5 The blockade by pertussis toxin of the anti-adrenergic effect of 10 μM adenosine in isolated ventricular myocytes. Sequential measurements: open columns, basal contractions; solid columns, plus isoprenaline (3 nM); cross-hatched columns, plus isoprenaline and adenosine. (a) Guinea-pig isolated myocytes, control $n = 6$. (b) Guinea-pig isolated myocytes pretreated with 1 $\mu g\,ml^{-1}$ pertussis toxin, $n = 6$. (c) Human isolated myocytes, control $n = 9$. (d) Human isolated myocytes pretreated with 5 $\mu g\,ml^{-1}$ pertussis toxin, $n = 6$. Responses are expressed as % cell shortening. * $P < 0.001$ cf the mean isoprenaline response.

the anti-adrenergic effect of 10 μM adenosine in the human myocytes ($n = 3$, data not shown).

In the guinea-pig myocytes, pertussis toxin preincubation at 35°C significantly lowered the EC_{50} values for isoprenaline in the absence of adenosine, from 1.44 ± 0.25 nM to 0.15 ± 0.06 nM ($P < 0.001$ cf 22°C or 35°C control myocytes, $n = 8$), but did not affect the maximum contraction amplitude to isoprenaline. Responses to high concentrations of calcium were unaffected by pertussis toxin treatment.

Discussion

In this study we have demonstrated that the effects of adenosine on myocytes isolated from cardiac ventricular tissue are consistent with those on intact whole heart (Schrader *et al.*, 1977) or papillary muscle (Hopwood *et al.*, 1987).

In guinea-pig and human myocytes adenosine had no effect on basal contractile responses, even following pertussis toxin pretreatment, although these cells could increase their contraction amplitude in response to a high concentration of calcium. Adenosine had a slight 'direct' effect in rat ventricular myocytes, increasing their basal contractile responses. It is possible that rat ventricular myocytes may contain the stimulatory A_2 receptors which have previously been described in uncontaminated rat heart sarcolemma preparations (Anand Srivastrava, 1985). However, the positive inotropic effect of adenosine was not always reversible, which is not consistent with the known effects of A_2-receptor agonists.

CPX has been shown to exhibit selective A_1-receptor affinity in rat brain membranes (Bruns *et al.*, 1987) and, more recently, has been shown to be A_1-selective in guinea-pig isolated tissues (Collis *et al.*, 1989). In our experiments CPX produced an approximately 10 fold shift in the anti-adrenergic response of adenosine in guinea-pig myocytes at a concentration of 3.5 nM. This is strong evidence that adenosine is mediating its anti-adrenergic effect via A_1-receptors, as CPX has been shown to bind to A_1-receptors with a K_i of 0.46 nM and to A_2-receptors with a K_i of 340 nM (Bruns *et al.*, 1987). This is consistent with recent work on the receptor involved in the anti-adrenergic effect of adenosine; for example, Bohm and co-workers have shown that adenosine produces its 'indirect' effect in human papillary muscles via A_1-adenosine receptors (Bohm *et al.*, 1989b).

The anti-adrenergic effect or 'indirect' effect of adenosine was shown to be less pronounced in rat myocytes than in guinea-pig myocytes and greater in the myocytes isolated from failing human heart. This may be attributable to species differences in receptors or G_i levels, or to the effects of disease. The anti-adrenergic effect via A_1-receptors in rat ventricular myocytes may be partially masked by a concomitant activation of A_2-receptors. However, this cannot account for differences between human and guinea-pig myocytes. It has already been determined that there is an increase of a 40 KDa pertussis toxin substrate that is thought to be G_i in the failing human heart (Feldman *et al.*, 1989). This increase in G_i in heart failure may account for the more pronounced anti-adrenergic effect seen in human myocytes compared to those isolated from normal guinea-pig hearts. Comparisons of myocytes from normal and failing human myocardium will be required to determine whether differences are disease- or species-related.

Pertussis toxin specifically inactivates inhibitory G-proteins by ADP-ribosylation of a specific cysteine residue located four amino acids from their carboxyl termini (Sunyer *et al.*, 1989). Our experiments have shown that pertussis toxin pretreatment of ventricular myocytes from guinea-pigs and failing human hearts resulted in abolition of the anti-adrenergic effect of adenosine. This confirms the role of G_i as the signal transducer in the 'indirect' effect of adenosine in ventricular muscle. Myocytes isolated from failing human heart required a greater quantity of pertussis toxin to block completely the anti-adrenergic effect of adenosine than did myocytes from normal guinea-pig heart. This may be attributable to species variation or it may be due to the increased levels of G_i seen in heart failure.

In the guinea-pig myocytes the effects of pertussis toxin on the isoprenaline contractile responses in the absence of adenosine were accompanied by a shift in the isoprenaline CRC to the left, so that the myocytes were more sensitive to β-adrenoceptor stimulation. We assume that this is not due to inhibition of the action of endogenous adenosine by the pertussis toxin because the superfused isolated myocyte is unlikely to be contaminated by endogenous adenosine. In our experimental setup a small number of myocytes (total volume approximately 5 nl) are contained within a 200 μl bath, with 10 solution changes per minute. Any endogenous adenosine produced by these cells will be minimal and will be rapidly washed away. Therefore, it would appear that there is some tonic inhibitory effect of G_i on the isoprenaline response in guinea-pig cardiac muscle.

In summary, the present study shows that adenosine receptors exist in the rat, guinea-pig and human ventricle muscle. In both human and guinea-pig ventricles, these receptors mediate only 'indirect' effects, whereas in rat they mediate both 'direct' and 'indirect' responses. The anti-adrenergic effect of adenosine in guinea-pig and human myocytes is mediated by A_1-adenosine receptors which are coupled to the inhibitory guanine nucleotide binding protein, G_i, this effect being blocked by pertussis toxin. We have established conditions whereby the effects of G_i may be abolished in human tissue, and the functional consequences of this studied. This will be of value in future experiments to determine the role of G_i in heart failure.

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References

ANAND SRIVASTRAVA, M.B. (1985). Regulation of adenylate cyclase by adenosine and other agonists in rat myocardial sarcolemma. *Arch. Biochem. Biophys.*, **243**, 439–446.

BELARDINELLI, L. & ISENBERG, G. (1983). Actions of adenosine and isoproterenol on isolated mammalian ventricular myocytes. *Circ. Res.*, **53**, 287–297.

BOHM, M., BRUCKNER, R., NEUMANN, J., SCHMIZ, W., SCHOLZ, H. & STARBATTY, J. (1986). Role of guanine nucleotide-binding protein in the regulation by adenosine of cardiac potassium conductance and force of contraction. Evaluation with pertussis toxin. *Naunyn Schmiedebergs Arch. Pharmacol.*, **332**, 403–405.

BOHM, M., BEUCKELMANN, D., BROWN, L., FEILER, G., LORENZ, B., NABAUAER, M., KEMKES, B. & ERDMANN, E. (1988). Reduction of beta-adrenoceptor density and evaluation of positive inotropic responses in isolated diseased human myocardium. *Eur. Heart J.*, **9**, 844–852.

BOHM, M., GIERSCHIK, P., UNGERER, M. & ERDMANN, E. (1989a). Coupling of adenosine receptors to a pertussis toxin-sensitive G protein in the human heart. *Eur. J. Pharmacol.*, **172**, 407–411.

BOHM, M., PIESKE, B., UNGERER, M. & ERDMANN, E. (1989b). Characterization of A1 adenosine receptors in atrial and ventricular myocardium from diseased human hearts. *Circ. Res.*, **65**, 1201–1211.

BRISTOW, M.R., GINSBURG, R., MINOBE, W., CUBICCIOTTI, R.S., SAGEMAN, W.S., LURIE, K., BILLINGHAM, M.E., HARRISON, D.C. & STINSON, E.B. (1982). Decreased catecholamine sensitivity and beta-adrenergic receptor density in failing human hearts. *New Engl. J. Med.*, **307**, 205–211.

BROWN, L., LORENZ, B. & ERDMANN, E. (1986). Reduced positive inotropic effects in diseased human ventricular myocardium. *Cardiovasc. Res.*, **20**, 516–520.

BRUNS, R.F., FERGUS, J.H., BADGER, E.W., BRISTOL, J.A., SANTAY, L.A., HARTMAN, J.D., HAYS, S.J. & HUANG, C.C. (1987). Binding of the A1-selective adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine to rat brain membranes. *Naunyn-Schmiedebergs. Arch. Pharmacol.*, **335**, 59–63.

COLLIS, M.G., STOGGALL, S.M. & MARTIN, F.M. (1989). Apparent affinity of 1,3-dipropyl-8-cyclopentylxanthine for adenosine A1 and A2 receptors in isolated tissues from guinea-pigs. *Br. J. Pharmacol.*, **97**, 1274–1278.

DOBSON, J.G., JR. (1978). Reduction by adenosine of the isoproterenol-induced increase in cyclic adenosine 3',5'-monophosphate formation and glycogen phosphorylase activity on rat heart muscle. *Circ. Res.*, **43**, 785–792.

FELDMAN, A.M., CATES, A.E., BRISTOW, M.R. & VAN DOP, C. (1989). Altered expression of alpha-subunits of G proteins in failing human hearts. *J. Mol. Cell Cardiol.*, **21**, 359–365.

GILMAN, A.G. (1987). G proteins: Transducers of receptor-generated signals. *Ann. Rev. Biochem.*, **56**, 615–649.

HARDING, S.E., VESCOVO, G., KIRBY, M., JONES, S.M., GURDEN, J. & POOLE-WILSON, P.A. (1988). Contractile responses of isolated rat and rabbit myocytes to isoproterenol and calcium. *J. Mol. Cell. Cardiol.*, **20**, 635–647.

HARDING, S.E., JONES, S.M., VESCOVO, G., O'GARA, P. & POOLE-WILSON, P.A. (1989). Beta-adrenoceptor desensitisation in single ventricular cells isolated from failing human hearts. *Circulation*, **80**, 1748. (Abstract)

HARDING, S.E., O'GARA, P., JONES, S.M., BROWN, L.A., VESCOVO, G. & POOLE-WILSON, P.A. (1990). Species dependence of contraction velocity in single isolated cardiac myocytes. *Cardiosci.*, **1**, 49–53.

HAZEKI, O. & UI, M. (1981). Modification by islet-activating protein of receptor-mediated regulation of cyclic AMP accumulation in isolated rat heart cells. *J. Biol. Chem.*, **256**, 2856–2862.

HENRICH, M., PIPER, H.M. & SCHRADER, J. (1987). Evidence for adenylate cyclase-coupled A1-adenosine receptors on ventricular cardiomyocytes from adult rat and dog heart. *Life Sci.*, **41**, 2381–2388.

HOPWOOD, A.M., HARDING, S.E. & HARRIS, P. (1987). An anti-adrenergic effect of adenosine on guinea-pig but not rabbit ventricles. *Eur. J. Pharmacol.*, **137**, 67–75.

MARTENS, D., LOHSE, M.J., RAUCH, B. & SCHWABE, U. (1987). Pharmacological characterization of A1 adenosine receptors in isolated rat ventricular myocytes. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **336**, 342–348.

NEWMAN, W.H., GROSSMAN, S.J., FRANKIS, M.B. & WEBB, J.G. (1984). Increased myocardial adenosine release in heart failure. *J. Mol. Cell. Cardiol.*, **16**, 577–580.

SCHRADER, J., BAUMANN, G. & GERLACH, E. (1977). Adenosine as inhibitor of myocardial effects of catecholamines. *Pflugers Arch.*, **372**, 29–35.

SUNYER, T., MONASTIRSKY, B., CODINA, J. & BIRNBAUMER, L. (1989). Studies on nucleotide and receptor regulation of Gi proteins: effects of pertussis toxin. *Mol. Endocrinol.*, **3**, 1115–1124.

TOMLINS, B., HARDING, S.E., KIRBY, M.S., POOLE-WILSON, P.A. & WILLIAMS, A.J. (1986). Contamination of a cardiac sarcolemmal preparation with endothelial plasma membrane. *Biochim. Biophys. Acta*, **856**, 137–143.

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The crucial role of physiological Ca^{2+} concentrations in the production of endothelial nitric oxide and the control of vascular tone

[1]P. Lopez-Jaramillo, [2]M.C. Gonzalez, R.M.J. Palmer & [3]S. Moncada

Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS

1 The effect of varying the extracellular Ca^{2+} concentration on the basal and acetylcholine (ACh)-induced release of nitric oxide (NO) from the rabbit aorta was investigated by use of a superfusion bioassay system.

2 Changes between 0.5 and 2.0 mM in the concentration of Ca^{2+} superfusing the detector bioassay tissues or perfusing endothelium-denuded donor aortae had no effect on the tone of these tissues.

3 Increasing the concentration of Ca^{2+} perfusing endothelium-containing donor aortae from zero to 1.25 mM caused a transient (24 ± 9 min), concentration-dependent basal release of NO, which was attenuated at higher concentrations of Ca^{2+} (1.5–2.0 mM).

4 The duration of the effect of Ca^{2+} on the basal release of NO was increased by a concomitant infusion of L-arginine (100 μM) through the donor aorta.

5 Changes in the concentration of Ca^{2+} between 0.5 and 2.0 mM had a similar biphasic effect on the release of NO induced by ACh, which was also maximal at 1.25 mM Ca^{2+}.

6 When Ca^{2+} was removed from the Krebs buffer perfusing the donor aorta, the basal release of NO declined within 2 min. In contrast, the release of NO induced by ACh declined progressively over 60 min.

7 Thus changes in the concentration of Ca^{2+} around the physiological range modulate the synthesis of NO by the vascular endothelium and consequently, vascular tone. This may account for the effects of dietary Ca^{2+} supplements on the control of some hypertensive states.

Introduction

The vascular endothelium produces nitric oxide (NO) (Palmer *et al.*, 1987) which is synthesized from the terminal guanidino nitrogen atom(s) of L-arginine (Palmer *et al.*, 1988a). Furthermore, this synthesis is inhibited by the L-arginine analogue N^G-monomethyl-L-arginine (L-NMMA; Palmer *et al.*, 1988b; Rees *et al.*, 1989a). The synthesis of NO by the vascular endothelium plays a significant role in the control of blood pressure (Rees *et al.*, 1989b) and flow in animals (Gardiner *et al.*, 1990) and man (Vallance *et al.*, 1989), This has led to the suggestion that the generation of NO from L-arginine maintains a vasodilator tone in the cardiovascular system (Rees *et al.*, 1989b; Moncada & Palmer, 1990).

The release of NO accounts for the biological actions of endothelium-derived relaxing factor (EDRF; for review see Moncada *et al.*, 1988). The release of EDRF and the endothelium-dependent vascular relaxation are dependent on transmembrane Ca^{2+} flux rather than on mobilization of intracellular Ca^{2+} stores (Luckhoff *et al.*, 1988; White & Martin, 1989). In addition, the endothelial NO synthase is a Ca^{2+}-dependent enzyme (Palmer & Moncada, 1989; Moncada & Palmer, 1990; Meyer *et al.*, 1989).

Epidemiological studies have shown that restricted dietary Ca^{2+} is associated with elevations in blood pressure (McCarron *et al.*, 1982; Kesteloot & Joossens, 1988), and with increased incidence of pregnancy-induced hypertension (PIH; Belizan & Villar, 1980). Studies with animal models of hypertension have verified this association (Ayachi, 1979; Peuler *et al.*, 1987) and have further suggested that increases in dietary Ca^{2+} lower the blood pressure (Furspan *et al.*, 1989; Hatton *et al.*, 1989). Clinical trials have confirmed that dietary Ca^{2+} supplements can lower blood pressure in some hypertensive individuals (Grobbee & Hofman, 1986) and in normotensive pregnant women (Lopez-Jaramillo *et al.*, 1989) and can also reduce the incidence of PIH (Lopez-Jaramillo *et al.*, 1990). The beneficial effect of Ca^{2+} supplementation was more evident in animal models of hypertension (McCarron *et al.*, 1981) and in those essential hypertensive patients (Resnick *et al.*, 1986) and pregnant women (Lopez-Jaramillo *et al.*, 1989) whose ionised Ca^{2+} concentration in serum was low, but which could be raised by Ca^{2+} supplementation.

Several hypotheses have been proposed to explain the mechanisms by which Ca^{2+} supplementation acts to reduce blood pressure; these include changes in smooth muscle membrane stability (Furspan & Bohr, 1986), natriuresis and associated changes in sodium homeostasis (Zemel *et al.*, 1988), depletion of phosphate (Lau *et al.*, 1984) and increases in prostacyclin synthesis (Lopez-Jaramillo *et al.*, 1987a,b). To date, however, none of these have been substantiated.

Because of all this we decided to investigate whether small variations in extracellular Ca^{2+} concentration around the physiological range alter the production of NO by the vascular endothelium and are, therefore, relevant to the understanding of the physiological regulation of vascular tone and its changes during conditions such as hypertension and PIH.

Methods

Bioassay of NO

Male New Zealand White rabbits (2.0–2.3 kg) were killed with an overdose of sodium pentobarbitone. A segment (6 cm) of the thoracic aorta was removed, trimmed free of adhering fat and connective tissue and placed in a perspex chamber. The tissue was then perfused intraluminally at 5 ml min^{-1}, by a Watson Marlow 101 U/R pump, with Krebs buffer, gassed with 95% O_2/5% CO_2 at 37°C, containing indomethacin

[1] Present address: Unidad de Metabolismo Mineral, Laboratorio de Investigaciones en Metabolismo y Nutricion, Facultad de Ciencias Medicas, Universidad Central del Ecuador, Quito, Ecuador.

[2] Present address: Departamento de Fisiologia, Facultad de Medicina, Universidad Autonoma de Madrid, Spain.

[3] Author for correspondence.

(5 μM) and different concentrations of Ca^{2+} (0.5, 0.75, 1.0, 1.25, 1.5 and 2.0 mM). This aorta was called the donor tissue. In some experiments this donor tissue was denuded of endothelium by gently rubbing the endothelial surface with a pipe cleaner as described previously (Rees *et al.*, 1989a).

For the bioassay, the effluent of the donor tissue was used to superfuse two spiral strips of rabbit thoracic aorta, denuded of endothelium (bioassay tissues), in a cascade as described previously (Rees *et al.*, 1989a). The Ca^{2+} concentration in the fluid superfusing these tissues was kept constant by mixing the effluent of the donor tissue with sufficient Ca^{2+} to maintain a concentration of 2 mM. After a period of 40 min equilibration, the bioassay tissues were contracted with a continuous infusion of 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$ (U46619; 50 nM). Atropine sulphate (0.2 μM) was continuously infused over the bioassay tissues (O.T.) in order to block any direct action of acetylcholine (ACh).

The tension of bioassay tissues was recorded with Harvard Bioscience isotonic transducers on a Linearcorder mark VII WR3101 (Graphtec). The amplification of the recorder was adjusted so that similar relaxations to glyceryl trinitrate (GTN; 20 nM) were observed in each bioassay tissue. In some experiments, L-arginine (100 μM), haemoglobin (Hb, 3.5, 6.25 nM), superoxide dismutase (SOD; 15 u ml^{-1}), L-NMMA (100 μM), D-NMMA (100 μM) or N-iminoethyl-L-ornithine (L-NIO; 100 μM) were added to the perfusate at a rate of 100 μl min^{-1}. The release of an unstable rabbit aorta-relaxing substance which could be potentiated by SOD and inhibited by Hb and by arginine analogues, which are known inhibitors of NO synthesis (Rees *et al.*, 1989a), was identified as NO.

The release of NO was stimulated in some experiments by ACh (1 μM), which was delivered as a 1 min infusion through an injection port immediately before the donor tissue. Exogenous NO (220 pmol) was given as a bolus injection over the bioassay tissues.

Materials

Nitric oxide (>99.98 pure, British Oxygen Corporation); $CaCl_2$ (BDH Analar), glyceryl trinitrate (Wellcome), indomethacin, ACh, SOD, atropine sulphate, L-arginine free base, Hb (Sigma), sodium pentobarbitone (May & Baker) and U46619 (Cayman Chemical) were obtained as indicated. L-NMMA, D-NMMA and L-NIO were synthesised by Dr H.F. Hodson of Wellcome Research Laboratories. All drugs used were administered in saline solution.

Statistics

Results are expressed as mean ± s.e.mean for n separate experiments. One way analysis of variance (ANOVA) was used to examine differences between the mean of the relaxation at the different Ca^{2+} concentrations. Contractions or relaxations of the bioassay tissues are expressed as % of the relaxation obtained in the uppermost bioassay tissue with a standard (20 nM) bolus injection of GTN.

Results

Effect of Ca^{2+} on basal release of NO

Changes in Ca^{2+} concentrations between 0.5 and 2 mM did not have a direct effect on the tone of the bioassay tissues, either when they were without tone, or when contracted with U46619 (n = 3 for each). In addition they had no effect on the actions of NO administered directly O.T. (n = 3). When the bioassay tissues were superfused with Ca^{2+}-free Krebs solution for longer than 1 h, a small concentration-independent contraction was observed with the first addition of Ca^{2+} (n = 5). No further change in the tone of the bioassay tissues was observed with subsequent addition of Ca^{2+} (0.5–2.0 mM). Furthermore, if the concentration of Ca^{2+} was changed between 0.5–2.0 mM in a donor tissue denuded of endothelium, there was no change in the tone of the bioassay tissues (n = 3).

When the concentration of Ca^{2+} perfusing a donor aorta with intact endothelium was changed from 2.0 to 1.25 mM, the release of an EDRF-like substance was observed (Figure 1). This substance was unstable, its stability was enhanced by SOD (15 u ml^{-1}; n = 3; data not shown). Furthermore, its action was antagonized by Hb (3.5, 6.25 nM; n = 3; Figure 1). Moreover, infusion of L-NMMA (100 μM) or L-NIO (100 μM), but not D-NMMA (100 μM), also abolished the release of NO (n = 3 for each). These results indicated that this EDRF-like substance was NO.

The release of NO which also occurred when the Ca^{2+} concentration was changed from zero to 1.25 mM resulted in the relaxation of the uppermost bioassay tissue, the tone of which was reduced by 51.4 ± 6.4% (n = 17) of that induced by a standard dose of GTN (20 nM). When the Ca^{2+} concentration was further increased to 1.5–2.0 mM, release of NO was reduced, such that the tone of the bioassay tissue was 7.9 ± 5.1% (n = 7) of the standard GTN dose.

The basal release of NO observed when the Ca^{2+} concentration perfusing the donor tissue was changed from zero to 1.25 mM was further increased when the donor tissue was perfused with L-arginine (100 μM), such that the relaxation of the uppermost bioassay tissue was 31.5 ± 18% (n = 5) greater than that obtained with 1.25 mM Ca^{2+} alone (Figure 2). Moreover, L-arginine increased the duration of the Ca^{2+}-induced release of NO from 24 ± 9 min to 42.5 ± 4 min (n = 5).

When Ca^{2+} was removed from the Krebs buffer perfusing the donor tissue, the tone of the uppermost bioassay tissue increased significantly compared to that in the presence of 1.25 mM Ca^{2+} (Figure 3).

Effect of Ca^{2+} on NO release induced by ACh

The release of NO induced by ACh (1 μM) was also dependent on the extracellular Ca^{2+} concentration. When Ca^{2+} was removed from the Krebs solution, the release of NO induced by ACh was progressively reduced and was abolished following perfusion of the donor tissue for 60 min in Ca^{2+}-free Krebs solution (Figure 3).

The release of NO induced by ACh was maximal in the presence of 1.25 mM Ca^{2+} (Figure 4). At 1.0 mM Ca^{2+} the release was reduced, but was not significantly different from that in the presence of 1.25 mM Ca^{2+}. At higher (1.5, 2.0 mM) or lower (0.5, 0.75 mM) Ca^{2+} concentrations, the release of NO

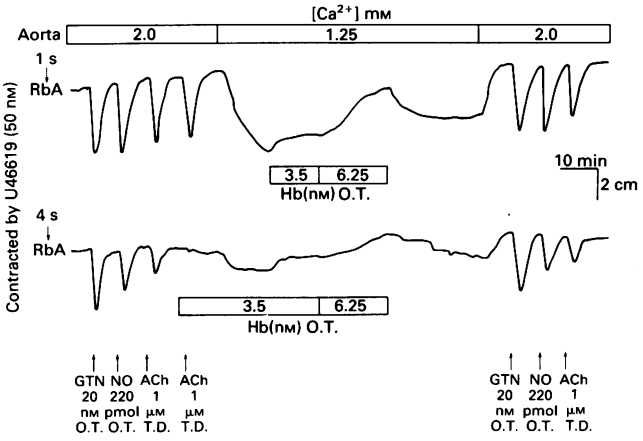

Figure 1 Effect of haemoglobin (Hb) on the action of nitric oxide (NO). Infusion of haemoglobin (3.5 or 6.25 nM) over the bioassay tissues (O.T.) inhibited the relaxation induced by NO released both by acetylcholine (ACh, 1 μM) and by decreasing the concentration of Ca^{2+} perfusing the donor aorta from 2.0 mM to 1.25 mM. Trace representative of 3 similar experiments. RbA = rabbit aorta, GTN = glyceryl trinitrate, T.D. = applied to the donor tissue.

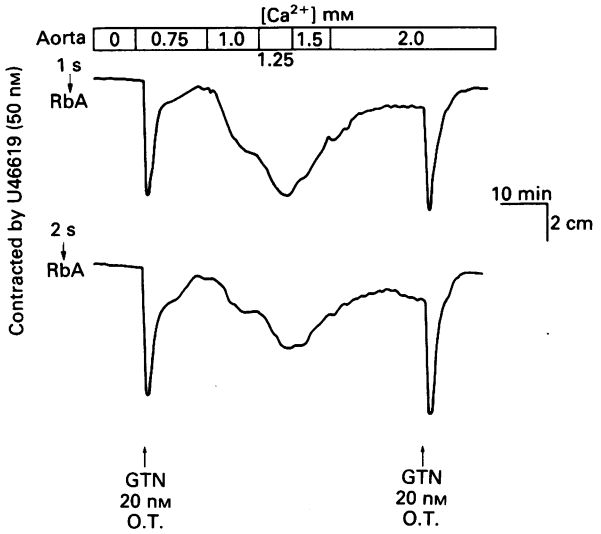

Figure 2 Effect of Ca^{2+} concentration on basal release of nitric oxide (NO) in the presence of 100 μM L-arginine. When the Ca^{2+} concentration perfusing a donor aorta was increased to 1.0 mM, the release of NO was observed and this was enhanced when the Ca^{2+} concentration was increased to 1.25 mM. When the Ca^{2+} concentration was further increased (1.5–2.0 mM), a decline in the release of NO was observed. Trace representative of 5 similar experiments. For key to abbreviations used see legend of Figure 1.

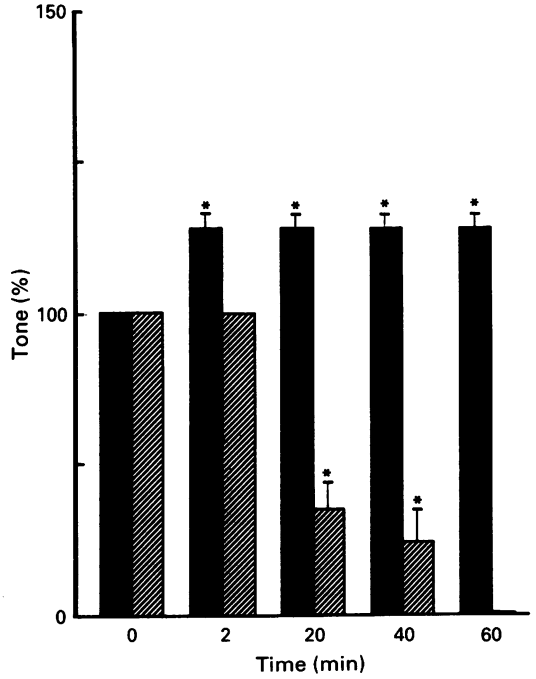

Figure 3 Change in tone of the bioassay tissues (solid columns) and in the relaxing response (hatched columns) to acetylcholine (ACh) with time following a change in Ca^{2+} concentration from 1.25 mM to zero. A significant increase in the tone of the uppermost bioassay tissue was observed 2 min after the removal of the Ca^{2+} in the donor tissue perfusate. The release of nitric oxide (NO) induced by ACh was progressively reduced following perfusion of the donor aorta for 60 min in Ca^{2+}-free Krebs solution. Changes in the basal tone of the uppermost bioassay tissue were expressed as a % of the relaxation induced by a standard dose of glyceryl trinitrate (GTN, 20 nM). Changes in the release of NO induced by ACh were expressed as a % of that in the presence of 1.25 mM Ca^{2+}. Each value is the mean of 5 separate determinations; bars show s.e.mean. * Indicates that the value differs significantly from the value observed at 1.25 mM Ca^{2+}.

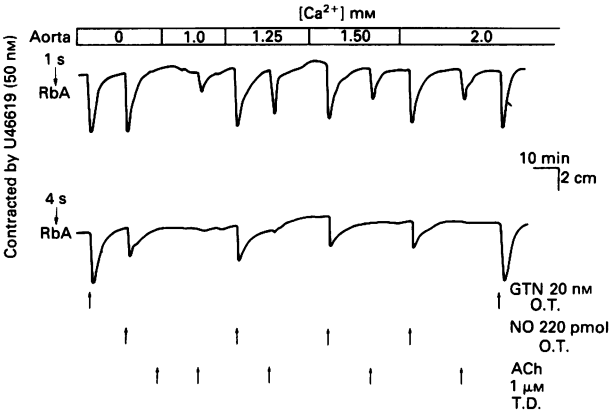

Figure 4 Effect of Ca^{2+} concentration on nitric oxide (NO) release induced by acetylcholine (ACh, 1 μM). The maximum release was observed when the Ca^{2+} concentration was 1.25 mM. At higher or lower Ca^{2+} concentrations, there was a significant reduction in NO release. Trace representative of 7 similar experiments. For key to abbreviations used see legend of Figure 1.

was significantly ($P < 0.05$) reduced compared to that in the presence of 1.25 mM Ca^{2+} (Figure 5).

Discussion

The present study confirms that the synthesis of endothelial NO is critically dependent on the extracellular concentration of Ca^{2+} and demonstrates that small changes in the concentration of this cation around the physiological range fundamentally affect NO release.

When donor aortae with intact endothelium were perfused with Krebs buffer containing Ca^{2+} concentrations within the physiological range, basal release of an EDRF-like substance was observed. At Ca^{2+} concentrations outside the physiological range, both above and below, there was a decline in this basal release. The vascular relaxing activity released was inhibited by Hb and by the inhibitors of NO synthase, L-NMMA and L-NIO, but not by D-NMMA, and was potentiated by SOD. These are all characteristics of NO (for review see Moncada *et al.*, 1988). Changes in Ca^{2+} concentration over the range used in the present study had no effect on the tone of the bioassay tissues, either under basal conditions or when contracted with U46619, and did not induce the release of a relaxing substance from endothelium-denuded donor

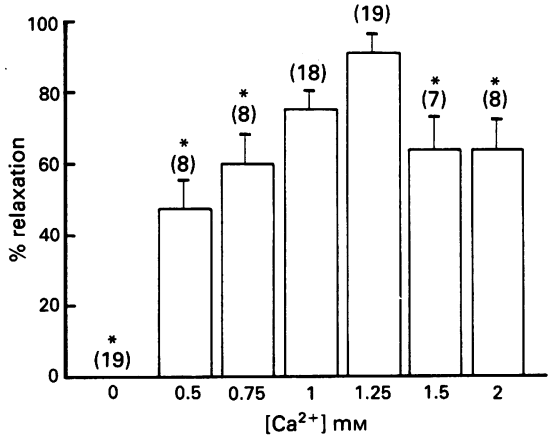

Figure 5 Relaxation of the uppermost bioassay tissue by nitric oxide (NO) released by acetylcholine (ACh, 1 μM). The donor tissue was perfused with Krebs solution containing different Ca^{2+} concentrations. Each value is the mean and bars show s.e.mean of n (number in parentheses) separate determinations. * Indicates that the value differs significantly from the value observed at 1.25 mM Ca^{2+}.

aortae, confirming that the endothelium is the site of action of Ca^{2+}. Since the endothelial NO synthase is Ca^{2+}-dependent (Meyer *et al.*, 1989; Moncada & Palmer, 1990), it is likely that these affects of Ca^{2+} are mediated by modulating the activity of this enzyme.

The magnitude of basal release of NO in the absence of extracellular Ca^{2+} cannot be determined by the present bioassay technique. However, Luckhoff *et al.* (1988) did not observe detectable NO release in the absence of extracellular Ca^{2+} using the activation of a soluble guanylate cyclase to detect NO. These findings suggest that the modulation of basal NO release by physiological Ca^{2+} concentrations will have a considerable effect on the resultant vascular tone. Furthermore, they indicate that in the majority of studies *in vitro*, which are carried out in the presence of 2.5 mM Ca^{2+}, the release of NO is not being studied under optimum conditions.

The mechanism whereby concentrations of Ca^{2+} above the physiological range attenuate the basal release of NO is not clear. It is possible that, as in the cerebellum (Knowles *et al.*, 1989), high Ca^{2+} concentrations might down-regulate the guanylate cyclase in the vascular smooth muscle of the detector tissues. This does not seem to be the case in smooth muscle cells, since the response of the bioassay tissues to NO was not affected by the Ca^{2+} concentration.

The release of NO by physiological Ca^{2+} concentrations was transient, but its duration could be significantly enhanced by exogenous L-arginine. These data indicate that, in addition to the concentration of Ca^{2+}, the availability of L-arginine contributes to the regulation of NO synthesis by the vascular endothelium.

The release of NO induced by ACh was also dependent on the extracellular Ca^{2+} concentration, being maximal in the physiological range and abolished in the absence of Ca^{2+}. Interestingly, the release of NO by ACh was observed at both low (0.5–0.75 mM) and high (1.5–2.0 mM) Ca^{2+} concentrations, conditions under which basal release of NO was not detectable. This suggests either that there are different mechanisms whereby Ca^{2+} enters the cell to stimulate the synthesis of NO, or that ACh is a more effective stimulus which can rely to some extent on intracellular Ca^{2+}. This suggestion is reinforced by the finding that, following transfer from physiological (1.25 mM) to zero Ca^{2+} concentration, the basal release of NO declines much more rapidly than the stimulated release.

At present, the physiological stimuli for the release of NO are not known. Mechanical factors, such as shear stress (Pohl *et al.*, 1986) and pulsatile flow (Rubanyi *et al.*, 1986), are postulated to be major stimuli for the release of NO. The entry of Ca^{2+} into endothelial cells may occur through channels that open in response to 'stretch' (Lansman *et al.*, 1987). Furthermore, Ando *et al.* (1988) have demonstrated an increase in cytosolic free Ca^{2+} levels in bovine aortic endothelial cells in culture subjected to controlled degrees of shear stress. It is likely, therefore, that the basal release of NO occurs as a consequence of the opening of this stretch-activated channel by the pulsatile flow used in our system. Furthermore, since ACh activates receptor-operated Ca^{2+} channels (Danthuluri *et al.*, 1988), it is likely that the consequences of activation of these two routes of Ca^{2+} entry into cells accounts for the differential effect of Ca^{2+} on basal and stimulated release of NO.

A physiological role for endothelium-derived NO in the control of vascular tone *in vivo* has recently been demonstrated in animals and man. Intravenous administration of L-NMMA induced a dose-dependent rise in blood pressure in animals (Rees *et al.*, 1989b; Whittle *et al.*, 1989; Gardiner *et al.*, 1990) and had a vasoconstrictor effect in the arterial system of the human forearm (Vallance *et al.*, 1989).

Individuals with essential hypertension tend to have low ionised Ca^{2+} concentrations in serum (McCarron, 1982; Resnick, 1987). Dietary Ca^{2+} supplementation trials in hypertensive populations have yielded variable results, although anti-hypertensive effects are seen in those subgroups of the hypertensive population who exhibit low ionised Ca^{2+} concentration in serum (Grobbee & Hofman, 1986; Resnick *et al.*, 1986). A decrease in serum ionised Ca^{2+} occurs during pregnancy (Pitkin & Gebhardt, 1977; Lopez-Jaramillo *et al.*, 1988). In pregnant women, dietary Ca^{2+} supplementation maintains the Ca^{2+} levels in the physiological range (Lopez-Jaramillo *et al.*, 1989) and significantly reduces blood pressure (Villar *et al.*, 1987; Marya *et al.*, 1987) and the incidence of PIH (Lopez-Jaramillo *et al.*, 1990). A similar effect of higher dietary Ca^{2+} has also been observed in the spontaneously hypertensive rat, but not Wistar-Kyoto rats. In these animals, Ca^{2+} supplementation increased serum ionised Ca^{2+} (Furspan *et al.*, 1989) and decreased blood pressure (Ayachi, 1979; Furspan *et al.*, 1989; McCarron *et al.*, 1981).

In summary, our results indicate that Ca^{2+} plays a crucial role in the modulation of vascular tone. The effect is mediated by the regulation of the synthesis of endothelial NO rather than by a direct effect on vascular smooth muscle. These findings may explain the paradoxical action of increased serum Ca^{2+} and the antihypertensive effect observed in some hypertensive states (McCarron *et al.*, 1987; Zaloga *et al.*, 1988; Furspan *et al.*, 1989). Furthermore, they suggest that small variations in the extracellular Ca^{2+} levels may be of relevance in the development of some forms of hypertension including PIH.

References

ANDO, J., KOMATSUDA, T. & KAMIYA, A. (1988). Cytoplasmic calcium response to fluid shear stress in cultured vascular endothelial cells. *In Vitro Cell Dev. Biol.*, **24**, 871–877.

AYACHI, S. (1979). Increased dietary calcium lowers blood pressure in the spontaneously hypertensive rat. *Metabolism*, **12**, 1234–1238.

BELIZAN, J.M. & VILLAR, J. (1980). The relationship between calcium intake and edema-proteinuria and hypertension-gestosis: an hypothesis. *Am. J. Clin. Nutr.*, **33**, 2202–2210.

DANTHULURI, N.R., CYBULSKY, M.I. & BROCK, T.A. (1988). ACh-induced calcium transients in primary cultures of rabbit aortic endothelial cells. *Am. J. Physiol.*, **255**, H1549–H1553.

FURSPAN, P.B. & BOHR, D.F. (1986). Calcium-related abnormalities in lymphocytes from genetically hypertensive rats. *Hypertension*, **8** (Suppl. II), 123–126.

FURSPAN, P., RINALDI, G.J., HOFFMAN, K. & BOHR, D.F. (1989). Dietary calcium and cell membrane abnormality in genetic hypertension. *Hypertension*, **13**, 727–730.

GARDINER, S.M., COMPTON, A.M., BENNETT, T., PALMER, R.M.J. & MONCADA, S. (1990). Control of regional blood flow by endothelium-derived nitric oxide. *Hypertension*, **15**, 486–492.

GROBBEE, D.E. & HOFMAN, A. (1986). Effect of calcium supplementation on diastolic blood pressure in young people with mild hypertension. *Lancet*, **ii**, 703–707.

HATTON, D.C., SCROGIN, K.E., METZ, J.A. & McCARRON, D.A. (1989). Dietary calcium alters blood pressure reactivity in spontaneously hypertensive rat. *Hypertension*, **13**, 622–629.

KESTELOOT, H. & JOOSSENS, J.V. (1988). Relationship of dietary sodium, potassium, calcium, and magnesium with blood pressure. Belgian Interuniversity Research on Nutrition and Health. *Hypertension*, **12**, 594–599.

KNOWLES, R.G., PALACIOS, M., PALMER, R.M.J. & MONCADA, S. (1989). Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 5159–5162.

LANSMAN, J.B., HALLAM, T.J. & RINK, T.J. (1987). Single stretch-activated ion channels in vascular endothelial cells as mechanotransducers? *Nature*, **325**, 811–813.

LAU, K., CHEN, S. & EBY, B. (1984). Evidence for the role of PO_4 deficiency in antihypertensive action of a high Ca^{2+} diet. *Am. J. Physiol.*, **246**, H324–H331.

LOPEZ-JARAMILLO, P., NARVAEZ, M. & YEPEZ, R. (1987a). Effect of calcium supplementation on the vascular sensitivity to angiotensin II in pregnant women. *Am. J. Obstet. Gynecol.*, **156**, 261–262.

LOPEZ-JARAMILLO, P., GUARNER, F. & MONCADA, S. (1987b). Effect of calcium and parathyroid hormone on prostacyclin synthesis by

vascular tissue. *Life Sci.*, **40**, 983–986.

LOPEZ-JARAMILLO, P., YEPEZ, R., NARVAEZ, M., GAIBOR, M., MOSCOSO, H. & CORRADO, A.P. (1988). Calcium metabolism in normal pregnant and pregnancy-induced hypertension. *J. Bras. Gynecol.*, **98**, 189–191.

LOPEZ-JARAMILLO, P., NARVAEZ, M., WEIGEL, R.M. & YEPEZ, R. (1989). Calcium supplementation reduces the risk of pregnancy-induced hypertension in an Andes population. *Br. J. Obstet. Gynaecol.*, **96**, 648–655.

LOPEZ-JARAMILLO, P., NARVAEZ, M., FELIX, C. & LOPEZ, A. (1990). Dietary calcium supplementation and prevention of pregnancy hypertension. *Lancet*, **335**, 293.

LUCKHOFF, A., POHL, U., MULSCH, A. & BUSSE, R. (1988). Differential role of extra- and intracellular calcium in the release of EDRF and prostacyclin from cultured endothelial cells. *Br. J. Pharmacol.*, **95**, 189–196.

MARYA, R.K., RATHHEE, S. & MANROW, M. (1987). Effect of calcium and vitamin D supplementation on toxaemia of pregnancy. *Gynecol. Obstet. Invest.*, **24**, 38–42.

McCARRON, D.A. (1982). Low serum concentrations of ionized calcium in patients with hypertension. *N. Engl. J. Med.*, **307**, 226–228.

McCARRON, D.A., MORRIS, C.D. & COLE, C. (1982). Dietary calcium in human hypertension. *Science*, **217**, 267–269.

McCARRON, D.A., MORRIS, C.D. & BUKASKI, R. (1987). The calcium paradox of essential hypertension. *Am J. Med.*, **82** (Suppl. 1B), 27–33.

McCARRON, D.A., YOUNG, N.N., UGORETZ, B.A. & KRUTZIK, S. (1981). Disturbances of calcium metabolism in the spontaneously hypertensive rat: Attenuation of hypertension by calcium supplementation. *Hypertension*, **4** (Suppl. I), 162–167.

MEYER, B., SCHMIDT, K., HUMBERT, R. & BOHME, E. (1989). Biosynthesis of endothelium-derived relaxing factor: a cytosolic enzyme in porcine aortic endothelial cells Ca^{2+}-dependently converts L-arginine into an activator of soluble guanylyl cyclase. *Biochem. Biophys. Res. Commun.*, **164**, 678–685.

MONCADA, S. & PALMER, R.M.J. (1990). The L-arginine:nitric oxide pathway in the vessel wall. In *Nitric oxide from L-Arginine: A Bioregulatory System.* ed. Moncada, S. & Higgs, E.A., pp. 17–31. Amsterdam: Elsevier.

MONCADA, S., RADOMSKI, M.W. & PALMER, R.M.J. (1988). Endothelium-derived relaxing factor: identification as nitric oxide and role in the control of vascular tone and platelet function. *Biochem. Pharmacol.*, **37**, 2495–2501.

PALMER, R.M.J. & MONCADA, S. (1989). A novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. *Biochem. Biophys. Res. Commun.*, **158**, 348–352.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

PALMER, R.M.J., ASHTON, D.S. & MONCADA, S. (1988a). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664–666.

PALMER, R.M.J., REES, D.D., ASHTON, D.S. & MONCADA, S. (1988b). L-Arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem. Biophys. Res. Commun.*, **153**, 1251–1256.

PEULER, J.D., MORGAN, D.A. & MARK, A.L. (1987). High calcium diet reduces blood pressure in Dahl salt-sensitive rats by neural mechanisms. *Hypertension*, **9** (Suppl. III), 159–165.

PITKIN, R.M. & GEBHARDT, M.P. (1977). Serum calcium concentrations in human pregnancy. *Am. J. Obstet. Gynecol.*, **127**, 775–778.

POHL, U., BUSSE, R., KUON, E. & BASSENGE, E. (1986). Pulsatile perfusion stimulates the release of endothelial autacoids. *J. Appl. Cardiol.*, **1**, 215–235.

REES, D.D., PALMER, R.M.J., HODSON, H.F. & MONCADA, S. (1989a). A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. *Br. J. Pharmacol.*, **96**, 418–424.

REES, D.D., PALMER, R.M.J. & MONCADA, S. (1989b). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 3375–3378.

RESNICK, L.M., NICHOLSON, J.P. & LARAGH, J.H. (1986). Calcium metabolism in essential hypertension: relationship to altered renin system activity. *Fed. Proc.*, **45**, 2739–2745.

RESNICK, L.M. (1987). Uniformity and diversity of calcium metabolism in hypertension. A conceptual framework. *Am. J. Med.*, **82** (Suppl. 1B), 16–25.

RUBANYI, G.M., ROMERO, J.C. & VANHOUTTE, P.M. (1986). Flow-induced release of endothelium-derived relaxing factor. *Am. J. Physiol.*, **250**, H1145–H1147.

VALLANCE, P., COLLIER, J. & MONCADA, S. (1989). Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet*, **ii**, 987–1000.

VILLAR, J., REPKE, J., BELIZAN, J. & PAREJA, G. (1987). Calcium supplementation reduces blood pressure during pregnancy: results of a randomized controlled clinical trial. *Obstet. Gynecol.*, **70**, 317–322.

WHITE, D.G. & MARTIN, W. (1989). Differential control and calcium-dependence of production of endothelium-derived relaxing factor and prostacyclin by pig aortic endothelial cells. *Br. J. Pharmacol.*, **97**, 683–690.

WHITTLE, B.J.R., LOPEZ-BELMONTE, J. & REES, D.D. (1989). Modulation of the vasodepressor actions of acetylcholine, bradykinin, substance P and endothelium in the rat by a specific inhibitor of nitric oxide formation. *Br. J. Pharmacol.*, **98**, 646–652.

ZALOGA, G.P., WILLEY, S., MALCOM, D., CHERNOW, B. & HOLADAY, J.W. (1988). Hypercalcemia attenuates the blood pressure response to epinephrine. *J. Pharmacol. Exp. Ther.*, **247**, 949–952.

ZEMEL, M.B., GUALDONI, S.M. & SOWERS, J.R. (1988). Reduction in total and extracellular water associated with calcium-induced natriuresis and the antihypertensive effect of calcium in blacks. *Am. J. Hypertension*, **1**, 70–72.

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The effects of Bay K 8644 and nifedipine on the responses of rat urinary bladder to electrical field stimulation, β,γ-methylene ATP and acetylcholine

Xuenong Bo & [1]Geoffrey Burnstock

Department of Anatomy and Developmental Biology and Centre for Neuroscience, University College London, Gower, Street, London WC1E 6BT

1 Bay K 8644 (0.33 nM to 1 μM) greatly increased the contractions of rat urinary bladder detrusor muscle induced by β,γ-methylene ATP (β,γ-MeATP, 10 μM) and by electrical field stimulation of the purinergic component (the cholinergic response was blocked by atropine).

2 The contractions induced by acetylcholine (ACh, 10 μM) and by electrical field stimulation of the cholinergic component (the purinergic response was blocked following desensitization by α,β-MeATP) were also potentiated by Bay K 8644, although to a lesser extent than the purinergic responses.

3 Nifedipine (1 nM to 3.3 μM) inhibited all the contractions induced by β,γ-MeATP, ACh and electrical field stimulation. However, while the responses to β,γ-MeATP and electrical field stimulation of the purinergic component were almost abolished, a substantial proportion of the responses to ACh and electrical field stimulation of the cholinergic component were nifedipine resistant.

4 The concentration-effect curves for the potentiation by Bay K 8644 of the responses to β,γ-MeATP, ACh and electrical field stimulation were shifted to the right by nifedipine (10 nM). At concentrations greater than 1 μM, Bay K 8644 inhibited contraction.

5 It is concluded that voltage-sensitive calcium channels play an important role in the excitatory mechanical action of P_{2x}-purinoceptor-mediated purinergic responses in the rat urinary bladder, while cholinergic-mediated responses are less dependent on such channels.

Introduction

The actions of adenosine 5'-triphosphate (ATP) as a neurotransmitter or a neuromodulator have been studied in various tissues (Burnstock, 1972; 1986; Gordon, 1986; White, 1988). However, the mechanisms coupling the activation of purinoceptors to the electrophysiological and biochemical changes in the target cells are less clear. It has been observed that ATP can affect the ionic permeability of the plasma membrane of many cell types (Kolb & Wakelam, 1983; Marchenko *et al.*, 1987; Inoue, 1990). Among the ions, calcium may be of importance in mediating the action of ATP (Yatani *et al.*, 1982; Dorn *et al.*, 1989; Nonotte *et al.*, 1989), and in guinea-pig and rat urinary bladder the contractions induced by ATP have been demonstrated to be dependent on extracellular calcium (Iacovou *et al.*, 1988; Bhat *et al.*, 1989). Nifedipine, a 1,4-dihydropyridine, blocks calcium channels and can preferentially inhibit the non-cholinergic component of motor transmission in rat urinary bladder (Iravani *et al.*, 1988). Recently, nifedipine was shown to antagonize the contraction and calcium influx evoked by ATP in guinea-pig urinary bladder (Katsuragi *et al.*, 1990).

In the present study, we have investigated the effects of Bay K 8644, a 1,4-dihydropyridine, which is a calcium channel activator, and nifedipine on the excitatory mechanical response of rat urinary bladder to electrical field stimulation of the purinergic and cholinergic components, and on the contractions elicited by β,γ-methylene ATP (β,γ-MeATP) and acetylcholine (ACh). The aim was to evaluate the relative calcium-dependence of purinergic and cholinergic actions in urinary bladder. In this experiment β,γ-MeATP, a stable analogue of ATP, was chosen to induce contractions instead of ATP since previous experiments have shown that β,γ-MeATP acts more selectively on P_{2X}-purinoceptors, the dominant subclass of purinoceptors in urinary bladders (Brown *et al.*, 1979; Hourani *et al.*, 1985).

[1] Author for correspondence.

Methods

Male Wistar rats (200–250 g) were killed by asphyxiation with CO_2. Bladders were quickly removed. Four mucosa-free detrusor strips (length 8–10 mm, width 1.5–2 mm) were cut from each bladder. Each strip was threaded through a pair of platinum-ring electrodes, with one end attached to a holder and the other to a Dynamometer UF1 isometric force transducer. The strips were equilibrated for 1 h in organ baths containing modified Krebs solution (mM: NaCl 133, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.6, NaH_2PO_4 1.4, $NaHCO_3$ 16.3, glucose 7.7, pH 7.4) bubbled with 95% O_2 and 5% CO_2 at 37°C. The muscle strips were initially loaded to a tension of 1 g. Mechanical activity was displayed on a Grass polygraph. Electrical field stimulation was achieved by delivering pulses of 0.3 ms duration at supramaximal voltage from Grass SD 9 stimulators. The strips were stimulated at frequencies of 2 Hz and 16 Hz for 10 s. Two of the four strips from the same bladder were incubated with atropine (1 μM) for 10 min to block the cholinergic response. The other two strips were exposed to α,β-MeATP (10 μM) to desensitize the purinergic response (Kasakov & Burnstock, 1983). Guanethidine (5 μM) was present in all the experiments. The responses to β,γ-MeATP (10 μM) and ACh (10 μM) were tested before and after the exposure to atropine and α,β-MeATP. β,γ-MeATP and ACh were washed away immediately after the maximum responses were obtained in order to avoid desensitization. (When the control responses were being measured 0.1% acetone was present in the incubation solution.) The effects of Bay K 8644 (0.33 nM to 3.3 μM) and nifedipine (1 nM to 3.3 μM) were measured after the strips had been incubated with the agents for 10 min. The interaction between Bay K 8644 and nifedipine was demon-

strated by incubating the strips with different concentrations of Bay K 8644 and nifedipine (10 nM).

All the drugs were dissolved in distilled water except Bay K 8644 and nifedipine, which were dissolved in acetone. As Bay K 8644 and nifedipine solutions are sensitive to light they were kept in the dark and the organ baths were tightly wrapped with black plastic sheets. The total volume of added solutions was less than 0.5% of the capacity of the organ bath.

Drugs

β,γ-MeATP (Sigma), α,β-MeATP (Sigma), Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate, a gift from Bayer AG), nifedipine (Bayer AG), atropine sulphate (Antigen Ltd), guanethidine (Ciba).

Statistical analysis

The responses were calculated as a percentage increase or reduction compared with the relevant control groups. The results are expressed as mean ± s.e.mean. EC_{50} and IC_{50} values were calculated with Litchfield and Wilcoxon methods. Student's *t* test was used to test for significant differences between groups. Probability values of less than 0.05 were considered significant.

Results

Bay K 8644 potentiated the responses of detrusor strips to electrical field stimulation, β,γ-MeATP and ACh in a concentration-dependent manner in the range 0.33 nM to 1 μM (Figure 1a–f). When the concentration of Bay K 8644 was above 1 μM, the maximal responses were reduced, so the concentration-effect curve became bell-shaped. Bay K 8644 had a much greater effect on the contractions induced by β,γ-MeATP than on those induced by ACh (Table 1). The responses of atropine-treated detrusor strips to electrical field stimulation were more strongly increased by Bay K 8644 than were those of α,β-MeATP-desensitized strips (Table 1). The EC_{50} values showed no significant differences (Table 2). Bay K 8644 also increased the resting tension and spontaneous activity in a concentration-dependent manner (in the range 0.33 nM to 1 μM).

Nifedipine inhibited all the responses tested (Figure 2a and b). The responses to β,γ-MeATP and electrical field stimulation in atropine-treated strips were nearly or completely abolished, while a proportion of the responses to ACh and electrical field stimulation in α,β-MeATP-desensitized strips was nifedipine-resistant (Table 1). The nifedipine-resistant contractions were completely abolished by atropine. There was no significant difference in IC_{50} values, except that the effect on the responses to electrical field stimulation at 16 Hz showed a higher IC_{50} because the response included a higher proportion of the nifedipine-resistant component. In contrast to the effect of Bay K 8644, nifedipine reduced the resting tension and spontaneous activity. When the concentration was above 100 nM, all the spontaneous contractions were abolished.

All the concentration-effect curves for Bay K 8644-induced

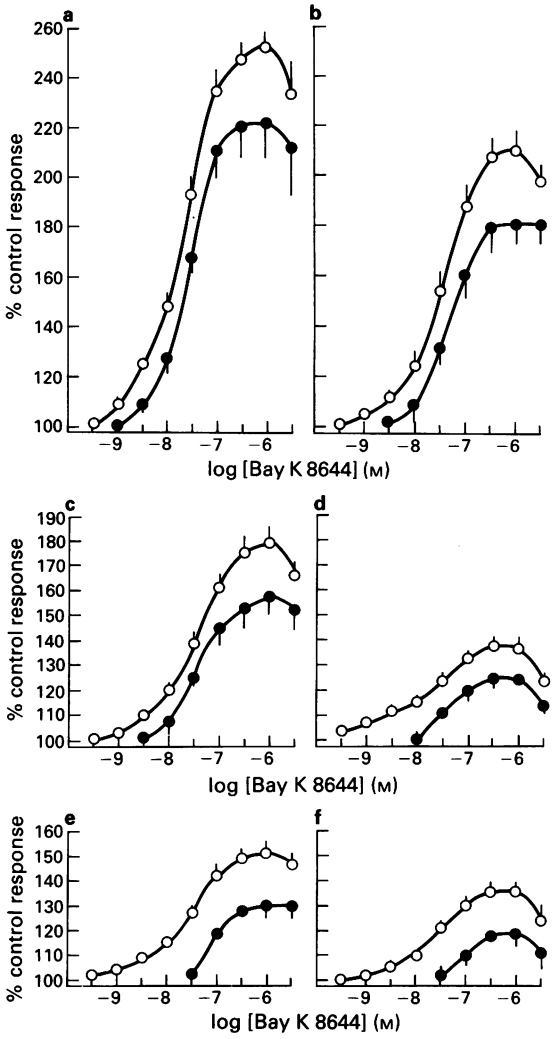

Figure 1 Concentration-response relationship for the effect of Bay K 8644 on the contraction of rat urinary bladder detrusor muscle induced by (a) β,γ-methylene ATP (β,γ-MeATP, 10 μM), (b) field stimulation of purinergic component at 2 Hz, (c) field stimulation of purinergic component at 16 Hz, (d) acetylcholine (ACh, 10 μM), (e) field stimulation of cholinergic component at 2 Hz, and (f) field stimulation of cholinergic component at 16 Hz, in the absence (○) and presence (●) of nifedipine (10 nM). Electrical field stimulation parameters: supramaximal voltage, 0.3 ms duration and 10 s stimulation. Cholinergic responses were blocked by atropine (1 μM), purinergic responses were desensitized by α,β-MeATP (10 μM). Each point represents the mean of 7 experiments; vertical lines show s.e.mean.

Table 1 The maximal effects of Bay K 8644 (0.33 nM to 3.3 μM) and nifedipine (1 nM to 3.3 μM) on the responses of rat urinary bladder detrusor to β,γ-methylene ATP (β,γ-MeATP, 10 μM), acetylcholine (ACh, 10 μM) and electrical field stimulation and the maximal effects of Bay K 8644 in the presence of nifedipine (10 nM)

			Field stimulation			
			Purinergic		*Cholinergic*	
	β,γ-MeATP	*ACh*	*2 Hz*	*16 Hz*	*2 Hz*	*16 Hz*
Bay K 8644	252 ± 5***	138 ± 4	210 ± 8***	179 ± 7***	151 ± 5	136 ± 4
Bay K 8644 + nifedipine	221 ± 4***	125 ± 2	181 ± 8***	158 ± 8***	130 ± 5	119 ± 5
Nifedipine	2 ± 1***	16 ± 2	0 ± 0***	2 ± 1***	6 ± 1	23 ± 3

Data shown are mean ± s.e.mean of the percentage of the control response, $n = 7$.
Significant difference between the relevant groups of β,γ-MeATP and ACh, field stimulation of purinergic and cholinergic components; *** $P < 0.001$.

Table 2 The EC_{50} values for the effect of Bay K 8644 (0.33 nM to 3.3 μM) on the responses of rat urinary bladder detrusor to β,γ-methylene ATP (β,γ-MeATP, 10 μM), acetylcholine (ACh, 10 μM) and electrical field stimulation in the absence and presence of nifedipine (10 nM) and the IC_{50} values for nifedipine (1 nM to 3 μM) on the response

			Field stimulation			
			Purinergic		*Cholinergic*	
	β,γ-MeATP	*ACh*	*2 Hz*	*16 Hz*	*2 Hz*	*16 Hz*
Bay K 8644	25 ± 3	17 ± 5††	26 ± 6	31 ± 5	25 ± 4†††	27 ± 3†††
Bay K 8644 + nifedipine	34 ± 5	43 ± 5	40 ± 9*	36 ± 6*	87 ± 9	71 ± 13
Nifedipine	33 ± 7	34 ± 4	44 ± 6	45 ± 6*	40 ± 5	69 ± 9

Data shown are mean values (in nM) ± s.e.mean, $n = 7$.
Significant differences between the relevant groups of β,γ-MeATP and ACh, field stimulation of purinergic and cholinergic components are indicated by * $P < 0.001$.
Significant differences between the groups in the absence and presence of nifedipine are indicated by †† $P < 0.01$, ††† $P < 0.001$.

potentiation of the responses to β,γ-MeATP, ACh and electrical field stimulation were shifted to the right in the presence of nifedipine (10 nM) (Figure 1a–f). The maximal responses were also suppressed. The EC_{50} values for the effect of Bay K 8644 on β,γ-MeATP and stimulation of purinergic nerves in the presence of nifedipine (10 nM) showed no significant difference compared with those in the absence of nifedipine, owing to the suppression of the maximal responses. Nevertheless, the EC_{50} values for the effect of Bay K 8644 on the responses to ACh and electrical field stimulation of the cholinergic component were still significantly increased in the presence of nifedipine (10 nM).

Discussion

The present study shows that contractions of rat urinary bladder elicited by β,γ-MeATP and electrical field stimulation of the purinergic nerves can be greatly potentiated by Bay K 8644, and nearly abolished by nifedipine. This provides further evidence that the excitatory mechanical action of purinergic nerves is dependent on extracellular calcium, and that the activation of P_{2X}-purinoceptors may lead to the opening of calcium channels. In guinea-pig and rat urinary bladder contractions induced by ATP are greatly reduced in low calcium calcium channels. In guinea-pig and rat urinary bladder the contractions induced by ATP are greatly reduced in low calcium or calcium-free solutions (Iacovou *et al.*, 1988; Bhat *et al.*, 1989). A recent study found that the contraction and calcium-influx evoked by ATP in guinea-pig urinary bladder smooth muscle can be antagonized by nifedipine (Katsuragi *et al.*, 1990), which is in good agreement with the results presented in this paper. Intracellular recording techniques have revealed that extracellular ATP can elicit fast inward currents in smooth muscle cells isolated from guinea-pig bladder (Marchenko *et al.*, 1987; Inoue, 1990). In other tissues, the role of calcium in the action of ATP and purinergic nerve stimulation has also been studied. In guinea-pig vas deferens it has been shown that, in contrast to the action of noradrenaline, nifedipine can block the action potentials and contractions elicited by electrical field stimulation and ATP, while Bay K 8644 augments the action potentials and contractions (Burnstock *et al.*, 1986; MacKenzie *et al.*, 1988). In the dog mesenteric artery, nifedipine can selectively inhibit the purinergic component of the contractions evoked by sympathetic nerve stimulation (Omote *et al.*, 1989), and in snail neurones nanomolar concentrations of extracellular ATP can activate membrane calcium channels (Yatani *et al.*, 1982). Also, P_2-purinoceptor-mediated calcium mobilization across the cell membrane has been observed in rat isolated alveolar type II cells (Dorn *et al.*, 1989), murine thymocytes (El-Moatassim *et al.*, 1989), human promyelocyteic cell line HL60 (Nonotte *et al.*, 1989), Ehrlich ascites tumour cells (Dubyak & De Young, 1985), pancreatic β-cells (Gylfe & Hellman, 1987) and rat parotid acinar cells (McMillian *et al.*, 1987).

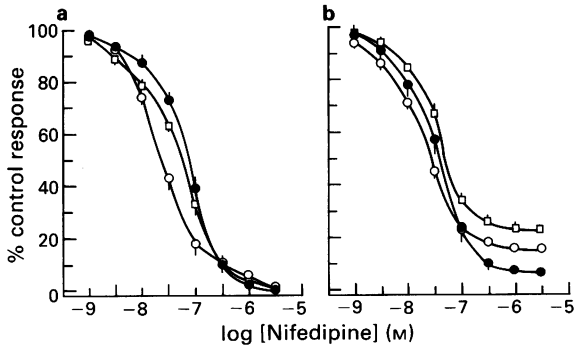

Figure 2 Concentration-response relationship for the effect of nifedipine on the contraction of rat urinary bladder detrusor muscle induced by (a) β,γ-methylene ATP (β,γ-MeATP, 10 μM, ○) and field stimulation of the purinergic component at 2 Hz, (●) and 16 Hz (□), (b) acetylcholine (ACh, 10 μM, ○) and field stimulation of the cholinergic component at 2 Hz (●) and 16 Hz (□). Electrical field stimulation parameters: supramaximal voltage, 0.3 ms duration, and 10 s stimulation. In (a) cholinergic responses had been blocked by atropine (1 μM); in (b) purinergic responses had been desensitized by α,β-MeATP (10 μM). Each point represents the mean of 7 experiments and vertical lines show s.e.mean.

Compared with the contractions evoked by β,γ-MeATP and electrical field stimulation of the purinergic component, the responses to ACh and electrical field stimulation of the cholinergic component were less affected by Bay K 8644. The inhibitory effects of nifedipine on the responses to ACh and cholinergic nerve stimulation were significant, but a proportion of the contraction was nifedipine-resistant. Previous results have indicated that nifedipine reduces the amplitude of nerve-mediated rat bladder contraction by about 70% (Maggi *et al.*, 1988) and the amplitude of nerve-mediated guinea-pig urinary bladder contractions by 62% (Adamson *et al.*, 1986). The nifedipine-resistant contractions were still cholinergic because they were abolished by atropine. In gastrointestinal systems, such as the mouse isolated distal colon, contractile activity induced by ACh is barely enhanced by Bay K 8644 and partially inhibited by nifedipine (Fontaine & Lebrun, 1988), and in rat duodenal smooth muscle the response induced by ACh is not modified by Bay K 8644 but can be significantly decreased by nifedipine (Coruzzi *et al.*, 1986). This indicates that the contractile response to ACh is only partially dependent on the dihydropyridine-sensitive calcium channels. However, the reason for the discrepancy between the sensitivities to Bay K 8644 and nifedipine is not clear at present.

All the concentration-effect curves for the potentiation by Bay K 8644 of the responses to β,γ-MeATP, ACh and electrical field stimulation were shifted to the right by nifedipine. The interaction between Bay K 8644 and nifedipine was not typically competitive because the maximal response to Bay K 8644 was suppressed. Increasing the concentration of Bay K 8644 did not surmount the inhibitory effect of nifedipine, on the contrary, when the concentration of Bay K 8644 exceeded

1 μM its effect became negative. This characteristic of Bay K 8644 has been observed in many experiments (Bechem & Schramm, 1987). The inhibitory effect of Bay K 8644 may not result from the use of a racemic compound because there is no obvious difference between the effects of the racemic compound and (−)-Bay K 8644 (Kass, 1987). One explanation is that there are two dihydropyridine receptors, i.e., excitatory and inhibitory, and when the concentration of Bay K 8644 is above a certain level it also activates the inhibitory receptors (Thomas *et al.*, 1984; Lee *et al.*, 1987). In guinea-pig heart, nifedipine even shows a small positive inotropic effect at low concentrations (about 4 nM) (Thomas *et al.*, 1984). These results suggest that Bay K 8644 and nifedipine are not pure agonists or antagonists of calcium channels. At high concentrations Bay K 8644 has been shown to interfere with intracellular activity, which may also account for the bell-shaped concentration-response curve of Bay K 8644 (Movsesian & Adelstein, 1984).

The voltage-sensitive calcium channels on excitable cell membranes have been classified into three subtypes, i.e., T, N and L. T- and L-type channels are important in smooth muscle activities (Miller, 1987). 1,4-Dihydropyridines primarily act on L-type channels (Nowycky *et al.*, 1985). From the present experiments the involvement of the L-type channels in the action of purinergic responses seems obvious, but a process linking the activation of P_{2X}-purinoceptors and the opening of calcium channels remains to be established. Receptor-operated calcium permeable channels activated by ATP have been found on the smooth muscle membrane of rabbit ear artery (Benham & Tsien, 1987). These channels, with a selectivity of 3:1 for calcium over sodium, can be opened at very negative potentials and are nifedipine-resistant. Purinoceptor-operated channels, which are permeable to many kinds of cations, have also been found in the single isolated cells of guinea-pig urinary bladder (Inoue, 1990). Thus, one possible mechanism is that ATP depolarizes the membrane by acting on the receptor-operated channels leading to the influx of calcium and other cations. Depolarization of the membrane will promote the opening of the voltage-sensitive calcium channels and elicit smooth muscle contraction. The existence of receptor-operated calcium permeable channels may also account for the phenomenon in guinea-pig vas deferens, that excitatory junction potentials and ATP- and α,β-MeATP-elicited depolarizations at low concentrations are insensitive to both Bay K 8644 and nifedipine, while action potential discharges and contractile responses are greatly affected by the two dihydropyridines (MacKenzie *et al.*, 1988). However, a direct link between P_{2X}-purinoceptors and the voltage-sensitive calcium channels cannot be excluded at present. The effect of P_{2Y}-purinoceptor activation on membrane ion permeability, which leads to hyperpolarization of the smooth muscle cell by selectively opening potassium channels (White, 1988; Burnstock, 1990), seems to be different from that of P_{2X}-purinoceptor activation.

In conclusion, the results indicate that voltage-sensitive calcium channels play an important role in the excitatory mechanical effect of P_{2X}-purinoceptor-mediated responses in rat urinary bladder, while the action of cholinergic nerves is less dependent on such channels.

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References

ADAMSON, G.M., CALLAHAN, S.M. & CREED, K.E. (1986). Effects of nifedipine on electrical and mechanical activity of the urinary bladder of the guinea pig. *Proc. Aust. Physiol. Pharmacol. Soc.*, **17**, 136P.

BECHEM, M. & SCHRAMM, M. (1987). Calcium agonists. *J. Mol. Cell. Cardiol.*, **19**, (Supplement II) 63–75.

BENHAM, C.D. & TSIEN, R.W. (1987). A novel receptor-operated Ca^{2+}-permeable channel activated by ATP in smooth muscle. *Nature*, **328**, 275–278.

BHAT, M.B., MISHRA, S.K. & RAVIPRAKASH, V. (1989). Differential susceptibility of cholinergic and noncholinergic neurogenic responses to calcium channel blockers and low Ca^{2+} medium in rat urinary bladder. *Br. J. Pharmacol.*, **96**, 837–842.

BROWN, C., BURNSTOCK, G. & COCKS, T. (1979). Effects of adenosine 5′-triphosphate (ATP) and β,γ-methylene ATP on the rat urinary bladder. *Br. J. Pharmacol.*, **65**, 97–102.

BURNSTOCK, G. (1972). Purinergic nerves. *Pharmacol. Rev.*, **24**, 509–581.

BURNSTOCK, G. (1986). The changing face of autonomic neurotransmission. *Acta Physiol. Scand.*, **126**, 67–91.

BURNSTOCK, G. (1990). Classification and characterization of purinoceptors. In *Purines in Cellular Signalling: Targets for New Drugs.* ed. Jacobson, K.A., Daly, J.W. & Manganiello, V. pp. 241–253. Springer: New York.

BURNSTOCK, G., MACKENZIE, I. & MANZINI, S. (1986). Regulation of voltage-dependent calcium channels in the guinea-pig vas deferens by the dihydropyridines nifedipine and Bay K 8644. *J. Physiol.*, **373**, 62P.

CORUZZI, G., POLI, E. & BERTACCINI, G. (1986). Bay K 8644 and acetylcholine: different interaction with calcium ions in the rat duodenal muscle. *Pharmacology*, **33**, 206–216.

DORN, C.C., RICE, W.R. & SINGLETON, F.M. (1989). Calcium mobilization and responses recovery following P_2-purinoceptor stimulation of rat isolated alveolar type II cells. *Br. J. Pharmacol.*, **97**, 163–170.

DUBYAK, G.R. & DE YOUNG, M.B. (1985). Intracellular Ca^{2+} mobilization activated by extracellular ATP in Ehrlich ascites tumor cells. *J. Biol. Chem.*, **260**, 10653–10661.

EL-MOATASSIM, C., MAURICE, T., MANI, J-C. & DORDAND, J. (1989). The $[Ca^{2+}]_i$ increase induced in murine thymocytes by extracellular ATP does not involve ATP hydrolysis and is not related to phosphoinositide metabolism. *FEBS Lett.*, **242**, 391–396.

FONTAINE, J. & LEBRUN, P. (1988). Pharmacological analysis of the effects of Bay K 8644 and organic calcium antagonists on the mouse isolated distal colon. *Br. J. Pharmacol.*, **94**, 1198–1206.

GORDON, J.L. (1986). Extracellular ATP: effects, sources and fate. *Biochem. J.*, **233**, 309–319.

GYLFE, E. & HELLMAN, B. (1987). External ATP mimics carbachol in initiating calcium mobilization from pancreatic β-cells conditioned by previous exposure to glucose. *Br. J. Pharmacol.*, **92**, 281–289.

HOURANI, S.M.O., WELLFORD, L.A. & CUSACK, N.J. (1985). L-AMP-PCP, an ATP receptor agonist in guinea-pig bladder, is inactive on taenia coli. *Eur. J. Pharmacol.*, **108**, 197–200.

IACOVOU, J.W., HILL, S.J. & BIRMINGHAM, A.T. (1988). Involvement of extracellular calcium in agonist-induced contraction of the guinea-pig detrusor. *Br. J. Pharmacol.*, **95**, 848P.

INOUE, R. (1990). Purinergic receptor-operated currents recorded from single isolated cells of guinea-pig urinary bladder. *J. Physiol.*, **424**, 22P.

IRAVANI, M.M., LUHESHI, G.N. & ZAR, M.A. (1988). Inhibition of noncholinergic motor transmission in isolated rat urinary bladder by nifedipine. *J. Physiol.*, **401**, 69P.

KASAKOV, L. & BURNSTOCK, G. (1983). The use of the slowly degradable analog, α,β-methylene ATP, to produce desensitization of the P_2-purinoceptor: effect on non-adrenergic, non-cholinergic responses of the guinea-pig urinary bladder. *Eur. J. Pharmacol.*, **86**, 291–294.

KASS, R.S. (1987). Voltage-dependent modulation of cardiac calcium channel current by optical isomers of Bay K 8644: implications for channel gating. *Circ. Res.*, **61**, (Suppl. I), 1–5.

KATSURAGI, T., USUNE, S. & FURUKAWA, T. (1990). Antagonism by nifedipine of contraction and Ca^{2+}-influx evoked by ATP in guinea-pig urinary bladder. *Br. J. Pharmacol.*, **100**, 370–374.

KOLB, H-A. & WAKELAM, M.J.O. (1983). Transmitter-like action of ATP on patched membranes of cultured myoblasts and myotubes. *Nature*, **303**, 621–623.

LEE, R.T., SMITH, T.W. & MARSH, J.D. (1987). Evidence for distinct calcium channel agonist and antagonist binding sites in intact cultured embryonic chick ventricular cells. *Circ. Res.*, **60**, 633–691.

MACKENZIE, I., MANZINI, S. & BURNSTOCK, G. (1988). Regulation of

voltage-dependent excitatory responses to α,β-methylene ATP, ATP and non-adrenergic nerve stimulation by dihydropyridines in the guinea-pig vas deferens. *Neurosci.*, **27**, 317–332.

MAGGI, C.A., MANZINI, S., PARLANI, M., CONTE, B., GIULIANI, S. & MELI, A. (1988). The effect of nifedipine on spontaneous, drug-induced and reflexly-activated contractions of the rat urinary bladder: evidence for the participation of an intracellular calcium store to micturition contraction. *Gen. Pharmacol.*, **19**, 73–81.

MARCHENKO, S.M., VOLKOVA, T.M. & FEDOROV, O.I. (1987). ATP-activated ionic permeability in smooth muscle cells isolated from the guinea-pig urinary bladder. *Neirofiziologiya*, **19**, 95–100.

McMILLIAN, M.K., SOLTOFF, S.P., CANTLEY, L.C. & TALAMO, B.R. (1987). Extracellular ATP elevates intracellular calcium in rat parotid acinar cells. *Biochem. Biophys. Res. Commun.*, **149**, 523–530.

MILLER, R.J. (1987). Multiple calcium channels and neuronal function. *Science*, **235**, 46–52.

MOVSESIAN, M.A. & ADELSTEIN, R.S. (1984). Inhibition of turkey gizzard myosin light chain kinase activity by Bay K 8644. *Eur. J. Pharmacol.*, **103**, 161–163.

NONOTTE, I., MATHIEU, M-N. & CHEVILLARD, C. (1989). P_2-purinergic-induced ionised calcium elevation in the human promyelocytic cell line HL60. *Br. J. Pharmacol.*, **97**, 238P.

NOWYCKY, M.C., FOX, A.F. & TSIEN, R.W. (1985). Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature*, **316**, 440–443.

OMOTE, S., KIGOSHI, S. & MURAMATSU, I. (1989). Selective inhibition by nifedipine of the purinergic component of neurogenic vasoconstriction in the dog mesenteric artery. *Eur. J. Pharmacol.*, **160**, 239–245.

THOMAS, G., GROB, R. & SCHRAMM, M. (1984). Calcium channel modulation: ability to inhibit or promote calcium influx resides in the same dihydropyridine molecule. *J. Cardiovasc. Pharmacol.*, **6**, 1170–1176.

WHITE, T.D. (1988). Role of adenine compounds in autonomic neurotransmission. *Pharmacol. Ther.*, **38**, 129–168.

YATANI, A., TSUDA, Y., AKAIKE, N. & BROWN, A.M. (1982). Nanomolar concentrations of extracellular ATP activate membrane Ca channels in snail neurones. *Nature*, **296**, 169–171.

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