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Effect of pertussis toxin on A_1-receptor-mediated inhibition of insulin secretion

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Previous studies have provided evidence for the presence on B cell membrane of adenosine receptors (P_1-purinoceptors) of the A_1-subtype which inhibit insulin secretion. In this work we have investigated the implication of a guanosine triphosphate (GTP) binding protein (G protein) in the A_1 purinoceptor-induced inhibition of insulin secretion from the isolated perfused pancreas of the rat. A group of rats was treated with pertussis toxin ($10\,\mu g\,kg^{-1}$, i.v.) 28 h prior to pancreas extirpation. This treatment totally abolished the 50% decrease in insulin secretion induced by (+)-N^6-phenylisopropyl adenosine ($1.65\,\mu M$), a P_1-purinoceptor agonist. These results indicate that the A_1-receptor-mediated inhibition of insulin secretion involves a pertussis toxin-sensitive G protein.

Introduction Recent pharmacological studies have shown that the inhibitory effects of stable analogues of adenosine on insulin secretion are mediated via activation of P_1-purinoceptors of the A_1-subtype (Hillaire-Buys *et al.*, 1987). In several systems A_1-receptor activation leads to a decrease in adenylate cyclase activity; in islets of Langerhans, P_1 agonists have also been reported to decrease cyclic AMP (Campbell & Taylor, 1982). Furthermore guanosine triphosphate (GTP) binding proteins (G proteins) have largely been implicated in the coupling of various receptors to adenylate cyclase (Ui, 1984). As concerns the inhibitory input to adenylate cyclase, the G protein involved (Gi) is known to be inactivated by pertussis toxin via ADP-ribosylation (Ui *et al.*, 1984). Therefore, in an attempt to investigate the coupling of the A_1 inhibitory receptor of the B cell with a G protein, we have examined the effect of pertussis toxin (PTX) on the ability of a stable adenosine analogue, (+)-N^6-phenylisopropyladenosine (D-PIA), to inhibit insulin secretion from the isolated perfused pancreas of the rat.

Methods Male Wistar rats weighing 350–370 g and fed *ad libitum* were used. Pertussis toxin ($10\,\mu g\,kg^{-1}$) or equivalent volume of vehicle was injected into the jugular vein under light pentobarbitone anaesthesia ($20\,mg\,kg^{-1}$). Twenty-eight hours later animals were anaesthetized with sodium pentobarbitone ($60\,mg\,kg^{-1}$) by intraperitoneal injection. The pancreas was isolated and perfused as previously described (Loubatières *et al.*, 1969). The pancreas was totally isolated from all neighbouring tissues and organs; it was perfused through its own arterial system with a Krebs-Ringer bicarbonate buffer containing purified bovine serum albumin ($2\,g\,l^{-1}$) and glucose (8.3 mM) throughout the experiment from time zero. A mixture of O_2 (95%) and CO_2 (5%) was bubbled through this medium at atmospheric pressure. The pH of the solution was 7.35–7.40. The preparation was maintained at 37.5°C. Each organ was perfused at a constant pressure. The pressure was selected so as to produce a flow rate of $2.5\,ml\,min^{-1}$ at the start of the experiment. The first sample was taken after an equilibration period of 45 min. The 60 min sample, taken just before the agonist administration, represents the reference sample. Each sample was taken over 1 min and immediately frozen for insulin radioimmunoassay. Insulin was assayed by the method of Herbert *et al.* (1965) using an antibody supplied by Miles Laboratories (Paris). ^{125}I-insulin was obtained from International CIS (Gif-sur-Yvette, France). The standard used was pure rat insulin (Novo, Copenhagen, Denmark) whose biological activity was $22.3\,\mu u\,ng^{-1}$. The intra- and inter-assay coefficients of variations were respectively 9.7% and 14.4%. The analytical sensitivity was $0.2\,ng\,ml^{-1}$.

The insulin output rate per minute was determined by multiplying the hormone concentration ($ng\,ml^{-1}$) by the outflow rate ($ml\,min^{-1}$). Insulin values are expressed in the text and figure as a percentage of the 60 min time value, taken as 100%.

The results are expressed as means ± s.e.mean. They were submitted to analysis of variance by the multiple comparison test (Zar, 1974).

(+)-N^6-phenylisopropyl adenosine (D-PIA; also known as S-PIA) was purchased from Boehringer (Mannheim, FRG, Cat. No. 713015) and pertussis toxin from List Biological Laboratories (Campbell, CA, U.S.A.).

Results Treatment of rats with pertussis toxin ($10\,\mu g\,kg^{-1}$) did not produce any change in animal body weight.

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The concentration of $1.5\,g\,l^{-1}$ glucose in the perfusion liquid induced a moderate stimulation of insulin secretion. At time 60 min, taken as 100%, the insulin output rate was 24.62 ± 6.51, 24.27 ± 5.14 and $23.10 \pm 8.20\,ng\,min^{-1}$ for control, untreated and PTX pretreated rats respectively. So, the *in vivo* treatment with PTX did not modify the insulin response to glucose $1.5\,g\,l^{-1}$.

Figure 1 shows that, in untreated rat pancreas, D-PIA at a concentration of 1.65 μM induced a fall in insulin output rate. This decrease was immediate and reached at 10 min about −50% of the basal value ($P < 0.001$). After stopping the infusion of D-PIA, the insulin output rate returned to the control value.

In the experiments performed with the PTX pretreated rats, the inhibitory effect of D-PIA on insulin secretion was completely abolished; insulin output profiles were comparable to those recorded in control rats.

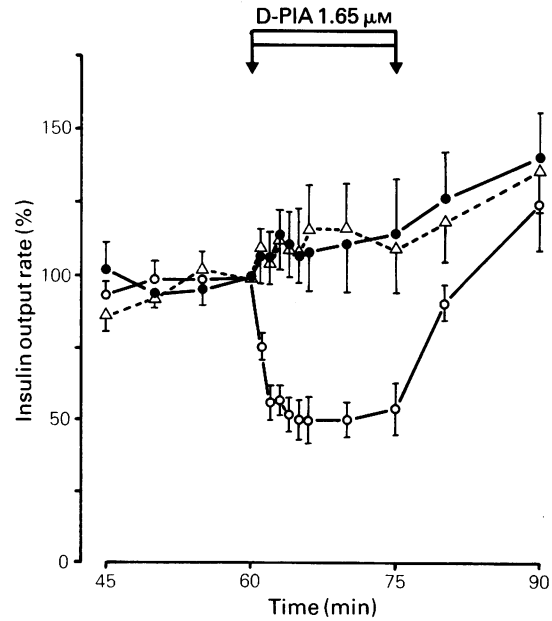


Figure 1 Effect of (+)-N^6-phenylisopropyl adenosine (D-PIA) 1.65 μM on insulin output rate in untreated (○) and pertussis toxin pretreated rats (●) ($n = 9$). Controls are shown, (△), $n = 5$. The perfusion medium contained 8.3 mM glucose. Insulin output rate is expressed as a percentage in relation to the value at time 60 min, taken as 100%. Each point represents the mean with s.e.mean shown by vertical bars.

Discussion Pertussis toxin is generally used as a tool to study the role of various G proteins in membrane transduction mechanisms. Our results clearly show that the inhibitory effect of D-PIA is abolished by pretreatment of rats with pertussis toxin. Thus the A_1 inhibitory receptor of the B cell is coupled with a PTX-sensitive G protein. It is known that pertussis toxin inactivates several GTP binding proteins (Stryer & Bourne, 1986) including the Gi protein involved in the inhibition of adenylate cyclase and other G proteins not involved in the regulation of this enzyme. Campbell & Taylor (1982) have shown that adenosine and its analogues decreased cyclic AMP levels in islets; consequently it can be suggested that the G protein involved in the inhibitory effects of A_1 purinoceptor agonists on the B cell is probably a Gi protein.

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An anti-inflammatory steroid inhibits tissue sensitization by IgE *in vivo*

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An anti-inflammatory corticosteroid, dexamethasone, was found to be a potent suppressor of cell sensitization induced by IgE antibody in a model of type I allergic inflammation *in vivo*. Low doses ($\lesssim 10^{-10}$ mol) of dexamethasone, administered intradermally to rabbits 90 min before injection of IgE into the same sites, suppressed local oedema induced by a local challenge with antigen 72 h later. The effect was not due to anti-inflammatory activity persisting in the skin site for the 3 day period. This novel potent activity of the corticosteroid may be an important component of its anti-allergic effect in diseases such as asthma.

Introduction The anti-inflammatory action of glucocorticoids such as dexamethasone in allergic reactions is well established (Schleimer, 1985), although the precise mechanisms involved are unclear. One possibility based on *in vitro* experiments is that glucocorticoids inhibit mediator release from inflammatory cells, although it has been reported that such inhibition does not occur in human lung mast cells (Schleimer *et al.*, 1983). While investigating mechanisms involved in a model of allergic type I inflammation *in vivo*, the passive cutaneous anaphylactic reaction (PCA) in the rabbit, a novel activity of dexamethasone was discovered. We demonstrate here that tissue sensitization induced by IgE is markedly inhibited by the drug. This may represent an important component of corticosteroid action in allergic diseases.

Methods PCA reactions were elicited in the shaved dorsal skin of rabbits (male NZW, 2.5–3.0 kg) as previously described (Hellewell *et al.*, 1987). Briefly, IgE-containing rabbit antiserum raised against bovine gamma globulin (BGG) was depleted of contaminating IgG by use of a protein A sepharose column, diluted three fold in saline and injected intradermally in 0.1 ml volumes. After a sensitization (or fixation) period of 72 h, animals were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}) intravenously and ^{125}I-rabbit serum albumin (5 μCi per rabbit) mixed with Evans blue dye (10 mg in 0.5 ml of pyrogen-free saline per rabbit) was injected intravenously. BGG dissolved in pyrogen-free saline (0.9%) was injected intradermally (1 μg 0.1 ml^{-1}) into sensitized skin sites to induce local oedema formation. After 30 min the animals were killed with a sodium pentobarbitone overdose, the dorsal skin was removed and injection sites were punched out and counted in a gamma counter together with plasma samples. Oedema formation in skin sites was then expressed in terms of μl plasma.

Ninety minutes before the injection of IgE, dexamethasone (Organon) or saline as control, was injected intradermally. Dexamethasone was tested in doses of 10^{-12}, 10^{-11} and 10^{-10} mol per 0.1 ml volume. In some experiments to investigate a possible relationship to phospholipase inhibition (Flower & Blackwell, 1979) the cyclo-oxygenase inhibitor indomethacin (Merck, Sharp and Dohme) and a 5-lipoxygenase inhibitor REV 5901 (pentyl-3-(2-quinolinylmethoxy) benzene methanol Revlon) were tested for inhibitory effects against tissue sensitization.

Reversed PCA reactions were elicited by injecting 100 μg per site of porcine anti-IgE (human) antibody (Nordic Immunological Laboratory) intradermally to cross-link naturally occurring IgE (Kravis & Henson, 1977). This induced an immediate oedema response and the effect of dexamethasone administered 73.5 h previously to the same sites was measured.

Results are expressed as mean ± s.e.mean. Six replicate injections were used for each treatment in each rabbit. A statistically significant difference between means was taken as $P \lesssim 0.05$ using the Wilcoxon rank sum test.

Results As shown in Figure 1, dexamethasone induced a dose-related inhibition of local oedema in the PCA reaction when administered intradermally 90 min before the injection of IgE. Dexamethasone was potent: a 59% inhibition was observed with a dose of 10^{-10} mol per site in this experiment. In a further study in six rabbits, dexamethasone at this dose induced an inhibition of 43 ± 5%, $P < 0.05$.

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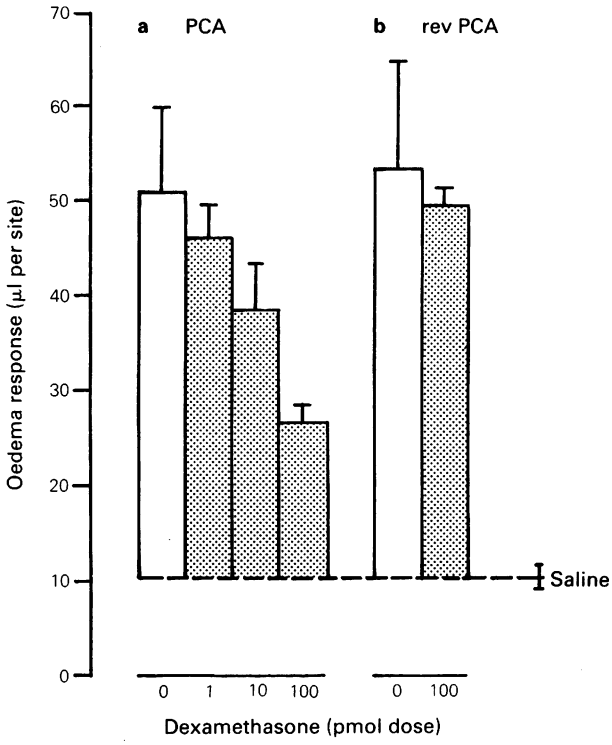


Figure 1 An experiment showing suppression of passive cutaneous anaphylactic (PCA) reactions by local administration of dexamethasone before sensitization (a). Inhibition is significant at 10 and 100 pmol doses ($n = 6$ sites, $P < 0.05$). In a further six rabbits dexamethasone induced a $43 \pm 5\%$ suppression ($P < 0.05$). Dexamethasone did not suppress the reversed PCA reaction (b).

Neither indomethacin (10^{-8} mol $0.1\,ml^{-1}$) nor REV 5901 (10^{-7} mol $0.1\,ml^{-1}$) significantly inhibited the PCA reaction when administered at the time of the IgE injection (results not shown).

It seemed possible (if unlikely) that the anti-inflammatory effect of the corticosteroid persisted in the skin for the 3 day antibody fixation period. Indeed, dexamethasone (10^{-10} mol per site) did suppress oedema in the PCA when administered 90 min before the local antigen challenge (i.e. PCA with saline pretreatment $78 \pm 11\,\mu l$, PCA with dexamethasone pretreatment $39 \pm 8\,\mu l$, $P < 0.05$ $n = 6$ rabbits). To test for persistence of the dexamethasone effect over 73.5 h, a reversed PCA was induced. As shown in Figure 1b the reversed PCA response was unaffected by steroid pretreatment for 73.5 h.

Discussion Low doses of a corticosteroid can inhibit tissue sensitization induced by IgE. Although dexamethasone could also suppress the PCA when administered at the end of the 3 day fixation period, possibly by an effect on microvascular permeability (Tsurufuji *et al.*, 1979), the results with the reversed PCA reaction show that a persistence of the anti-inflammatory effect over 3 days in the skin would not account for the inhibition of oedema observed. Further, as the anti-IgE cross-links naturally-occurring IgE fixed to skin mast cells, this suggests that the corticosteroid can inhibit antibody fixation, but cannot displace antibody once fixed.

It would be technically difficult to carry out similar experiments in man. Inhibitory effects of corticosteroids on Fc receptors of human leukocytes have, however, been observed *in vitro* (Crabtree *et al.*, 1979; Spiegelberg *et al.*, 1979; Yodoi *et al.*, 1981). We suggest, therefore, that inhibition of sensitization by IgE involving mast cell Fc receptors and possibly those of other tissue cells may be an important component of the action of corticosteroids in diseases such as allergic asthma.

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Anaesthetic potencies of primary alkanols: implications for the molecular dimensions of the anaesthetic site

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1 We have redetermined the anaesthetic potencies (EC_{50}s) for a series of primary alkanols, to resolve uncertainties about the molecular dimensions of the anaesthetic site resulting from the use of data from different laboratories.

2 For each alkanol, concentration-response relationships for loss of righting reflex (LRR) were plotted for over one hundred tadpoles, and the median effective concentrations determined. Aqueous concentrations present during potency assays were determined independently, and for alkanols with chain length greater than nonanol, correction was made for depletion from the aqueous phase.

3 The EC_{50}s were found to decrease logarithmically with increasing number of carbon atoms in the hydrocarbon chain of the alkanol (C_N), such that, on average, each additional methylene group was associated with an approximately four fold increase in potency.

4 The relationship between log EC_{50} and C_N was best described by the quadratic equation, $\log EC_{50} = 0.022\ (\pm 0.0038)\ C_N^2 + 0.76\ (\pm 0.051)\ C_N + 3.7\ (\pm 0.14)$ ($r^2 = 0.9951$).

5 A previously described correlation between the apparent changes in the free energy of binding of an additional methylene group both to luciferase and to the sites for LRR in tadpoles was not confirmed.

6 A cut-off in potency beyond dodecanol was established in experiments where tadpoles were maintained in supersaturated solutions of tridecanol for 20 h without demonstrable LRR.

7 These findings indicate that the soluble enzyme firefly luciferase does not adequately model the anaesthetic site. Specifically, there are discrepancies in the position of cut-off, and the apparent changes in the free energy of binding, per methylene group, of an alkanol to luciferase do not parallel that for tadpoles.

Introduction

It is still not known whether general anaesthetics produce their effects by acting on central nervous system neurotransmitter receptor proteins directly, or indirectly through the lipids which surround these receptors. One approach to resolve these alternatives is to apply pharmacological criteria derived from observations of anaesthesia in animals to well-characterized model systems. For example, a particularly useful criterion is the 'cut-off' in anaesthetic potency, that is, the aburpt loss of potency among the higher molecular weight members of homologous families of anaesthetics. The primary alkanols are one such series that can be used to probe structure-activity relationships in these model systems. In animals, cut-off was thought to occur beyond tridecanol at tetradecanol (Meyer & Hemmi, 1935; Pringle *et al.*, 1981), but it remains to be established whether cut-off occurs due to depletion of the aqueous alkanol concentration or loss of intrinsic anaesthetic activity (Lee, 1976).

Proponents of the protein-based theories have used data obtained with the lipid-free enzyme, firefly luciferase, to argue that anaesthetics act directly at central nervous system receptors. Specifically, luciferase not only manifests a cut-off in inhibitory potency for the primary alkanols and alkanes, but also demonstrates changes in the free energy for binding of alkanols which parallel those found in

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animals (Franks & Lieb, 1985). However, the validity of this latter point has recently been questioned (Elliott & Haydon, 1986) because the potencies of the primary alkanols *in vivo* are not known with a high degree of precision. Problems with these values in the literature are that: (i) data have never been derived in a single laboratory for the complete homologous series of n-alkanols (Vernon, 1913; Meyer & Hemmi, 1935; Pringle *et al.*, 1981); (ii) the potencies were roughly estimated by a 'bracketing' method rather than quantal concentration-response analysis, so that no standard errors could be derived; and (iii) measurements of the actual free concentration of alkanol in solution are lacking. This is especially important for the higher molecular weight compounds where adsorption might lead to significant depletion of the free concentration. Although such values in the literature may be useful for a first approximation, they are inadequate to define the fine structure of their molecular targets. Therefore, we have systematically determined the loss of righting reflex (LRR) potencies in tadpoles for the complete homologous series of primary aliphatic alkanols. With these values, we show that *in vivo* potency data from tadpoles do not parallel the inhibition of firefly luciferase by the primary alkanols, and we firmly establish the position of the cut-off in anaesthetic potency for the series of primary alkanols.

Methods

Early pre-limb bud tadpoles, *Rana pipiens*, approximately 1.0–1.5 cm in length (Carolina Biological Supply Co., Burlington, NC), were used for determination of anaesthetic concentration-response curves as previously described (Alifimoff *et al.*, 1987). Briefly, groups of five tadpoles were placed in covered 100 ml beakers in neutral, oxygenated, aqueous solutions of alkanols prepared in twice distilled water. No tadpole was used more than once. Anaesthesia was defined as the loss of righting reflex (LRR). After allowing 15 to 240 min for equilibration with the alkanol, the tadpoles were tipped manually with a flame polished glass pipette. Unresponsiveness for greater than 5 s was scored as LRR, and this test was repeated at 15 min intervals. After each experiment, all tadpoles were placed in containers of neutral distilled water until recovery of righting reflex was confirmed.

[^{14}C]-tetradecanol was assayed for its ability to induce LRR in three ways. First, two groups of five tadpoles each were incubated for 20 h in separate beakers containing 100 ml of oxygenated, saturated solutions, and then assayed for LRR. Samples of these solutions were obtained for liquid scintillation counting at 30 min intervals for the first 4 h, and then again at 20 h. Second, ten tadpoles were incubated in a beaker containing 500 ml of an oxygenated, supersaturated solution for 72 h; both LRR and aqueous concentration were assayed at 24 h intervals. Thirdly, to determine whether tetradecanol shifted the concentration-response curve of another anaesthetic, tadpoles were preincubated in an oxygenated, supersaturated solution of tetradecanol for 48 h (50 ml of solution per animal), then assayed for their responses to [^{14}C]-octanol. Tridecanol was also assayed for its ability to induce LRR. Two groups of ten animals were incubated in 1000 ml of an oxygenated, saturated solution of tridecanol and in 1000 ml of a supersaturated solution prepared from an ethanolic stock solution. Tadpoles were assayed for LRR at hourly intervals for 6 h and then again at 20 h. Samples were obtained for determination of free aqueous concentrations by gas chromatography at the start of the experiment and at 20 h.

Concentrations of alkanols present during assays were verified either by gas chromatography or liquid scintillation spectroscopy. Concentrations of the short chain alkanols (C_1–C_9; C_N = number of carbon atoms in the hydrocarbon chain of the alkanol) were measured with a Beckman GC 72B Gas Chromatograph (Porapak P column packing (Waters Co., Milford, MA) with column temperatures ranging from 130 to 215°C), while concentrations of undecanol and tridecanol were measured on a Varian 3700 Gas Chromatograph (Dexsil-300 column packing (Supleco Inc., Bellfonte, PA) with column temperatures from 140 to 200°C). Actual concentrations of the short chain alkanols were within 5% of their nominal values during the time course of assays. For the long-chain alkanols (C_{10}–C_{14}), the relationship between nominal and measured concentrations during concentration-response assays was more complex, and is described in the Results section.

For all agents, concentration-response assays were performed using a minimum of 10 animals at each of 5 different concentrations. Entire studies were repeated at least once, and the data pooled. The respective effective concentrations (EC_{50}) and slopes were obtained using a logistic method for quantal responses (Waud, 1972).

With the more potent agents, it was convenient to make aqueous solutions of alkanols from ethanolic stock solutions. To determine whether this made any difference in potency, the EC_{50} of [^{14}C]-octanol was determined when alkanol solutions were prepared (i) by dilution from ethanolic stock solutions, (ii) directly in water or (iii) from a [^{14}C]-alkanol directly in water. EC_{50}s obtained for octanol by each method were compared. The estimated variance in the respective EC_{50} values was calculated from the

Table 1 Time-course of [^{14}C]-tetradecanol depletion during a concentration-response assay

Time (h)	[^{14}C]-*tetradecanol* (μmol l^{-1} water)	% *LRR*
0	48.5*	0
24	43.1	0
48	22.2	0
72	12.7	0

See Methods for details. LRR = loss of righting reflex.
* Saturated solubility = 1.5 μM (Bell, 1973).

standard errors; the sum of these then yielded the estimated variance of the difference in the EC_{50} between the octanol prepared from an ethanolic stock solution, and radiolabelled or unlabelled octanol prepared directly in water. The ratio of these differences to their respective standard errors was then referred to a standard normal distribution. Statistical significance was assumed at the level of $P < 0.05$.

Drugs and solutions

All alkanols were the highest quality available from their respective manufacturers. Aqueous solutions of methanol (Mallinckrodt, Paris, KY; purity 99.8%), ethanol (Pharmco, Dayton, NJ; >99%), propanol (Mallinckrodt, St. Louis, MO; 99.8%), butanol (Baker, Phillipsburg, NJ; 99.7%), pentanol (Aldrich, Milwaukee, WI; 99%), and hexanol (Sigma, St. Louis, MO; 99%) were prepared in gas-tight vials by stirring weighed aliquots of each compound into twice distilled water for 1 h at room temperature. Solutions of heptanol (Eastman Kodak, Rochester, NY; 99%) and octanol (NuChek Prep, Elysian, MN; >99%) were prepared by stirring for longer (>12 h) in gas-tight vials at 40°C. Solutions of undecanol and tridecanol (NuChek Prep; >99%) were prepared from ethanolic stock solutions. Gas chromatography revealed no impurities in saturated stock solutions of these compounds.

[^{14}C]-octanol (4.5 Ci mol^{-1}), [^{14}C]-decanol (7.5 Ci mol^{-1}), [^{14}C]-dodecanol (56 Ci mol^{-1}) (ICN Biomedicals, Irvine, CA) and [^{14}C]-tetradecanol (58 Ci mol^{-1}) (NEN, Boston, MA) were used to determine free aqueous concentrations of the longer chain alkanols during concentration-response assays. Radiochemical purities were >98.5% as determined by thin layer chromatography, high performance liquid chromatography and/or autoradiography by the manufacturer. Aqueous solutions of each radiolabelled alkanol were prepared by diluting concentrated stock solutions made in ethanol; final ethanol concentrations were always <25 mM. Samples were counted in Liquiscint (National Diagnostics, Manville, NJ), and efficiency was determined using

Table 2 Loss of righting reflex potency of primary alkanols in *Rana pipiens* tadpoles at 20 ± 1°C

Alcohol	EC_{50} ± s.e.	*Slope* ± s.e.	n/*T*
Methanol	590 ± 41 mM	2.7 ± 0.44	3/170
Ethanol	190 ± 16 mM	2.3 ± 0.31	4/270
Propanol	73 ± 2.4 mM	7 ± 1.1	3/170
Butanol	10.8 ± 0.77 mM	2.2 ± 0.34	3/230
Pentanol	2.9 ± 0.11 mM	5.0 ± 1.2	3/150
Hexanol	570 ± 37 μM	3.9 ± 0.72	2/120
Heptanol	230 ± 11 μM	6 ± 1.1	2/140
Octanol	57 ± 2.5 μM	5 ± 1.0	3/150
Octanol[a]	55 ± 3.1 μM	3.6 ± 0.50	4/220
Octanol[b]	59 ± 3.1 μM	5 ± 1.1	3/170
Nonanol	37 ± 2.4 μM	2.3 ± 0.40	4/250
Decanol[a,b]	12.6 ± 0.48 μM	5.4 ± 0.81	3/170
Undecanol[a]	8.1 ± 0.81 μM	2.5 ± 0.49	2/110
Dodecanol[a,b]	4.7 ± 0.33 μM	3.1 ± 0.47	3/160
Tridecanol[a]	Not anaesthetic		
Tetradecanol[a]	Not anaesthetic		
Range:	126,383 ×		

EC_{50} was determined after 15–30 min for methanol through to nonanol; after 60 min for decanol and undecanol, and after 120 min for dodecanol. See Methods for curve-fitting procedure and estimation of standard errors. All concentrations were verified by gas chromatography unless otherwise noted.
n = number of experiments, T = total number of animals in *n* experiments.
[a] Aqueous alkanol solutions were prepared from concentrated ethanolic stock solutions; the final concentration of ethanol was <25 mM. See Methods for details.
[b] A [^{14}C]-alkanol was used to measure the aqueous concentrations.

quenched samples of [^{14}C]-toluene as an external standard.

Results

The short-chain alkanols (C_1–C_9) produced a reversible LRR in tadpoles which plateaued in 10 min and remained unchanged when assayed at 15 and 30 min. For decanol and undecanol, a steady LRR response was not observed until 60 min; for dodecanol, 120 min was required. Accordingly, the EC_{50} values presented for decanol and undecanol were obtained at 60 min, while the EC_{50} value for dodecanol was obtained at 120 min. In contrast, tridecanol did not produce LRR during 20 h of exposure to both saturated and supersaturated solutions. Tetradecanol, on the other hand, did not produce LRR in tadpoles within 72 h even when concentrations in excess of saturation were present (Table 1). At intervals greater than 72 h, tetradecanol was irreversibly toxic. However, preincubation of tadpoles in tetradecanol for 48 h, did not shift their response to octanol (EC_{50}: 52 ± 5.2 with preincubation vs 59 ± 3.1 μM without preincubation; $P > 0.19$).

For octanol, results monitored by gas chromatography were in close agreement with those obtained using [^{14}C]-octanol (Table 2). However, the free aqueous concentrations of [^{14}C]-dodecanol decreased by about 15% during 2 h of incubation with tadpoles (Figure 1). Depletion of [^{14}C]-dodecanol from aqueous solution continued between 2 and 4 h, but resulted in little further change in either the aqueous concentration or in EC_{50} values (4.7 ± 0.33 (s.e.) vs 4.6 ± 0.73 μM, determined at 2 and 4 h, respectively, $P > 0.80$). In comparison, free aqueous concentrations of [^{14}C]-tetradecanol decreased by approximately 18% and 30% at 2 and 4 h, respectively. Depletion of free aqueous concentrations continued so that there was approximately 13, 50, and 68% depletion of decanol, dodecanol and tetradecanol, respectively, at 20 h.

Final concentrations of ethanol present during concentration-response assays for the long-chain alkanols (C_{10}–C_{14}) were always <25 mM; however, even as much as 40 mM ethanol does not produce LRR in tadpoles. Moreover, when results from assays performed in the presence and absence of 25 mM ethanol were compared (Table 2), it was clear that small concentrations of ethanol did not shift the concentration-response curves of other alkanols. There was no significant difference in either the EC_{50} or slope of concentration-response curves for octanol in the presence or absence of 25 mM ethanol (EC_{50}: 55 ± 3.1 vs 57 ± 2.5 μM, respectively, $P > 0.61$; slope: 3.6 ± 0.50 vs 5 ± 1.0 respectively, $P > 0.66$). Likewise, the EC_{50} (59 ± 3.1 μM, $P > 0.62$) and slope (5 ± 1.1, $P > 0.16$) for [^{14}C]-octanol were not different (Table 2).

The EC_{50}s for the series of primary alkanols, standard errors, and slopes are presented in Table 2. The EC_{50}s decreased in the series from methanol through to dodecanol, such that dodecanol was some 126,000 times more potent than methanol. The log concentration-response curves were sigmoidal with slopes ranging from 2.3 ± 0.40 to 7 ± 1.1 (Figure 2), but no systematic trends (for example, as a function of C_N) were observed. Similar degrees of

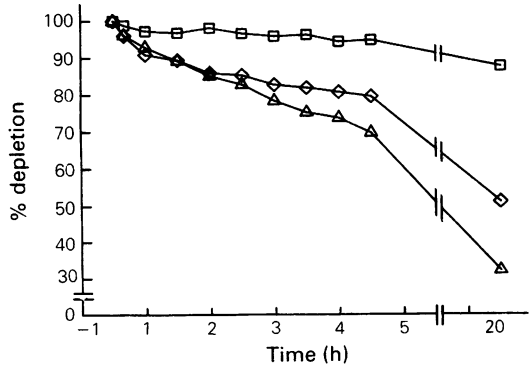


Figure 1 Time-course for depletion of long-chain alkanols from aqueous solution; decanol (□), dodecanol (◇), and tetradecanol (△). Each point represents the mean of five separate determinations during a single experiment under the conditions of the concentration-response assay (see Methods for details). In all cases, the standard deviations were <10% of the mean values presented.

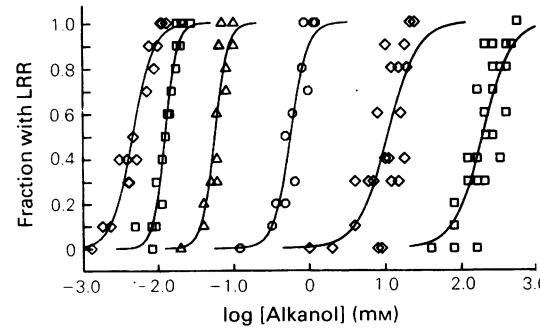


Figure 2 Log concentration-response relationships for the primary alkanols with an even number of carbon atoms. Dodecanol is the left-most, while ethanol is the farthest to the right. Curves were fitted according to the method of Waud (1972). Each symbol represents the mean response of ten animals, scored quantally as described in Methods. In some instances, symbols overlap. The total number of animals tested appears in Table 2. LRR = loss of righting reflex.

variation in the slopes of concentration-response curves for tadpoles have been previously found in our laboratory (Dodson *et al.*, 1985; Alifimoff *et al.*, 1987).

The coefficients of variation in EC_{50} values (s.e./EC_{50}) × 100%) ranged from 3.3 to 9.9%, which are comparable to those previously obtained by us (Pringle *et al.*, 1981; Dodson *et al.*, 1985; Alifimoff *et al.*, 1987) and by others measuring either righting response or minimum alveolar concentration (MAC) in mice (Deady *et al.*, 1980), or MAC in man (Saidman *et al.*, 1967). A similar degree of variation was found between experiments. For example, the mean of five individual ethanol concentration-response experiments which were fitted independently was 190 ± 22 mM (s.e.).

The relationship between EC_{50} and the alkanol carbon chain length (C_N) was logarithmic (Figure 3a), and a least-squares line fitted to log EC_{50} versus C_N had a slope of −0.49 ± 0.024 (s.e.) ($r^2 = 0.9764$). The deviations of the observed values for EC_{50} from the fitted line were assessed by calculating the residual of each point (Figure 3b). This suggested that the data might be better fitted by a quadratic rather than a linear equation. Fitting the data to a quadratic equation (log EC_{50} = 0.022 (±0.0038) C_N^2 ± 0.76 (±0.051) C_N + 3.7 (±0.14) ($r^2 = 0.9951$)) resulted in a significantly better fit ($P < 0.002$; F test).

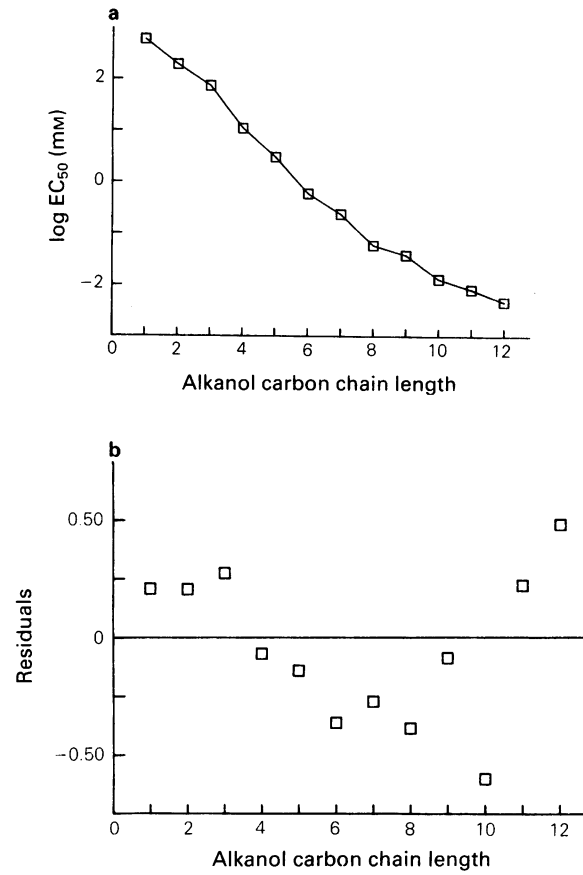


Figure 3 (a) Relationship between log EC_{50} and the number of carbons in the hydrocarbon chain of the primary alkanols (C_N). Error bars are smaller than the symbols. (b) Residual analysis of (a). The residuals (y axis) are the difference between the observed value of log EC_{50} and the calculated value from a linear fit of the data in (a).

Discussion

This study was undertaken to resolve deficiencies in the anaesthetic potency values for the primary alkanol series available from the literature. The problems mentioned in the Introduction have been addressed in this study as follows: (i) the EC_{50}s presented in Table 2 represent the only complete series of anaesthetic potencies for primary alkanols from a single laboratory; (ii) the potencies were derived by analysis of quantal concentration-response curves so that estimates of standard errors around both EC_{50}s and slopes could be made; and (iii) free aqueous concentrations of all alkanols were monitored either by gas chromatography or liquid scintillation spectroscopy. Finally, monitoring the free concentrations allowed the exact position of the cut-off in anaesthetic potency of primary alkanols to be established.

Any model of the anaesthetic site should share the pharmacological properties of general anaesthesia itself, including pressure reversal (Lever *et al.*, 1971); lack of stereoselectivity between enantiomeric pairs of secondary alkanols (Alifimoff *et al.*, 1987); and cut-off (Janoff & Miller, 1982). Anaesthetic cut-off has been observed in several series of homologous compounds, including the primary alkanols, alkanes, and fluorocarbons (for a review see Miller, 1987). While luciferase has also been shown to manifest a cut-off in inhibitory potency for the primary alkanols, this occurs at a higher molecular weight member of the series (beyond hexadecanol) than in animals (beyond dodecanol). One conceivable explanation for this discrepancy involves the depletion of anaesthetic from the aqueous phase, wherein the cut-off *in vivo* might actually occur at a longer chain length than previously observed. Our data, however, clearly establish that cut-off *in vivo* is not an artifact of depletion and that it occurs at tridecanol. This was demonstrated by the inability of both tridecanol and tetradecanol to produce LRR. Tridecanol did

Table 3 Comparison of anaesthetic potencies of the primary alkanols obtained by different investigators

C_N	Vernon (1913) (mM)	Meyer & Hemmi (1935) (mM)	Pringle *et al.* (1981) (mM)	Present study (mM)
1	990	—	—	590
2	410	330	120	190
3	104	110	54	73
4	22.3	30	12	11
5	—	7.0	—	3
6	0.9	—	0.7	0.57
7	0.35	0.38	—	0.23
8	0.127	0.13	0.06	0.057
9	—	0.025	—	0.037
10	—	0.010	0.013	0.013
11	—	0.005	—	0.0081
12	—	0.0075	0.0054	0.0047
13	—	—	0.037	Not anaesthetic

Dashed lines indicate that values were not determined.

not induce LRR after exposure of tadpoles for 20 h to either saturated or supersaturated aqueous solutions of tridecanol. Even after 20 h of exposure to supersaturated solutions, aqueous concentrations still remained above saturated solubility (Bell, 1973). Likewise, tetradecanol did not induce LRR despite (i) long-term exposure of tadpoles to solutions containing in excess of 0.9 μM, and (ii) exposure for 72 h to supersaturated solutions of [^{14}C]-tetradecanol (Table 1). Although 74% of the total amount of [^{14}C]-tetradecanol initially added was depleted during the 72 h, the aqueous phase still contained more tetradecanol than the published saturated solubility of approximately 1.5 μM (Bell, 1973).

The observed percentage depletion increased with the alkanol chain length. *A priori*, this might arise from increasing lipophilicity and surface activity coupled with decreasing aqueous solubility. Simple calculations show that uptake into the fatty tissues of the tadpoles would account for much of the depletion within the range of error of the published lipid-buffer partition coefficients (Sallee, 1978; Franks & Lieb, 1986).

When our results are compared to those previously obtained by Pringle *et al.* (1981), the majority of their values are within the precision of our measurements. However, the values for both ethanol and propanol observed by Pringle *et al.* (1981) were significantly lower than in the present study. In addition, they demonstrated that tridecanol was a partial anaestheic, but in the present study, it did not induce LRR even after prolonged exposure to supersaturated solutions. Because the purities of the anaesthetics used in that study as well as their free aqueous concentrations were not monitored by gas chromatography, we can only explain the discrepancies by possible contamination with potent higher molecular weight anaesthetic alkanols.

That the relationship between log EC_{50} and C_N is best described by a quadratic equation rather than a straight line can be interpreted to mean that the approach to cut-off is gradual rather than abrupt, whereas the phenomenon of cut-off itself is even more abrupt than previously appreciated, in that potency disappears completely after dodecanol, i.e., there are no partial anaesthetics. This gradual approach to cut-off implies that the intrinsic anaesthetic efficacy of dodecanol per methylene group is less than that of the other alkanols. Whether this somewhat gradual loss of potency per methylene group occurs in other homologous series of anaesthetics which display cut-off remains to be investigated.

While the approach to cut-off is gradual for the primary alkanols in that the higher molecular weight members of the series significantly deviate from a linear relationship, we found no such deviations from linearity in the region of hexanol and heptanol. Indeed a fit of the EC_{50}s of propanol through octanol showed a strong linear correlation ($r^2 = 0.9912$). This is in contrast to the results of Franks & Lieb (1985) who compared a collection of data for tadpole anaesthesia with the inhibitory potencies of primary alkanols on luciferase (IC_{50}). They observed that the chain-length dependence of the IC_{50}s levelled off in the region of hexanol and heptanol (i.e., these two alkanols were approximately equal in potency) and that the change in the free energy of binding ($\Delta\Delta G°$) of alkanols to luciferase appeared to parallel that found in animals. This was interpreted to mean that the alkanol binding site on luciferase, and the *in vivo* anaesthetic site, share

similar molecular dimensions. However, recalculation of their $\Delta\Delta G°$ with our self-consistent set of EC_{50}s shows that the reported correlation between anaesthetic activity in tadpoles and luciferase does not exist (Figure 4). The disparity between our EC_{50}s and those used by Franks & Lieb (1985) (Vernon, 1913; Meyer & Hemmi, 1935) are made apparent in Table 3. It appears that Vernon's and Meyer's laboratories employed different anaesthetic endpoints (suppression of spontaneous movement vs suppression of reflex response, respectively) as well as different indices of relative potencies (EC_{100} vs EC_{50}, respectively). In addition, as pointed out by Elliott & Haydon (1986), the value shown for pentanol (Vernon, 1913) is actually an estimate based on that of isopentanol. Thus, it would seem that combining these two sets of data for the purposes of molecular level arguments is inappropriate.

Linearity of log EC_{50} versus C_N for the lower molecular weight members of the series has been observed in mice (LRR for ethanol through hexanol, $r = 0.993$ (Lyons *et al.*, 1981)) and the freshwater shrimp *Gammarus* (butanol through octanol, $r^2 = 0.998$, Elliott *et al.*, 1987). In the latter study, hexanol and heptanol were not equipotent, as they are shown to be for luciferase. Thus whatever such anomalies observed in luciferase may imply about the molecular features of the alkanol site *in vitro*, inferences regarding the site *in vivo* must be made with caution.

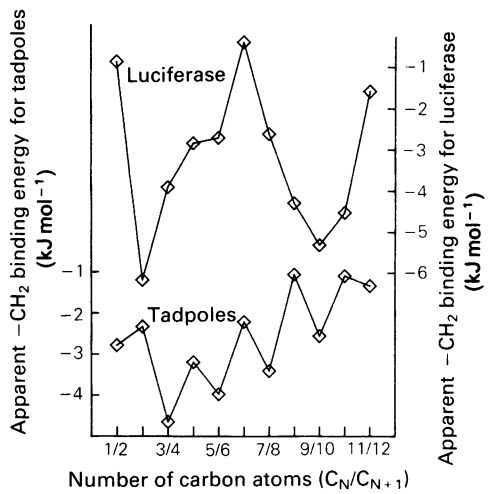


Figure 4 The apparent changes in the free energy of binding ($\Delta\Delta G°$) for a methylene group to luciferase (top) and for loss of righting reflex (LRR) in tadpoles (bottom) as a function of C_N/C_{N+1}. Note that the vertical axis scale for luciferase is shown on the right while that for tadpoles is shown on the left. Points were calculated using the differences in thermodynamic free energy,

$$\Delta\Delta G° = -RT \log[EC_{50}(N)/EC_{50}(N+1)]$$

for luciferase inhibition and LRR. The EC_{50} values for luciferase inhibition are from Franks & Lieb (1985 and personal communication). The EC_{50} values for LRR are from Table 2.

One previous explanation for cut-off, derived from lipid based theories of anaesthesia, was based on the reported decrease in the membrane/buffer partition coefficient for tetradecanol, such that the maximum membrane concentration obtained was less than that necessary for anaesthesia (Pringle *et al.*, 1981). However, recent data obtained in cholesterol-containing bilayers (Franks & Lieb, 1986) revealed that partition coefficients continue to increase beyond dodecanol, making it unlikely that cut-off is a consequence of inability to achieve a sufficient membrane concentration. Therefore, either biomembranes behave differently in this respect from lipid bilayers, which seems unlikely, or the alternative explanation, that cut-off may arise from a loss in the intrinsic pharmacological efficacy must be considered (Pringle *et al.*, 1981; Janoff & Miller, 1982). Indeed, observation of a gradual loss of lipid disordering potency, or even an increase in order, by long chain alkanols in spin labelled lipid bilayers (Richards *et al.*, 1978) and postsynaptic membranes (L. Firestone, Miller, K.W., unpublished observations) support the latter alternative.

In summary, we present LRR potency values for the complete series of anaesthetic primary alkanols. In contrast to the previously published values, the aqueous concentrations of all the alkanols were monitored, and the EC_{50} values were obtained from quantal concentration-response relationships. The EC_{50}s show a relationship between potency and alkanol chain length from methanol through to dodecanol which is best described by a quadratic equation, suggesting that the *approach* to cut-off is gradual. However, after dodecanol potency abruptly cuts off in that there are no partial anaesthetics. Depletion of longer chain alkanols, while significant, is unable to account for the potency cut-off.

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Contractile responses to calcium chloride in rat aortic rings bathed in K^+-free solution are resistant to organic calcium antagonists

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1 In rat aortic rings, devoid of functional endothelium, suspended in a modified Krebs solution (KCl: 0 mM; $CaCl_2$: 0.63 mM), addition of $CaCl_2$ (0.89–10 mM) produced concentration-related increases in tension (E_{max} = 2.38 ± 0.10 g, EC_{50} = 2.31 ± 0.15 mM, n = 36).

2 The Ca^{2+} evoked contractile responses were not modified by cinnarizine (10 μM), diltiazem (1 μM), ryanodine (10 μM), verapamil (1 μM), or the dihydropyridines, nitrendipine (1 μM) and (−)-Bay K 8644 (0.003–0.3 μM).

3 Cobalt chloride (0.1–1 mM) competitively antagonized the Ca^{2+} concentration-response curve; the Schild plot (slope 1.08 ± 0.04), gave a pA_2 value of 3.3 ± 0.01 (n = 27). Nickel chloride (0.5–1 mM) displaced the Ca^{2+} concentration-response curve to the right, without an effect on the maximum response. Cadmium chloride (3–30 μM) depressed the maxima of concentration-response curves to Ca^{2+} with an IC_{50} of 15.5 ± 1.1 μM (n = 6).

4 Monochlorobenzamil (100 μM), a Na^+-Ca^{2+} exchange inhibitor, failed to modify the Ca^{2+}-induced contractions.

5 In conclusion, Ca^{2+} evoked concentration-related contractile responses of rat aortic rings bathed in a K^+-free medium; these effects were attenuated by the divalent cations cobalt, nickel and cadmium, but not modified by several organic calcium antagonists. The lack of effect of diltiazem verapamil and the dihydropyridines would suggest that, under these experimental conditions, extracellular Ca^{2+} enters the cytosol via pathways which are distinct from the slow (L-type) calcium channels.

Introduction

Contractile responses to calcium of smooth muscle preparations, preincubated in a Ca^{2+}-free bathing medium and exposed to a depolarizing concentration of KCl are often used to identify compounds possessing calcium channel antagonistic properties (Spedding & Cavero, 1984; Godfraind *et al.*, 1986). The rationale of this test is that the elevated concentration of K^+ produces cellular depolarization, which is accompanied by the opening of voltage-operated calcium channels, allowing extracellular calcium ions to enter the cytosol.

In the absence of extracellular K^+, the membrane of rat vascular smooth muscle has been demonstrated to be in a slightly depolarized state (Hermsmeyer, 1976). This finding may account for our observation that the calcium agonist, (−)-Bay K 8644, can induce Ca^{2+}-dependent contractile responses of rat aortic ring preparations bathed in K^+-free salt solution (Cavero & Lawson, 1987).

The aim of this study was to characterize pharmacologically the contractile responses of rat aortic rings, suspended in a K^+-free bathing medium, when exposed to increasing concentrations of Ca^{2+}.

Methods

The thoracic aorta of male Sprague-Dawley rats (300–350 g) was removed, cut into 6 rings (3 mm in length) and the endothelium destroyed by gently

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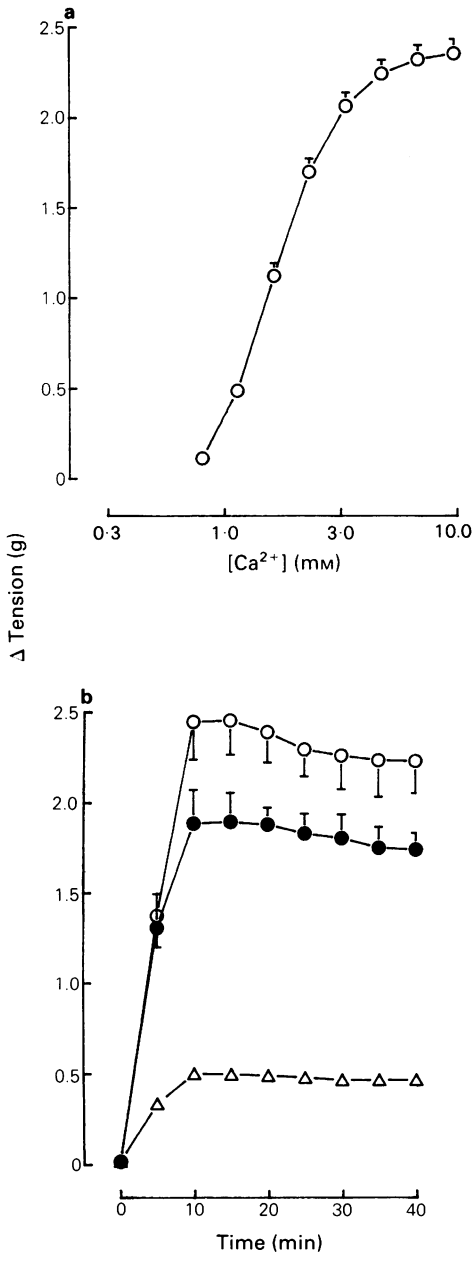


Figure 1 Effects of Ca^{2+} on rat aortic rings bathed in K^+-free medium containing 0.63 mM $CaCl_2$. (a) Cumulative concentration-response curve to Ca^{2+} (0.89–10 mM): $E_{max} = 2.38 \pm 0.10$ g, $EC_{50} = 2.31 \pm 0.15$ mM; $n = 36$. (b) Time-course effects of single Ca^{2+} additions (to achieve final bath concentration of 1.25 (△), 2.5 (●) and 5.0 (○) mM). The steady-state responses were significant increases in tension ($P < 0.05$: paired t test; $n = 6$ per group). Data represent mean values with vertical bars indicating s.e.mean.

rubbing the intimal surface with a small wooden stick (1.5 mm diameter). Each preparation was suspended between platinum hooks in 25 ml of K^+-free Krebs solution (composition in mM: NaCl 117, $CaCl_2$ 0.63, NaH_2PO_4 1.0, $MgSO_4 \cdot 7H_2O$ 1.2, glucose 11.5, $NaHCO_3$ 25) maintained at 37°C and gassed with 95% O_2 and 5% CO_2. An initial load of 2 g was applied to each preparation and maintained throughout a 30–45 min equilibration period. Tension was recorded on a Graphtec Linearcorder (Mk VII, WR 3101) via force-displacement transducers (Grass FT03c).

The lack of a relaxant response to acetylcholine (2 μM), in preparations previously contracted with noradrenaline (0.02 μM), indicated the absence of a functional endothelium (Furchgott & Zawadzki, 1980).

Cumulative concentration-response curves to $CaCl_2$ (0.89–10 mM) were constructed on preparations 20 min after exposure to (−)-Bay K 8644 (0.003–0.3 μM), cromakalim (10 μM), cadmium (3–30 μM), cobalt (0.1–1.0 mM), diltiazem (1.0 μM), monochlorobenzamil (100 μM), nickel (0.5–1.0 mM), nitrendipine (1.0 μM) or verapamil (1.0 μM) and 30 min after exposure to cinnarizine (10 μM) or ryanodine (10–30 μM). Control experiments (i.e., preparations pretreated with the corresponding vehicle) were carried out in a matched ring from the same animal; only one concentration-response curve to Ca^{2+} was obtained from each preparation.

Analysis of results

The EC_{50} values (i.e., the concentration of $CaCl_2$ required to produce 50% of the maximal response) and the E_{max} (maximal response evoked by $CaCl_2$) were calculated for each individual concentration-response curve to $CaCl_2$. pA_2 values were determined by the method of Arunlakshana & Schild (1959). pK_B values were calculated for individual concentrations of antagonist tested (Furchgott, 1972). All values in the text are expressed as means ± s.e.mean for n determinations.

Drugs and solutions

The following drugs were used: acetylcholine bromide (Sigma), (−)-Bay K 8644 (methyl-1,4-dihydro - 2,6 - dimethyl - 3 - nitro - 4 - (2 - tri - fluoromethylphenyl)-pyridine-5-carboxylate, synthesized by Dr Gueremy, Rhône-Poulenc Santé, Vitry-sur-Seine, France), cromakalim (synthesized by Dr Walsh, May & Baker Labs., Dagenham, U.K.), cadmium chloride (Prolabo, France), cinnarizine (synthesized by Dr Sarret, Rhône-Poulenc Santé), cobalt chloride (Prolabo, France), diltiazem chlorhy-

Table 1 Effects of calcium channel antagonists and other pharmacological agents on the contractile responses to Ca^{2+} of rat aortic rings, bathed in K^+-free medium containing 0.63 mM $CaCl_2$

Antagonist	*Conc.* (μM)	n	EC_{50} (mM)	E_{max} (g)
Control		36	2.31 ± 0.15	2.38 ± 0.10
Nitrendipine	1.0	5	2.94 ± 0.26	2.16 ± 0.14
Diltiazem	1.0	5	2.99 ± 0.27	2.07 ± 0.17
Verapamil	1.0	5	2.26 ± 0.18	2.42 ± 0.27
Cinnarizine	10.0	6	2.36 ± 0.18	2.26 ± 0.27
Monochlorobenzamil	100	9	2.67 ± 0.39	2.33 ± 0.13
Cromakalim	10.0	6	2.20 ± 0.14	1.98 ± 0.19
Ryanodine	10	6	1.87 ± 0.13	2.06 ± 0.2
	30	6	1.45 ± 0.1*	2.05 ± 0.11
(−)-Bay K 8644	0.003	8	2.39 ± 0.11	2.42 ± 0.25
	0.03	8	2.61 ± 0.23	2.22 ± 0.3
	0.3	8	2.12 ± 0.15	2.37 ± 0.32

Cumulative concentration-response curves to Ca^{2+} were constructed after exposure of the preparations to one of the treatments, or the corresponding vehicle (control). For each concentration-response curve to Ca^{2+}, EC_{50} and E_{max} values were calculated and are expressed as means ± s.e.mean for each treatment group. An asterisk indicates responses that were significantly ($P < 0.05$) different from the corresponding control.

drate (Synthélabo, Paris, France), monochlorobenzamil (synthesized by M. Peyronel, Rhône-Poulenc Santé), nitrendipine (synthesized by Dr Aloup, Rhône-Poulenc Santé), verapamil (synthesized by M. Hannotin, Rhône-Poulenc Santé) and ryanodine (Agrisystem International, Pennsylvania, U.S.A.).

Stock solutions (1 mg ml^{-1}) of nitrendipine, cromakalim and (−)-Bay K 8644 were prepared in absolute ethyl alcohol. Cinnarizine was dissolved in 0.01 N isethionic acid. All other drugs were dissolved, and all dilutions made, in distilled water.

Results

Effects of Ca^{2+} on aortic rings suspended in K^+-free bathing solution

In aortic rings suspended in a K^+-free bathing medium, containing 0.63 mM Ca^{2+}, cumulative addition of $CaCl_2$ (0.89–10 mM) produced concentration-related contractile responses (Figure 1). Addition of single concentrations of $CaCl_2$ to the bathing medium, to achieve final Ca^{2+} concentrations of 1.25, 2.5 or 5.0 mM, evoked contractile responses that attained their respective maxima within 10 min which were maintained for over 30 min (Figure 1).

Effects of inorganic calcium channel antagonists on the contractile responses to Ca^{2+}

Cobalt (0.1–1.0 mM) and nickel (0.5–1 mM) displaced the Ca^{2+} concentration-response curves to the right (Figures 2 and 3). The maximum responses to Ca^{2+} were not modified by Ni^{2+}; however, in the presence of Co^{2+}, the maxima to Ca^{2+} could not be determined due to solubility limitations. The Schild plot for Co^{2+} gave a pA_2 value of 3.3 ± 0.01 ($n = 27$) and

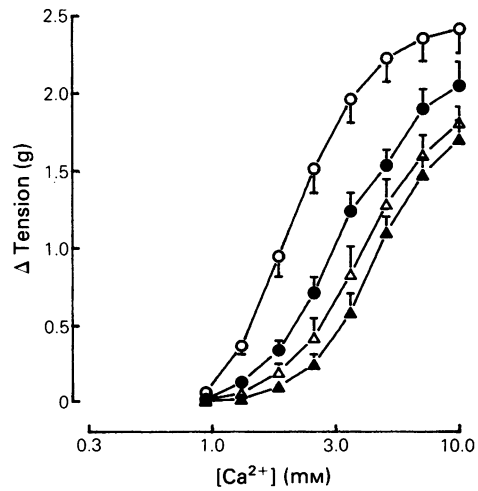


Figure 2 Effects of cobalt on the contractile responses to Ca^{2+} of rat aortic rings. Cumulative concentration-response curves to Ca^{2+} were constructed after exposure to Co^{2+} (0.0 (○); 0.1 (●); 0.3 (△) and 1.0 (▲) mM) ($n = 9$ per group) on preparations suspended in a K^+-free bathing medium containing 0.63 mM $CaCl_2$. Data represent mean values with vertical bars indicating s.e.mean.

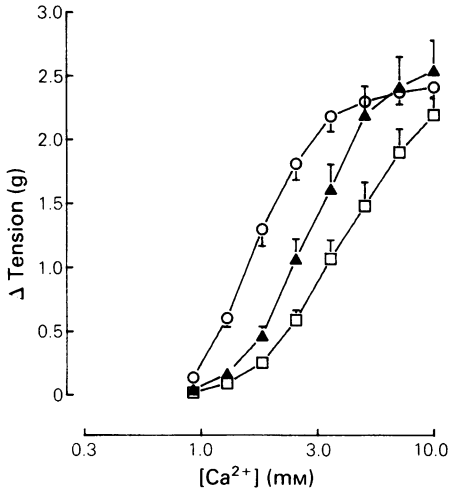


Figure 3 Effects of nickel on the contractile responses to Ca^{2+} of rat aortic rings. Cumulative concentration-response curves to Ca^{2+} were constructed after exposure to Ni^{2+} (0.0 (○); 0.5 (▲) and 1.0 (□) mM ($n = 6$ per group) on preparations suspended in a K^+-free bathing medium containing 0.63 mM $CaCl_2$. Data represent mean values with vertical bars indicating s.e.mean.

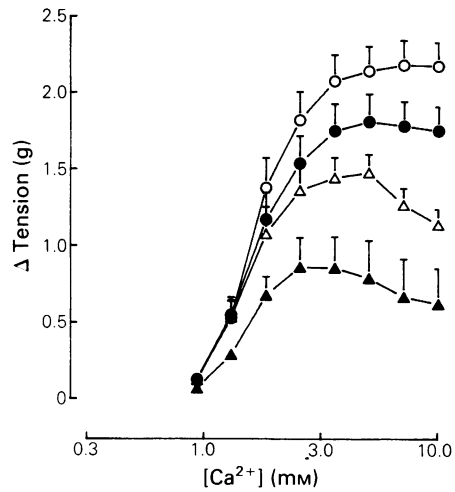


Figure 4 Effects of cadmium on the contractile responses to Ca^{2+} of rat aortic rings. Cumulative concentration-response curves to Ca^{2+} were constructed after exposure to Cd^{2+} (0.0 (○); 3 (●); 10 (△) and 30 (▲) μM) ($n = 6$ per group) on preparations suspended in a K^+-free bathing medium containing 0.63 mM $CaCl_2$. Data represent mean values with vertical bars indicating s.e.mean.

was characterized by a slope not significantly different from unity (1.08 ± 0.04). The pK_B values for 0.5 and 1.0 mM Ni^{2+} were 3.15 ± 0.08 ($n = 6$) and 2.94 ± 0.1 ($n = 6$), respectively.

Cadmium (3–30 μM) produced a concentration-related attenuation of the maximum contractile responses to Ca^{2+} of rat aortic rings in a K^+-free bathing medium (Figure 4). The IC_{50} (concentration required to reduce the E_{max} (2.19 ± 0.20 g) of Ca^{2+} by 50%) for Cd^{2+} was 15.5 ± 1.1 μM ($n = 6$).

Effects of organic pharmacological agents on the contractile responses to Ca^{2+}

Concentration-response curves for the contractile effect of Ca^{2+} were not modified by diltiazem (1 μM), cinnarizine (10 μM), verapamil (1 μM), nitrendipine (1 μM), (−)-Bay K 8644 (0.003–0.3 μM), monochlorobenzamil (100 μM) or cromakalim (10 μM) (Table 1).

Ryanodine (30 μM), an intracellular calcium antagonist (Fabiato, 1985), displaced the Ca^{2+} concentration-response curve to the left, with no significant change in the maximal response (Table 1).

Discussion

Calcium produced concentration-related contractile responses of the rat aortic ring preparation suspended in a potassium-free bathing medium. Under these experimental conditions vascular smooth muscle is in a slightly depolarized state (Hermsmeyer, 1976) and therefore the Ca^{2+}-induced contractions could be mediated by a voltage-sensitive mechanism. The responses to Ca^{2+}, in the absence of K^+, were attenuated by the divalent cations Co^{2+}, Ni^{2+}, and Cd^{2+} at concentrations which block Ca^{2+}-channels (Nachshen, 1984; Lansman *et al.*, 1986). These results, therefore, suggest the involvement of an influx of extracellular Ca^{2+} into the cytosol via voltage-operated Ca^{2+}-channels (VOCs).

Calcium channels, which open in response to membrane depolarization to admit extracellular calcium into the cytosol, have been classified into three types (T, N and L) on the basis of their gating properties and pharmacological sensitivities (Nowycky *et al.*, 1985). The T-type channel is characterized by a transient time-course due to rapid inactivation (20–50 ms) and a sensitivity to blockade by nickel ions. The L-type or 'slow' calcium channel is slowly inactivated (> 500 ms) and is the only type sensitive to the dihydropyridines. Another rapidly inactivated (50 ms) channel, the N-type, in contrast to the T-channel, is sensitive to ω-conotoxin from *Conus geographus*. Ca^{2+} influx through N-type channels is more sensitive to Cd^{2+} than to other divalent cations (e.g. Ni^{2+}, Co^{2+} or Mn^{2+})

(Nachshen, 1984). The evidence for multiple types of calcium channels has been reviewed in detail by Tsien *et al.* (1987a,b).

In vascular smooth muscle cells, however, only two types of Ca^{2+} channel have been identified electrophysiologically; an L-type channel and a rapidly inactivated dihydropyridine-resistant channel (possibly T-type) (Loirand *et al.*, 1976; Aaronson *et al.*, 1986; Bean *et al.*, 1986).

The contractile response of the rat aorta is not due to an influx of Ca^{2+} ions through L-type VOCs as the dihydropyridines (nitrendipine and (−)-Bay K 8644) failed to modify these responses. Under these experimental conditions, the calcium agonist (−)-Bay K 8644 can evoke contractile responses (Cavero & Lawson, 1987) which are abolished by low (< 30 nM) concentrations of nitrendipine (Lawson, personal observation). Thus, although L-type channels are not responsible for the Ca^{2+}-induced contraction, they can still be activated. Interestingly, cinnarizine, like verapamil and diltiazem, was inactive in this study; the transmembrane transport mechanism involved in the responses to Ca^{2+} appears to be insensitive to organic calcium channel antagonists. The pA_2 and pK_B values determined for Co^{2+} and Ni^{2+}, respectively, suggest that these cations have similar relative affinities for this Ca^{2+} channel. The apparent competitive inhibition may be a consequence of Ni^{2+} or Co^{2+} replacing the Ca^{2+} current, rather than a direct interaction with the channel. Cadmium, however, evoked a non-competitive inhibition of the contraction to Ca^{2+} which would suggest that this divalent cation interacts irreversibly with the channel. The inhibition by cadmium of the contraction to Ca^{2+} might suggest the involvement of N-type channels, which have so far only been identified in neurones (Tsien *et al.*, 1987b). However, further studies are needed to determine which dihydropyridine-resistant VOC is involved in the contractile response to Ca^{2+}.

The absence of extracellular K^+ will augment intracellular sodium concentrations due to total inhibition of Na^+,K^+-ATPase activity in vascular smooth muscle (Blaustein, 1977). The increased $[Na]_i$ will lead to an enhanced activation of Na^+-Ca^{2+} exchange and an elevation in the concentration of free intracellular Ca^{2+}. Under the conditions used in these experiments, the Na^+-Ca^{2+} exchange mechanism is possibly fully activated, therefore the contractile response associated with an elevation of $[Ca^{2+}]_o$ is probably not a result of the translocation of Ca^{2+} into the cytosol by this mechanism. Although further support for this conclusion is provided by the failure of monochlorobenzamil, a Na^+-Ca^{2+} exchange inhibitor (Siegl *et al.*, 1984), to modify the Ca^{2+}-evoked contractions, the involvement of this mechanism cannot be entirely eliminated.

Ryanodine inhibits calcium-induced release of intracellular stores of Ca^{2+} in cardiac and vascular smooth muscle (Fabiato, 1985; Ito *et al.*, 1986). The failure of ryanodine to inhibit the contractions to Ca^{2+} would indicate that these responses were the result of an influx of extracellular Ca^{2+} into the cytosol and not a release of this divalent cation from internal stores. However, high concentrations (30 μM) of ryanodine enhanced the contractile effects produced by an increase in the extracellular calcium concentration. This observation is compatible with the findings that under certain conditions ryanodine releases Ca^{2+} from sarcoplasmic reticulum of cardiac muscle (Hunter *et al.*, 1983; Meissner, 1986). However, further work is needed to determine the mechanism involved in these effects of ryanodine.

Finally, cromakalim, a K^+ channel activator, (Cook, 1988) failed to modify the contractile responses to Ca^{2+}. Cromakalim has been shown to inhibit contractions of rabbit aortic smooth muscle associated with an influx of extracellular Ca^{2+} resistant to dihydropyridines (Cook, 1988).

In conclusion, in rat aortic ring preparations bathed in a K^+-free medium, Ca^{2+} evoked dihydropyridine-resistant contractions. These effects appear to result from an influx of Ca^{2+} ions into smooth muscle cells through calcium channels, which are distinct from L-channels, and do not involve the Na^+-Ca^{2+} exchange or intracellular Ca^{2+} stores.

Therefore, the rat aorta bathed in a K^+-free salt solution may provide a functional model for characterization, and determination of the physiological role of dihydropyridine-insensitive calcium channels in vascular smooth muscle.

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Actions of bradykinin and related peptides on rabbit coeliac artery rings

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1 Rabbit coeliac artery rings were mounted in tissue baths containing Krebs solution at 37°C in order to determine whether their response to bradykinin is initiated by B_1- or B_2-receptors. Tension was recorded isometrically.

2 Phenylephrine contracted the tissue. Subsequent addition of bradykinin or des Arg^{10}-kallidin caused relaxation which was not dependent on an intact endothelium. Des Arg^{10}-kallidin, a B_1-receptor selective agonist, was more potent than bradykinin.

3 [β-Thienyl alanyl^{6,9}, D-Phe^8]-kallidin and [Leu^9]-des Arg^{10}-kallidin antagonized bradykinin and des Arg^{10}-kallidin. [Leu^9]-des Arg^{10}-kallidin, a B_1-receptor selective antagonist, was more potent than [$Thi^{6,9}$, D-Phe^8]-kallidin, a less selective drug that acts on both B_1- and B_2-receptors.

4 Kinin-induced relaxation was reversibly antagonized by ibuprofen (a cyclo-oxygenase inhibitor) and by 5-(N,N-hexamethylene)amiloride (an inhibitor of Na^+/H^+ exchange). Ibuprofen caused a parallel shift to the right of a semi-logarithmic plot of the agonist concentration-effect relationship, whereas the amiloride analogue depressed the maximum response and reduced the slope.

5 We conclude that bradykinin and des Arg^{10}-kallidin relax rabbit coeliac artery by combining with B_1-receptors. The response is mediated by a cyclo-oxygenase product and may be influenced by cellular Na^+/H^+ exchange.

Introduction

Bradykinin relaxes rabbit coeliac artery. This response is unusual in that it is inhibited by cyclo-oxygenase inhibitors (Aiken, 1974), and does not depend on the presence of an intact endothelium (Cherry *et al.*, 1982). Prostacyclin (PGI_2) production by rabbit coeliac artery is stimulated by bradykinin, and PGI_2 is probably the mediator of the relaxation caused by bradykinin in this tissue (Förstermann *et al.*, 1986).

Structure-activity studies of analogues of bradykinin on a variety of tissues have provided evidence of two kinds of bradykinin receptor, termed B_1 and B_2 (Regoli & Barabé, 1980). In the present study we investigated the effects of several bradykinin-related peptides on rabbit coeliac artery rings, to determine whether the response to bradykinin is initiated by a B_1- or B_2-receptor. Compounds studied included des Arg^{10}-kallidin, a selective B_1-receptor agonist, [Leu^9]-des Arg^{10}-kallidin, a selective B_1-receptor antagonist, and [β-Thienyl alanyl^{6,9}, D-Phe^8]-kallidin ([$Thi^{6,9}$, D-Phe^8]-kallidin), a non-selective B_1- and B_2-antagonist (Regoli & Barabé, 1980; Regoli *et al.*, 1986).

Bradykinin stimulates prostaglandin synthesis by activation of phospholipase and liberation of arachidonic acid (Hong & Levine, 1976). There is evidence that Na^+/H^+ exchange can regulate receptor-mediated phospholipase A_2 activation (Sweatt *et al.*, 1986). We used 5-(N,N-hexamethylene)amiloride to investigate whether Na^+/H^+ exchange is implicated in the bradykinin receptor-effector linkage in rabbit coeliac artery. This drug is a potent inhibitor of Na^+/H^+ exchange (Simchowitz & Cragoe, 1986) that inhibits Ca^{2+}-stimulated PGI_2 synthesis by rat aorta (Ritter *et al.*, 1987a). We also studied ibuprofen, to determine the effect of this cyclo-oxygenase inhibitor on the mechanical response.

Methods

Preparation of arterial rings

New Zealand white rabbits (2.5–5.8 kg) of either sex were killed with pentobarbitone (120 mg kg^{-1} i.v.). The coeliac artery was removed immediately, rinsed

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with ice-cold Krebs solution and cut with scissors into transverse rings (approximately 2.5 mm). Care was taken to avoid contact with the intimal surface, except in experiments in which the endothelium was deliberately removed by rubbing with filter paper (Furchgott & Zawadzki, 1980). Rings were mounted on pairs of stainless steel hooks and placed in 15 ml all-glass jacketed tissue baths containing Krebs solution at 37°C. Tension changes were recorded using an isometric force transducer and pen recorder (Grass). Basal tension was adjusted to 2 g, and the tissue allowed to equilibrate for at least 90 min before the start of each experiment. Two rings mounted in separate tissue baths were studied simultaneously to provide matched controls.

Measurement of agonist and antagonist potencies

After the equilibration period, phenylephrine was added to the bath (3×10^{-6} M). This caused a contraction that reached a plateau within 1–3 min and generally remained stable for at least 15 min. Functional integrity of the endothelium was established by determining the response to methacholine (Furchgott & Zawadzki, 1980). Relaxant agonists (bradykinin or des Arg^{10}-kallidin) were added during the plateau phase, and caused a fall in tension toward a new plateau. When this was reached a further dose was added. In some preparations further doses of agonist were applied. After the response to the final dose had reached a plateau, drugs were washed out of the tissue bath, each wash being accomplished by three changes of the bath volume with Krebs solution. Responses were quantified as % relaxation, and plotted against agonist concentration using a semi-logarithmic scale. The concentration of agonist required to cause 50% relaxation (EC_{50}) was estimated by interpolation. Five or more minutes after washout the same dose of phenylephrine was re-applied, followed as before by cumulative additions of the same or a different agonist. Antagonists were studied after obtaining a control dose-response curve, and were added to the tissue bath 5 min before the subsequent dose of phenylephrine (applications of antagonists for longer periods were found not to cause greater degrees of block). The dose of agonist (bradykinin or des Arg^{10}-kallidin) was increased so that in the presence of antagonist responses were kept similar to the control responses in the absence of antagonist. Fifteen minutes after washout phenylephrine was again applied and a second control dose-response curve to agonist determined to establish that the sensitivity of the preparation was stable.

The effect of antagonists that caused a parallel shift of the log dose-response relation was quantified as a dose-ratio (DR), the value of which was taken as the ratio of the concentration of agonist required to produce 50% relaxation in the presence of antagonist to that required to produce the same response in the absence of antagonist. In experiments with [Leu^9]-des Arg^{10}-kallidin and [Thi^{6,9}, D-Phe^8]-kallidin, the apparent dissociation equilibrium constant (K_D) of the antagonist was calculated from the molar concentration of the antagonist peptide [B], and the value of the DR, using the expression $DR - 1 = [B]/K_D$ (Gaddum, 1937).

Bathing solution and drugs

The bathing fluid was Krebs solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 2.4, $NaHCO_3$ 25, glucose 11.1. The solution was continuously gassed with 95% O_2/5% CO_2, resulting in a pH of 7.4. Phenylephrine, bradykinin, ibuprofen, methacholine and amiloride were obtained from Sigma, 5-(N,N-hexamethylene)amiloride was prepared by previously published methods (Cragoe *et al.*, 1967; 1981). Amiloride and its analogue were dissolved in dimethyl sulphoxide on the day of each experiment. Equal volumes of dimethyl sulphoxide were added to the control preparation. Peptides other than bradykinin were kindly supplied by Professor D. Regoli (Université de Sherbrooke, Canada). All peptides were dissolved in distilled water. PGI_2 was a generous gift from Wellcome Research Laboratories (Beckenham, U.K.).

Analysis

Results are shown as mean ± s.e.mean. Differences were compared by paired or non-paired *t* test as appropriate, and considered significant when $2P < 0.05$.

Results

Bradykinin and des Arg^{10}-kallidin each relaxed phenylephrine-contracted coeliac artery rings. The presence of an intact endothelium, indicated by relaxation to methacholine, was not necessary for relaxation responses to either of these kinins (Figure 1). The sensitivity to kinins of arteries from different rabbits varied considerably, but des Arg^{10}-kallidin was consistently more potent than bradykinin. The EC_{50} of bradykinin was $6.1 \pm 2.8 \times 10^{-7}$ M ($n = 22$) while that of des Arg^{10}-kallidin was $6.4 \pm 2.8 \times 10^{-9}$ M ($n = 8$).

[Leu^9]-des Arg^{10}-kallidin and [Thi^{6,9}, D-Phe^8]-kallidin had no agonist activity, but each antagonized responses to bradykinin and to des Arg^{10}-

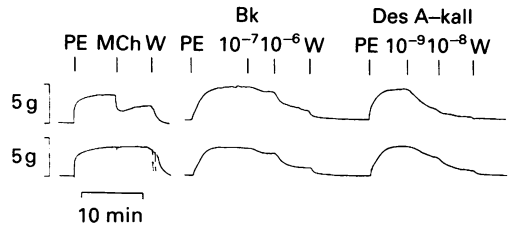


Figure 1 Effects of bradykinin and des Arg^{10}-kallidin on rabbit coeliac artery rings. Representative original tracings of isometric tension from (above) an intact ring and (below) an adjacent ring denuded of endothelium and studied simultaneously. Phenylephrine (PE) (3×10^{-6} M) contracted the tissues, which relaxed after washout (W). Methacholine (MCh) (10^{-5} M) relaxed only the intact ring. Bradykinin (Bk) (10^{-7} M, 10^{-6} M) relaxed both rings, as did des Arg^{10}-kallidin (des A-kall; 10^{-8} M, 10^{-9} M). (Representative of 6 experiments).

kallidin, but not to PGI_2 (Figure 2) [Leu^9]-des Arg^{10}-kallidin was significantly more potent than [Thi^{6,9}, D-PhE^9]-kallidin, with a mean apparent K_D approximately a hundred times lower than that of [Thi^{6,9}, D-Phe^8]-kallidin (Table 1). Values of the apparent K_D of each antagonist were similar whether bradykinin or des Arg^{10}-kallidin was used as agonist (Table 1).

Ibuprofen also antagonized responses to bradykinin in a reversible manner, causing a parallel shift to the right of a semi-logarithmic plot of the agonist dose-response relationship (Figure 3). Doubling the concentration of ibuprofen from 2×10^{-6} M to 4×10^{-6} M caused an increase in dose-ratio from 16.8 ± 6.2 ($n = 7$) to 222.9 ± 20.0 ($n = 3$). 5-(N,N-hexamethylene)amiloride also antagonized bradykinin-induced relaxation, but unlike ibuprofen it reduced the maximum effect and the slope of the dose-response curve (Figure 4). 5-(N,N-hexamethylene)amiloride (10^{-6} M) caused a mean inhibition of bradykinin-induced relaxation of $61.3 \pm 5.9\%$ ($n = 12$, $2P < 0.005$), but had no significant effect on responses to PGI_2 (mean increase $6.9 \pm 9.5\%$, $n = 5$).

Discussion

These findings with bradykinin-related peptides, both agonists and antagonists, indicate that brady-

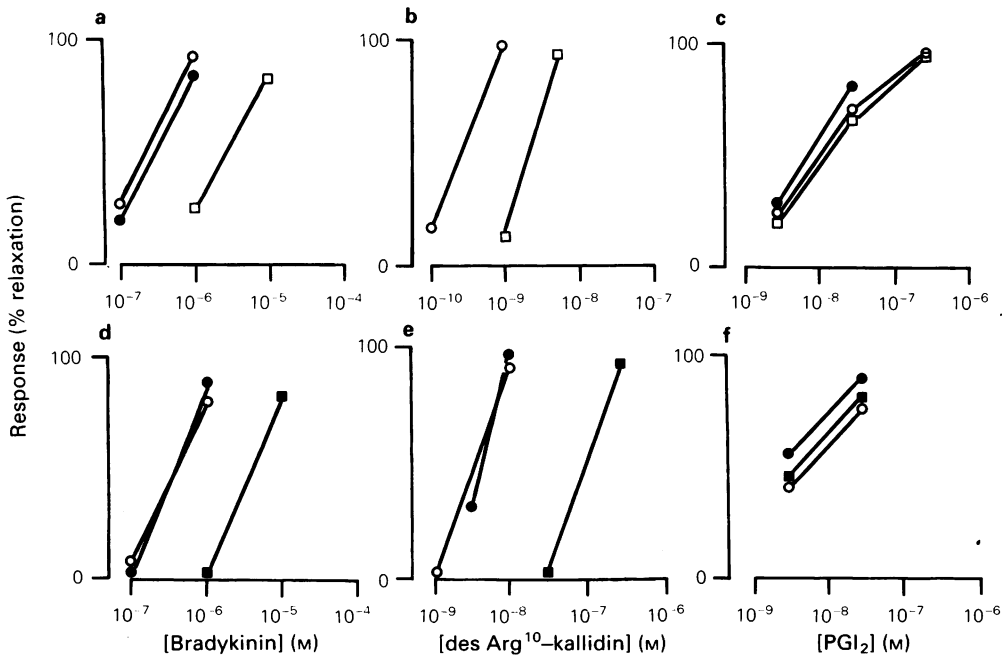


Figure 2 Effects of kinin-antagonists on relaxations of phenylephrine-contracted rings of rabbit coeliac artery in Krebs solution at 37°C. Six individual representative experiments are presented. Responses (% relaxation) are plotted against the molar concentration of agonist on a semi-logarithmic scale. Responses were measured before (○) and usually also after (●) exposure of the tissue to antagonists. In the presence of [Leu^9]-des Arg^{10}-kallidin, 5×10^{-8} M (□) or of [Thi^{6,9}, D-Phe^8]-kallidin 1.5×10^{-6} M (■), higher doses of bradykinin (a,d) and of des Arg^{10}-kallidin (b,e) were required to cause similar responses to those obtained in the control period, whereas neither antagonist appreciably affected sensitivity to prostacyclin (PGI_2) (c,f).

Table 1 Potencies of antagonists of kinin-induced relaxation in rabbit coeliac artery

Agonist	*Apparent dissociation constant* (K_D) *of antagonist* (M) [*Leu*^{9}]-*des Arg*^{10}-*kallidin*	[*Thi*^{6,9}, D-*Phe*^{8}]-*kallidin*
Bradykinin	$4.1 \pm 1.2 \times 10^{-9}$ (7)	$3.0 \pm 0.9 \times 10^{-7}$* (11)
Des Arg^{10}-kallidin	$2.9 \pm 1.2 \times 10^{-9}$ (9)	$2.4 \pm 1.3 \times 10^{-7}$* (5)

Data are presented as means ± s.e.mean of *n* (value in parentheses) results. Potencies of the antagonists [Leu^9]-des Arg^{10}-kallidin and [Thi^{6,9}, D-Phe^8]-kallidin are expressed as their mean apparent dissociation equilibrium constants, K_D (M). K_D was determined using the dose-ratio method with bradykinin and des Arg^{10}-kallidin as agonists on phenylephrene-contracted rings of rabbit coeliac artery in Krebs solution at 37°C. The estimated values of K_D for [Leu^9]-des Arg^{10}-kallidin using the two agonists did not differ significantly from one another, and the estimates of the K_D for [Thi^{6,9}, D-Phe^8]-kallidin were likewise similar. With either agonist the K_D of [Leu^9]-des Arg^{10}-kallidin differed significantly from that of [Thi^{6,9}, D-Phe^8]-kallidin (*$2P < 0.05$).

kinin relaxes rabbit coeliac artery rings by combining with a B_1-receptor. Thus the B_1-selective agonist des Arg^{10}-kallidin (Regoli & Barabé, 1980) is about 100 times more potent than bradykinin which is relatively selective for B_2-receptors (Regoli & Barabé 1980). The selective B_1-receptor antagonist [Leu^9]-des Arg^{10}-kallidin (Regoli & Barabé, 1980) is a potent antagonist of bradykinin and of des Arg^{10}-kallidin. The non-selective B_1- and B_2-receptor antagonist [Thi^{6,9}, D-Phe^8]-kallidin (Regoli *et al.*, 1986), is also an effective antagonist but is approximately 100 times less potent than [Leu^9]-des Arg^{10}-kallidin. Responses to bradykinin do not depend on the presence of intact endothelium, confirming the findings of Cherry *et al.* (1982) and of Förstermann *et al.* (1986). Relaxation of coeliac artery rings caused by des Arg^{10}-kallidin is also independent of endothelium, implying that the B_1-receptors that cause relaxation of this tissue are on vascular smooth muscle or other non-endothelial cells.

B_1-receptors have previously been described in several vascular tissues in cats and rabbits, including the anterior mesenteric vein and the aorta which are contracted by bradykinin (Regoli & Barabé, 1980), and the rabbit mesenteric artery which is relaxed by des Arg^{10}-kallidin (Churchill *et al.*, 1986). In these tissues the sensitivity to agonists increases progressively *in vitro* over six hours (Regoli *et al.*, 1978).

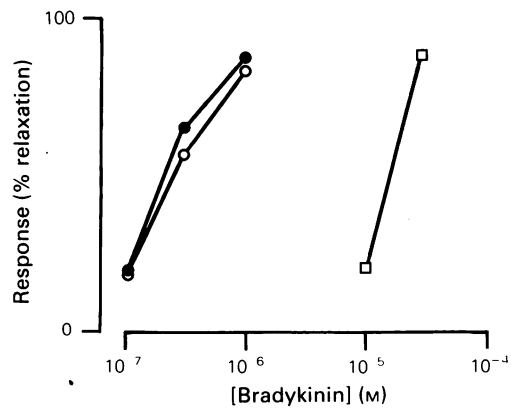


Figure 3 Effect of ibuprofen (4×10^{-6} M) on the sensitivity to bradykinin of a ring of phenylephrine-contracted rabbit coeliac artery in Krebs solution at 37°C. Responses (% relaxation) are plotted against the molar concentration of bradykinin on a semi-logarithmic scale. Responses were measured before (○), after (●) and during (□) exposure of the tissue to ibuprofen. Ibuprofen caused a surmountable antagonism from which the tissue recovered after the inhibitor was washed out of the bath (Representative of 3 experiments).

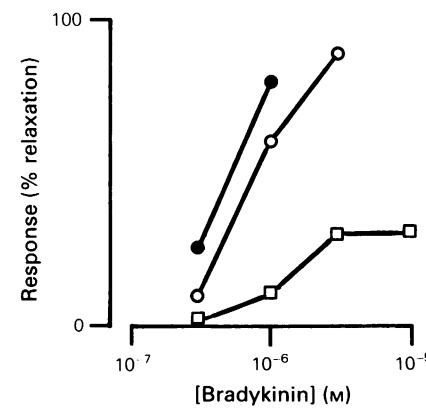


Figure 4 Effect of 5-(N,N-hexamethylene)amiloride (10^{-6} M) on the sensitivity to bradykinin of a ring of rabbit coeliac artery in Krebs solution (37°C) pre-contracted with phenylephrine. Responses (% relaxation) are plotted against the molar concentration of bradykinin on a semi-logarithmic scale. Responses were measured before (○), after (●) and during (□) exposure of the tissue to 5-(N,N-hexamethylene)amiloride, which reduced the slope and maximum of the response curve in a reversible manner. (Representative of 10 experiments).

Such an increase in sensitivity was not evident in our experiments in which quantitative measurements were made over a shorter interval. The final control dose-response curve was usually similar to the original one, although the sensitivity of the preparation was sometimes somewhat increased or reduced.

Bradykinin-induced relaxation of rabbit coeliac artery has previously been shown to be antagonized by the cyclo-oxygenase inhibitors indomethacin and fenoprofen (Aiken 1974; Cherry *et al.*, 1982; Förstermann *et al.*, 1986), and our findings with ibuprofen confirm that a cyclo-oxygenase product mediates the relaxation caused by bradykinin in this tissue. Förstermann *et al.* (1986) provided evidence that PGI_2 is the likeliest candidate for this role. Prostaglandin synthesis by fibrosarcoma cells is stimulated by bradykinin via B_2-receptors (Becherer *et al.*, 1982). The present findings imply that bradykinin may also be linked to prostaglandin synthesis by way of B_1-receptors.

Somewhat surprisingly, the inhibition caused by ibuprofen can be surmounted by increasing the concentration of bradykinin. Quantitatively, however, antagonism by ibuprofen is not competitive, an increase in ibuprofen concentration causing a strikingly disproportionate increase in dose-ratio. A possible explanation of the surmountable nature of the antagonism caused by ibuprofen is that the tissue possesses a considerable receptor reserve. In the absence of cyclo-oxygenase inhibitor, bradykinin may need to occupy only a small fraction of its receptors in order to cause sufficient PGI_2 production to induce a near-maximal response, and this rate of PGI_2 synthesis may itself be less than the maximum of which the tissue is capable. In the presence of ibuprofen, an increase in bradykinin concentration, and hence receptor occupancy, could restore the rate of PGI_2 formation, despite a reduction in cyclo-oxygenase activity, by increasing the concentration of substrate (free arachidonate). If this explanation is correct, a sufficiently high concentration of ibuprofen will eventually reduce the slope of the log dose-response relation (Furchgott, 1964), although the very high agonist concentration required made it impractical to test this.

Receptor-mediated phospholipase A_2 activation in human platelets is regulated by Na^+/H^+ exchange (Sweatt *et al.*, 1986), and H^+ activity is implicated in the control of PGI_2 synthesis by vascular tissue (Ritter *et al.*, 1987a,b). The involvement of PGI_2 in the response of rabbit coeliac artery to bradykinin (Förstermann *et al.*, 1986), raises the question whether Na^+/H^+ exchange participates in the early events that link receptor occupation to relaxation in this tissue. The present finding that 5-(N,N hexamethylene)amiloride (10^{-6} M) inhibits bradykinin-induced relaxation of rabbit coeliac artery supports this possibility. We cannot absolutely exclude the possibility of some other pharmacological action of 5-(N,N-hexamethylene)amiloride, but amiloride, which is much less potent as an inhibitor of Na^+/H^+ exchange than the 5-substituted analogue (Johnson *et al.*, 1976; Simchowitz & Cragoe, 1986), does not significantly inhibit bradykinin-induced relaxation at 10^{-6} M (data not shown).

The findings could be explained if B_1-receptor occupation by an agonist independently influences both phospholipase and Na^+/H^+ exchange. The latter process might then act permissively on phospholipase activation if this is dependent on a lower than basal intracellular H^+ concentration (cf Sweatt *et al.*, 1986). Inhibition of Na^+/H^+ exchange would then be expected to inhibit responses to bradykinin non-competitively, as observed. It may prove possible to test this model more directly in cultured cells.

This study was supported by the British Heart Foundation. Miss Audrey Douglas provided excellent secretarial assistance. We thank Dr N. Turner for his advice.

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Actions of the $GABA_B$ agonist, (−)-baclofen, on neurones in deep dorsal horn of the rat spinal cord *in vitro*

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1 The electrophysiological actions of the $GABA_B$ agonist, (−)-baclofen, on deep dorsal horn neurones were studied using an *in vitro* preparation of the spinal cord of 9–16 day old rat.

2 On all neurones tested, (−)-baclofen (100 nM–30 μM) had a hyperpolarizing action which was associated with a reduction in apparent membrane input resistance. The increase in membrane conductance was dose-dependent and had a Hill coefficient of 1.0.

3 The (−)-baclofen-activated hyperpolarization persisted in the presence of bicuculline (50 μM) and Mg^{2+} (20 mM).

4 The reversal potential of the hyperpolarizing event was estimated at 102 mV and was made less negative by increasing the external concentration of potassium ions.

5 Over the same concentration range, (−)-baclofen also depressed the polysynaptic composite excitatory postsynaptic potentials (e.p.s.ps) evoked in these neurones by electrical stimulation of the dorsal root entry zone.

6 The potassium channel blockers caesium, applied intracellularly, and barium, applied extracellularly, depressed the postsynaptic response to baclofen but not its effect on e.p.s.ps.

7 We propose that (−)-baclofen has more than one mechanism of action in spinal dorsal horn: a postsynaptic action mediated via an increase in potassium conductance and a presynaptic action that is not associated with potassium channels and may be mediated via calcium channels. Since previous studies have demonstrated little effect of (−)-baclofen on transmitter release in spinal cord, it is possible that the postsynaptic hyperpolarizing action of (−)-baclofen may account for its clinical potency as an anti-spastic agent.

Introduction

γ-Aminobutyric acid (GABA) is widespread as an inhibitory neurotransmitter within the CNS. There is considerable evidence to suggest that its receptors can be divided into two subtypes, $GABA_A$ and $GABA_B$. Muscimol, isoguvacine and 3-aminopropanesulphonic acid are selective agonists at the $GABA_A$ receptor whereas the *p*-chlorophenyl derivative of GABA, (−)-baclofen, is a highly selective agonist for the $GABA_B$ site. Furthermore, bicuculline is a potent, competitive antagonist at the $GABA_A$ receptor but has little activity at the $GABA_B$ receptor.

It is widely documented that $GABA_A$ receptor agonists exert their actions via an increase in membrane chloride conductance. The ionic conductance which results from $GABA_B$ receptor activation is, however, less well established. In isolated dorsal root ganglia, (−)-baclofen does not affect membrane potential or conductance but causes a shortening of the calcium action potential (Desarménian *et al.*, 1984; Schlichter *et al.*, 1984). This may be the result of a reduction in the amplitude of a slowly inactivating calcium current in these neurones (Robertson & Taylor, 1986). In hippocampus (Newberry & Nicoll, 1984) and substantia nigra (Pinnock, 1984), activation of the $GABA_B$ receptor results in postsynaptic membrane hyperpolarization associated with an increase in potassium conductance.

(−)-Baclofen is a highly effective anti-spastic agent at the level of the spinal cord (Fehr & Bein, 1974). *In vivo* studies have shown that applied microelectro-

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phoretically (Curtis *et al.*, 1974; Henry & Ben-Ari, 1976; Curtis *et al.*, 1981; Davies, 1981) or intravenously (Piercey & Hollister, 1979) it has a potent depressant action on the firing of neurones in dorsal horn which may be the result of either a reduction in the release of excitatory transmitter or the depression of the response to this transmitter via a postsynaptic mechanism. Of particular interest is the observation that baclofen will depress the response of dorsal horn neurones to exogenous excitants (Davies, 1981), implying the existence of a postsynaptic mechanism of action. Intracellular recording from motoneurones has failed to show any effect of systemic baclofen on membrane conductance although synaptic activity is markedly depressed (Pierau & Zimmerman, 1973; Fox *et al.*, 1978). Iontophoretic administration of baclofen to these same neurones does, however, reduce both excitability and membrane input resistance (Fox *et al.*, 1978).

Autoradiographic techniques have detected $GABA_B$ binding sites throughout spinal grey matter but demonstrate a clear band of high density binding to the dorsal horn. This is located mainly in laminae 2 and 3, but laminae 1 and 4 also appear to have a considerable number of binding sites (Price *et al.*, 1984). The effect of baclofen on spinal dorsal horn neurones is thus likely to be important in terms of its functional role.

We have therefore examined the action of baclofen on neurones in dorsal horn using an *in vitro* slice preparation of spinal cord from which we could obtain stable intracellular recordings. Some of these results have already been presented in abstract form (Allerton *et al.*, 1988).

Methods

Slice preparation

Methods used were adapted from those of Randić & co-workers (Miletić & Randić, 1980; Murase *et al.*, 1982). Experiments were performed on 9–16 day old Wistar rats. The animals were anaesthetized with ether and cooled by placing on ice for 5–10 min. When the animal was cool to the touch and the breathing was shallow, a laminectomy was performed to expose the lower thoracic and lumbosacral spinal cord together with the dorsal roots. A 1–1.5 cm length of cord was quickly excised with attached dorsal rootlets, the dura removed and each end of the cord cut to leave a 5–8 mm length of lumbar enlargement. Transverse slices (400 μm) were cut with a Vibratome (Lancer) and incubated in oxygenated artificial cerebro-spinal fluid (ACSF) at 36°C for at least 45 min before use.

Intracellular recording techniques

Slices were transferred to a recording chamber where they were continuously perfused with oxygenated artificial cerebrospinal fluid (ACSF). The slice rested upon Sylgard and was completely submerged in ACSF which flowed through the bath at a rate of $3\,ml\,min^{-1}$. It was observed from above under × 80 magnification and the recording electrode was placed in the deep dorsal horn under visual control with a micromanipulator. A bipolar electrode was placed on the dorsal root entry zone in a similar manner.

Drugs at a known concentration were dissolved in oxygenated ACSF and applied directly to the slice via the perfusion system by means of a three way tap. There was a dead time of approximately 1 min between turning the tap and the drug containing solution entering the bath. The flow rate through the bath did not change during this procedure. Since the volume of the bath was only 0.5 ml and the slice was completely submerged, equilibration took place rapidly. Drug removal was achieved simply by returning to drug-free ACSF.

Conventional intracellular recording techniques were used. Glass recording electrodes were filled with 3M KCl and had d.c. tip resistances of 40–100 MΩ when measured in physiological saline. Recording electrodes were placed in the region identified as the dorsal horn of the spinal cord under visual control, deep to the translucent band of the substantia gelatinosa (Rexed's lamina 2). The electrode was connected to a pre-amplifier with the facility for current injection (Digitimer Neurolog NL102). Membrane potential and injected current were amplified and displayed on an oscilloscope (Tektronix 5110) and chart recorder (Gould 2400S). Data were also recorded on digital tape (Sony) for future analysis.

Constant amplitude hyperpolarizing current pulses were injected into the neurone through the recording electrode to allow monitoring of membrane resistance. For initial impalement, pulses of 0.2 nA amplitude and 25 ms duration were injected at a rate of 10 Hz. As the stability of the recording became apparent, the rate of injection was reduced to 0.3 Hz and pulse duration increased (if necessary) to between 25 and 100 ms until saturation of the membrane capacitance occurred as visualized by the display of voltage on an oscilloscope. The time constant of the membrane could be established from analysis of electrotonic responses using this facility on the SCAN VI.2 (Single Channel Analysis) programme.

Current-voltage relationships could be established by variation of the amplitude of the hyperpolarizing pulses and measurement of the resulting voltage displacement (that is, the maximum potential to which

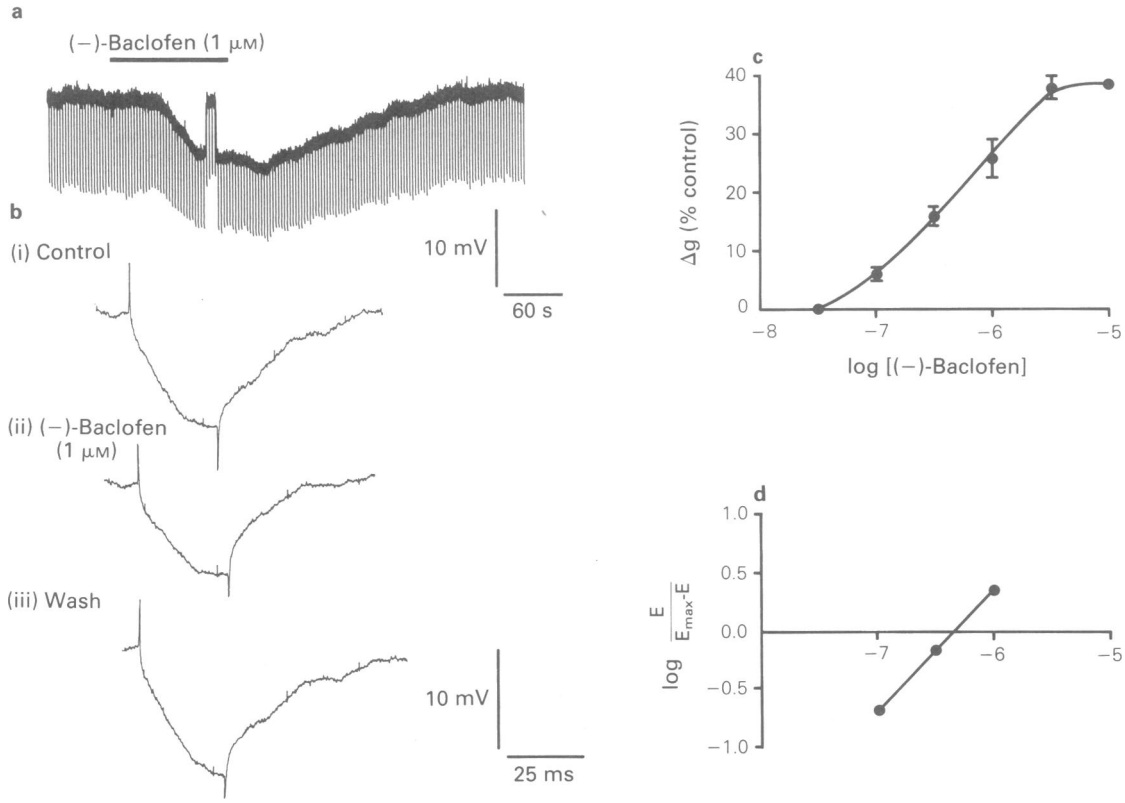


Figure 1 (−)-Baclofen, applied at concentrations of between 30 nM and 10 μM, produced hyperpolarizations in all dorsal horn neurones tested. (a) Chart recorder trace of a deep dorsal horn neurone with a resting membrane potential of −60 mV. Constant amplitude hyperpolarizing current pulses were injected into the neurone through the recording electrode producing the downward voltage deflections observed on the trace which gave an indication of membrane input resistance. (−)-Baclofen was applied for the period indicated by the solid bar, producing a hyperpolarization and a decrease in apparent input resistance. The change in input resistance was still observed when the membrane potential was restored to its original value by injection of depolarizing current showing that it is a drug-induced change and not merely a result of membrane rectification. (b) Membrane voltage response to injection of a 0.2 nA square current pulse (i) under control conditions, (ii) in the presence of (−)-baclofen (1 μM) and (iii) after washout of (−)-baclofen. These responses were elicited on the same neurone illustrated in (a) and therefore represent downward voltage deflections seen on a shorter time scale. Each represents a single voltage response captured on a storage oscilloscope and plotted out on a chart recorder. It can be seen that the membrane capacitance was fully charged and the electrode balanced with the bridge circuit. The change in conductance observed in the presence of the drug from these records is the same as that observed in (a) showing that the frequency response of the pen recorder did not attenuate the voltage response to square current pulses in this case. (c) Dose-dependency of the change in membrane conductance seen in the presence of (−)-baclofen. The mean values plotted are derived from the neurone illustrated in (a) and (b) and from three further neurones. The ordinate scale represents the change in conductance as a percentage of control conductance, while the abscissa scale expresses the logarithm of the concentration of (−)-baclofen. Each point represents the mean of the change observed on the same 4 neurones, except for that at the highest concentration of (−)-baclofen where the value shown is a mean of (−)-baclofen application to 2 of these 4 neurones; vertical bars show s.e.mean. (d) Transformation of the data shown in (b) to a Hill plot. The ordinate scale expresses $\log E/E_{max} - E$, where E is the change in conductance seen at the concentration of (−)-baclofen indicated on the abscissa scale and E_{max} is the maximum change in conductance seen in the presence of the drug (i.e. the response to 3 μM). The gradient of this line, the Hill slope, is 1.0.

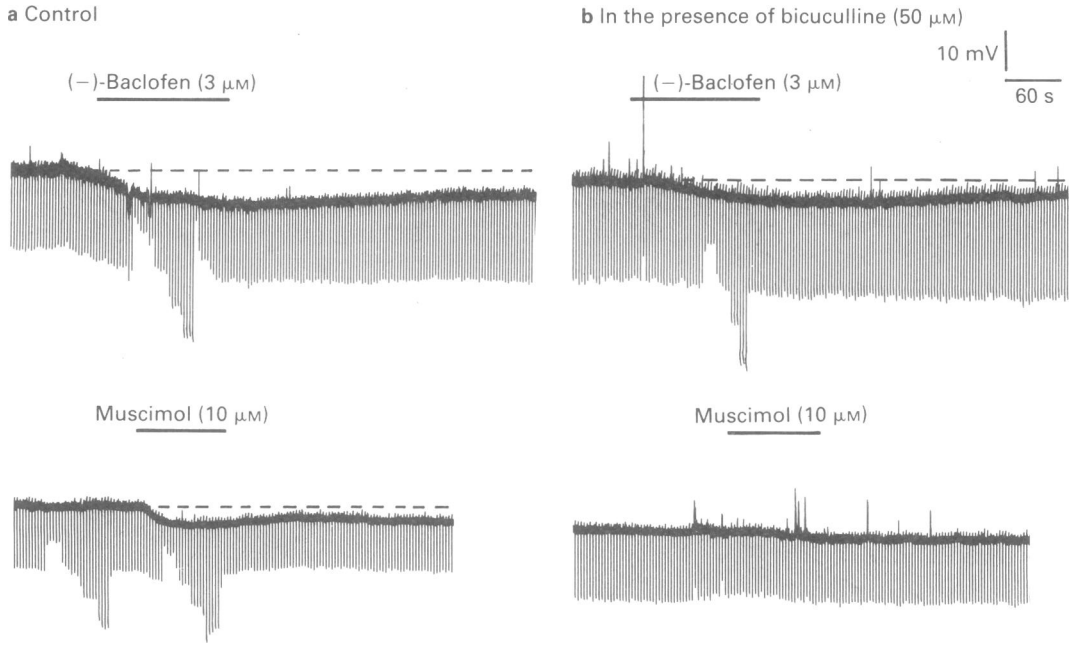


Figure 2 The hyperpolarization seen on application of (−)-baclofen persisted in the presence of a concentration of bicuculline known to block $GABA_A$ receptors. (a) The response of two different neurones to (−)-baclofen at a concentration of 3 μM and muscimol at a concentration of 10 μM respectively applied for the period indicated by the solid bar. (b) In the presence of bicuculline (50 μM) the response to muscimol but not (−)-baclofen was abolished. A hyperpolarization was still seen on application of (−)-baclofen although this response was reduced in the presence of bicuculline. Note also that in both neurones apparent input resistance was increased in the presence of bicuculline, presumably due to antagonism of the effects of endogenous GABA. The irregularity in the size of the electrotonic pulses was due to the variation in the amplitude of the injected current pulses that was necessary during IV plot construction. The maximum conductance change produced by (−)-baclofen on this neurone was a 30% increase during plotting of the IV curve. Note that the amplitude of current injections after the IV curve was not returned exactly to the pre-drug value. Distortion seen at the peak of the response was due to the slow chart speed. Measurements of amplitude were made at a higher speed that did not show this inaccuracy.

the membrane was displaced during passage of current). Voltage displacements were calculated by averaging 3 or more deflections measured on a chart recorder running at a speed of 0.5 mm s^{-1}. These deflections were of the same magnitude as the voltage response observed on the storage oscilloscope ensuring that the frequency response of the pen recorder had not influenced the apparent magnitude of the responses (see Figure 1). At speeds slower than this some distortion could arise but was observable (as in Figure 2).

Stimulation of dorsal rootlets with a bipolar stimulating electrode (pole separation 0.5 mm, insulated to within 0.5 mm of the tip) was used to obtain orthodromic activation of synapses in the dorsal horn region of spinal cord slices. Excitatory postsynaptic potentials (e.p.s.ps) were measured either directly after capture on the digital storage oscilloscope or after capture and plotting out on a pen recorder. Alternatively, e.p.s.ps were analysed off-line using the e.p.s.p analysis facility on SCAN VI.2.

Drugs and solutions

Composition of ACSF used for slice preparation and incubation was (mM): NaCl 124, KCl 5, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.3, $NaHCO_3$ 26 and glucose 10.

Composition (mM) of ACSF used for recording and drug application: NaCl 127, KCl 1.9, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.3, $NaHCO_3$ 26 and glucose 10.

Drugs used were: 4-aminopyridine (Sigma), (−)-baclofen hydrochloride (Ciba-Geigy), (+)-baclofen hydrochloride (Ciba-Geigy), barium chloride (Sigma), (−)-bicuculline methobromide (Sigma), caesium chloride (Sigma), muscimol (Sigma), tetraethylammonium bromide (Sigma).

Results

Results were obtained from a total of 46 neurones located in the deeper laminae (laminae 3–6 of Rexed) of spinal dorsal horn. The mean resting potential of the neurones studied was 67 ± 1 mV (mean ± s.e.mean, n = 38) and the mean apparent input resistance 80 ± 5 MΩ ($n = 39$). The mean membrane time constant was 14.3 ± 1.1 ms ($n = 10$). These membrane properties were determined with a KCl-filled electrode.

Postsynaptic action of (−)-baclofen

In all neurones tested, application of (−)-baclofen (100 nM–30 μM) resulted in membrane hyperpolarization associated with a reduction in apparent input resistance. Full recovery from the drug could generally be obtained after 10 to 20 min wash-out with drug-free saline and desensitization of the response was not apparent on repeat applications of (−)-baclofen to the same neurone. The increased membrane conductance seen in the presence of (−)-baclofen was dose-dependent. Transformation of the data to a Hill plot resulted in a Hill slope of 1.0 (Figure 1). (+)-Baclofen at a concentration of 10 μM had no effect ($n = 3$).

The direct nature of the interaction of (−)-baclofen with receptors on the post-synaptic membrane was confirmed by application of (−)-baclofen in the presence of a high concentration (20 mM) of magnesium ions. Such a concentration of magnesium completely prevents synaptic transmission within the slice but did not affect the response to (−)-baclofen ($n = 3$).

Since (−)-baclofen may have weak $GABA_A$ properties at higher concentrations, it was important to ensure that the observed hyperpolarization was not merely due to activation of the $GABA_A$ receptor. (−)-Baclofen was therefore applied in the presence of the $GABA_A$ antagonist bicuculline (50–100 μM) and the pharmacological effects of this substance were seen to persist. At these concentrations of bicuculline, however, some reduction (although never complete abolition) of the response to baclofen occurred in most neurones. The same concentration of bicuculline abolished completely the effects of the selective $GABA_A$ agonist muscimol (10 μM) (Figure 2).

In order to obtain some indication of the ionic event involved in $GABA_B$ activation, examination of the reversal potential of the event was made. The amplitude of the (−)-baclofen-induced hyperpolarization was reduced in a potential-dependent manner by the injection of hyperpolarizing current into the neurone under study. At a membrane potential of −100 mV, the hyperpolarizing response was abolished indicating that the reversal potential for the event lay around this value (Figure 3).

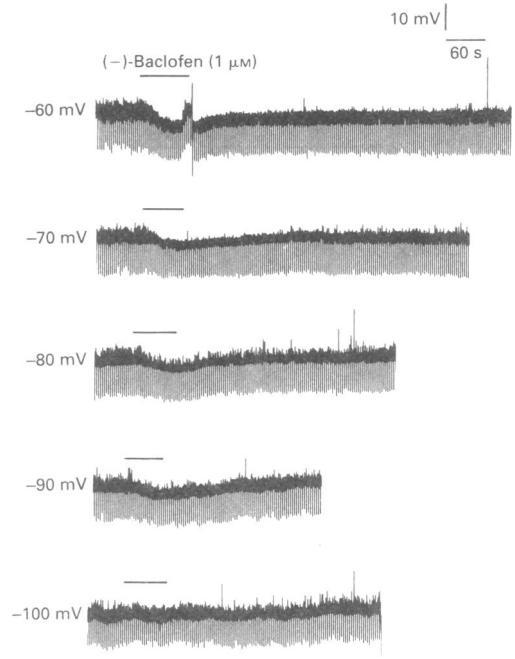


Figure 3 The amplitude of the hyperpolarization seen on application of (−)-baclofen was dependent on membrane potential. A single dose of the drug was applied for the period indicated by the solid bar to a deep dorsal horn neurone whose membrane potential was held at different potentials below rest by injection of hyperpolarizing current. As the membrane was made more hyperpolarized so the amplitude of the drug-induced hyperpolarization was reduced. The membrane potential could not be driven beyond the reversal potential as the relatively high resistance electrodes needed for this study would not pass sufficient current. As more current was passed through the electrode so its ability to monitor hyperpolarizing voltage deflections was reduced as the signal:noise ratio was degraded. The maximum conductance change produced by (−)-baclofen on this neurone was 25%.

A further indication of the reversal potential was obtained by the construction of current-voltage relationships for these neurones in drug-free and (−)-baclofen containing ACSF. The point at which the two lines intersect represents the potential at which no polarizing effect of (−)-baclofen would be observed, that is the reversal potential. Obtained in such a fashion, the reversal potential for (−)-baclofen was found to be -102 ± 2 mV (mean ± s.e.mean, $n = 7$). This was significantly different (Student's t test, $P < 0.01$) from the reversal potential found using a similar procedure on the same neurones for muscimol, -83 ± 3 mV ($n = 5$) (Figure 4).

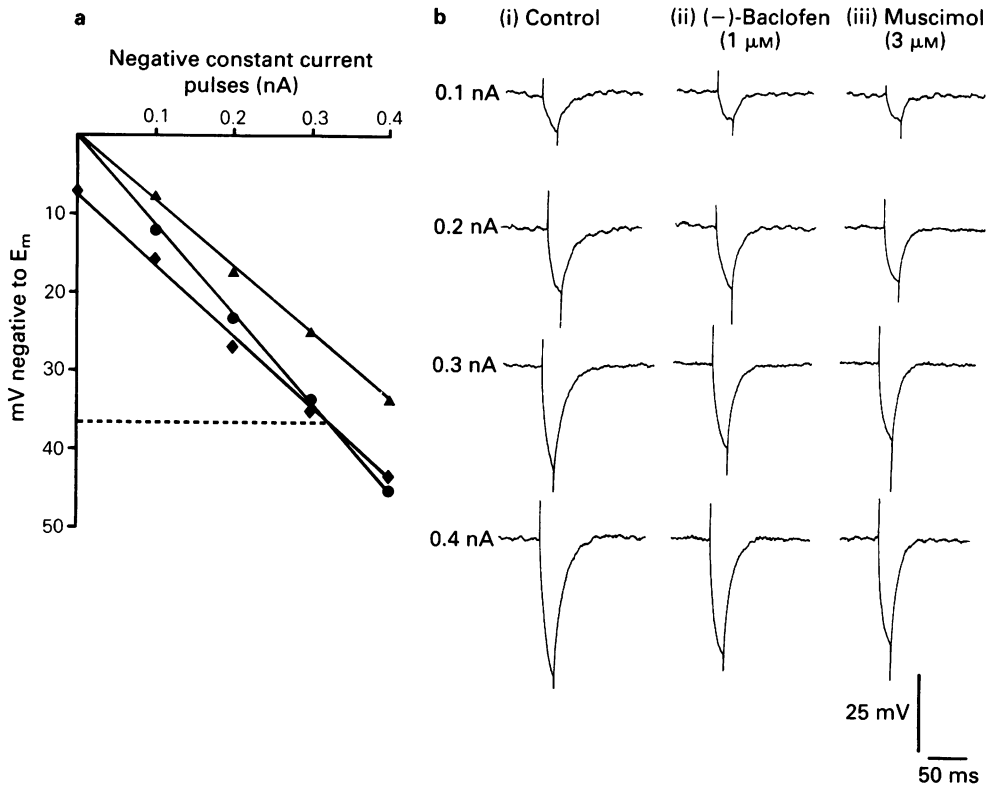


Figure 4 (a) Current-voltage plots were used to estimate the reversal potentials for (−)-baclofen and muscimol on the same neurone. Hyperpolarizing current pulses were injected into the neurone and the resulting voltage displacement measured as described in Methods. On the graph shown, the abscissa scale shows the amplitude of the injected current pulses and the ordinate scale the amplitude of the resulting voltgage displacement relative to the resting potential, E_m. The current-voltage plot obtained in the presence of muscimol (3 μM) (▲) intersects with the plot made under control conditions (●) at the resting membrane potential of −68 mV while that constructed in the presence of (−)-baclofen (1 μM) (◆) intersects with the control 37 mV negative to E_m. Since these points represent the potentials at which no polarizing actions of the drugs would be observed (i.e. the reversal potential), it can be seen that the reversal potentials for muscimol and (−)-baclofen on this neurone are quite distinct. That for muscimol can be estimated at −68 mV, while that for (−)-baclofen is approximately −105 mV. (b) Examples of voltage displacements obtained in response to injection of square current pulses of 0.1 to 0.4 nA in amplitude during construction of the IV plot shown in (a). Voltage responses illustrated were made (i) under control conditions, (ii) in the presence of 1 μM (−)-baclofen and (iii) in the presence of 3 μM muscimol. In (ii) voltage displacements were superimposed upon a 7 mV hyperpolarization. Records shown were captured off-line on a storage oscilloscope and plotted out on a chart recorder.

At the higher injected currents saturation of the membrane capacitance may not have been complete and thus graph (a) may not be accurate at these high currents. It is noteworthy, however, that linearity is preserved and our main conclusion, that reversal potentials for muscimol and baclofen differ, is unaffected by this consideration.

Estimates of the reversal potential suggested that activation of a potassium conductance may be responsible for the hyperpolarizing action of (−)-baclofen. If so, the reversal potential should vary according to the external concentration of potassium ions. It was shown that the reversal potential for (−)-baclofen was made less negative by increasing the external concentration of potassium ions, ($n = 2$) (Figure 5). On extrapolation of the relationship between external potassium concentration and the (−)-baclofen reversal potential, it could be seen that a tenfold change in the external potassium concentration would produce approximately the 60 mV shift in the reversal potential predicted from the Nernst equation for a pure potassium-mediated event.

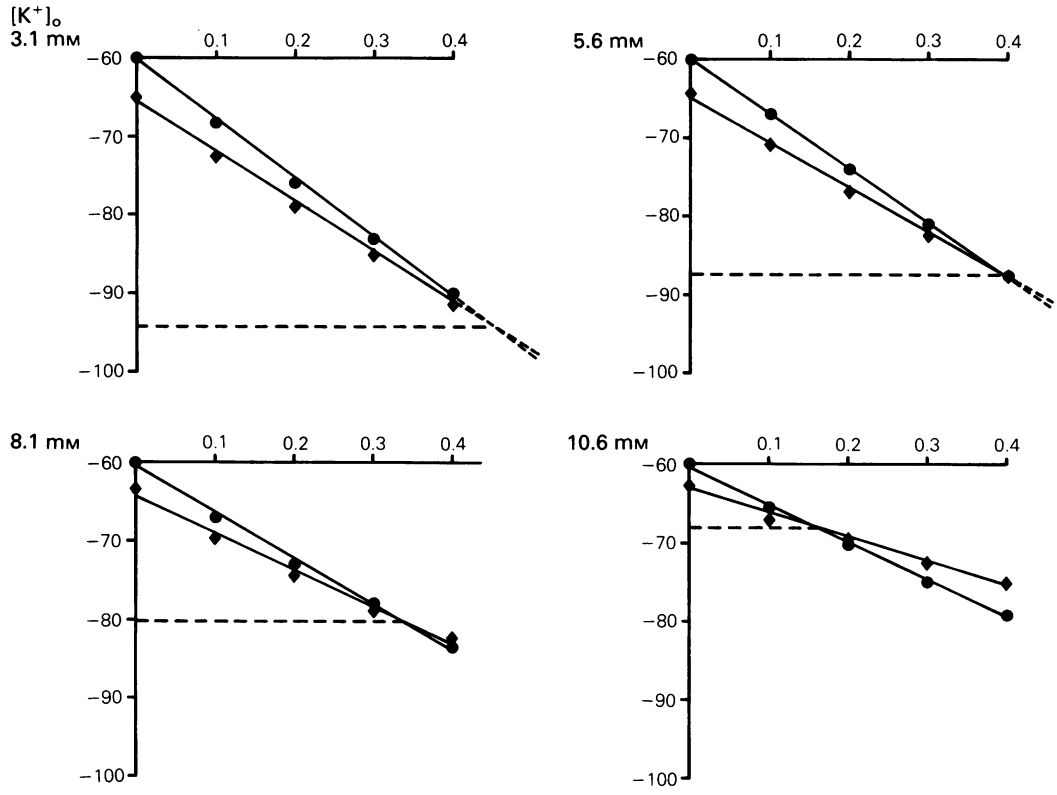


Figure 5 Estimates of the reversal potential for (−)-baclofen made on a single neurone with variation of the external concentration of potassium ions. $[K^+]_o$. External potassium concentration was increased from 3.1 mM to 5.6, 8.1 and 10.6 mM by addition of 1 M KCl to the perfusion medium. Membrane depolarization occurred so the membrane was brought back to its resting potential of −60 mV by current injection. Current-voltage plots were then constructed as described previously in control (●) and (−)-baclofen (1 μM) containing media (◆). The abscissa scale shows the amplitude of the hyperpolarizing current pulses injected into the neurone in nA and the ordinate scale the maximum potential in mV to which the membrane was displaced during these pulses. Again, the intersection of the two lines represents the reversal potential of (−)-baclofen. As the concentration of external potassium ions is raised so the reversal potential for (−)-baclofen becomes less negative.

Effect of (−)-baclofen on synaptic transmission

Polysynaptic composite excitatory postsynaptic e.p.s.ps can be evoked in deep dorsal horn neurones by electrical stimulation of the dorsal root entry zone. They may be up to 30 mV in amplitude and up to 350 ms in duration.

(−)-Baclofen (100 nM–30 nM) depressed the e.p.s.p. in all neurones tested ($n = 15$). This effect occurred along with the postsynaptic response although did not always follow the same time-course. E.p.s.p. depression was dose-dependent and larger than would be predicted if the change was merely due to the increase in postsynaptic membrane conductance (Figure 6(i)).

Effect of potassium channel blockers on the response to (−)-baclofen

Since an increase in membrane conductance can alone produce a reduction in e.p.s.p. amplitude, it was useful to be able to show that e.p.s.p. depression occurred in the absence of any postsynaptic effect. This was achieved by filling electrodes with 3 M CsCl as a substitute for 3 M KCl. As diffusion of ions occurs from the tip of the electrode into the impaled neurone, caesium ions are able to block potassium channels from the intracellular surface of the membrane. Under these conditions the postsynaptic action of baclofen was either substantially reduced or completely abolished, although depression of the

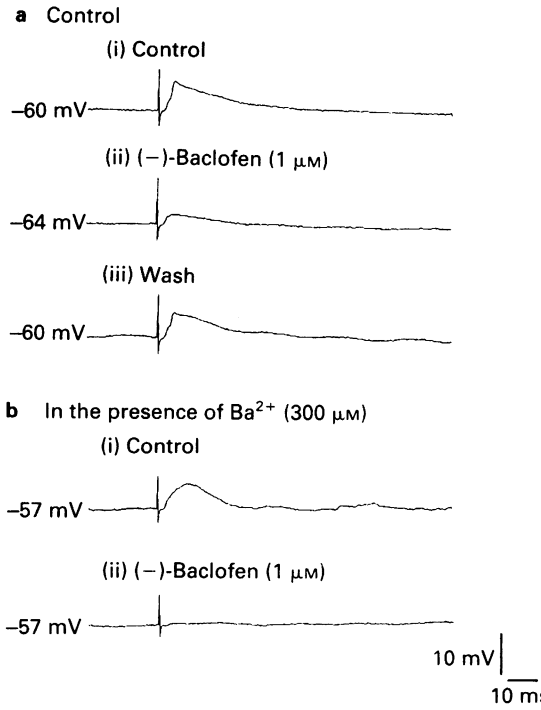


Figure 6 (–)-Baclofen depresses the polysynaptic composite e.p.s.p. evoked in dorsal horn neurones by electrical stimulation of the dorsal root entry zone. This effect persists in the presence of a concentration of barium known to block the hyperpolarizing response to baclofen. E.p.s.ps shown are the average of 10 e.p.s.ps analysed off-line. (a) Shows the control response of the e.p.s.p. to 1 μM (–)-baclofen. The knockdown of the e.p.s.p. seen on application of (–)-baclofen was superimposed upon a hyperpolarization of 4 mV and an increase in membrane conductance. (b) Shows the response of the e.p.s.p. evoked in the same neurone in the presence of 300 μM extracellular barium. Preincubation with barium had taken place for 10 min before (–)-baclofen application and no hyperpolarizing response of the neurone to (–)-baclofen was observed. Depression of the e.p.s.p. was however still observed under these conditions. The stimulus artifact in (b) is smaller as the amplitude of the stimulus was reduced to prevent action potential firing owing to the more excitable state of the neurone. A satisfactory return of the e.p.s.p. to control after (–)-baclofen application could not be obtained in the presence of barium as increasing potassium channel blockade made the neurone highly excitable and electrical stimulation resulted in burst firing of action potentials.

e.p.s.p. was still observed ($n = 6$). Blockade of the postsynaptic action of (–)-baclofen was a time-dependent phenomenon and it generally took up to 60 min of impalement with a caesium-filled electrode before complete abolition of the response occurred. Injection of caesium into the neurone and the resulting potassium channel blockade caused a gradual depolarization of the cell membrane over time.

Extracellular application of barium ions is also known to block potassium channels. Administration of barium chloride at a concentration of 300 μM in the perfusate markedly reduced the postsynaptic action of (–)-baclofen while not affecting its ability to depress e.p.s.ps (Figure 6b(ii)). Barium itself produced a depolarization of the neuronal membrane concomitant with an increase in apparent input resistance. Additionally, barium produced an increase in neuronal activity which could be depressed by both (–)-baclofen and a high concentration (20 mM) of magnesium ions.

In an attempt to learn more about the type of potassium current activated post-synaptically by (–)-baclofen, tetraethylammonium ions (TEA) at a concentration of 10 mM and 4-aminopyridine (4-AP) at a concentration of 50 μM were applied in the perfusate. Although both had effects on cell membrane properties (depolarization and firing of action potentials) neither drug affected the response to (–)-baclofen.

Discussion

The $GABA_B$ agonist (–)-baclofen exerts a direct hyperpolarizing action on the postsynaptic membrane of neurones in deep dorsal horn of spinal cord. The direct interaction of (–)-baclofen with receptors on the postsynaptic membrane is confirmed by the persistence of the effect in the presence of 20 mM Mg^{2+} which blocks synaptic transmission within the slice. The Hill slope associated with such a postsynaptic action is 1.0 implying that it is the result of (–)-baclofen binding to a single site. Estimates of the reversal potential of the postsynaptic event and its modulation by the external potassium concentration indicate that it is mediated by activation of a potassium conductance. This is supported by its sensitivity to intracellularly applied caesium and extracellularly applied barium.

A similar postsynaptic action of (–)-baclofen has been reported in other areas of CNS and is perhaps best characterized in hippocampus. Here, $GABA_B$ receptor activation induces a postsynaptic potassium current which is voltage-dependent and rectification is seen at both depolarized and hyperpolarized potentials (Inoue *et al.*, 1985a; Newberry & Nicoll, 1985). This contrasts with the conductance activated by (–)-baclofen in spinal dorsal horn neurones which, under our experimental conditions, does not rectify over the voltage range studied. However, it should be noted that under control conditions, membrane rectification is observed in hippocampal but

not spinal neurones and so the difference in voltage properties of the (−)-baclofen-evoked potassium currents may merely be a reflection of a difference between the two neurone populations.

In the hippocampal slice, the (−)-baclofen-activated current is blocked by micromolar concentrations of 4-AP (Inoue *et al.*, 1985a) whereas in the spinal cord slice concentrations of 4-AP up to 50 μM had no effect on the response to (−)-baclofen. It should, however, be noted that in hippocampal cultures the potassium current activated by (−)-baclofen was not affected by 4-AP (Brown & Gahwiler, 1987). The insensitivity of the (−)-baclofen response in dorsal horn neurones to TEA is consistent with previous findings in both hippocampal slices (Inoue *et al.*, 1985a) and cultures (Brown & Gahwiler, 1987).

The ionic event associated with postsynaptic $GABA_B$ receptor activation by (−)-baclofen is quite distinct from the chloride-mediated event associated with activation of the $GABA_B$ receptor and distinct reversal potentials for the two events can be obtained on the same dorsal horn neurones. There is however some depression of the response to (−)-baclofen by the $GABA_A$ receptor antagonist bicuculline. This phenomenon has also been observed in hippocampus (Inoue *et al.*, 1985b; Newberry & Nicoll, 1985) and may be the result of lack of absolute pharmacological selectivity of either agent or the ability of bicuculline to block potassium channels at the concentrations used (Heyer *et al.*, 1982).

The second observable effect of (−)-baclofen on deep dorsal horn neurones is the depression of the polysynaptic composite e.p.s.p. evoked by dorsal root stimulation. This still occurs where the postsynaptic response has been blocked by caesium and therefore cannot merely be due to the concomitant increase in postsynaptic membrane conductance.

It is possible that e.p.s.p. depression is the result of baclofen opening a potassium conductance on cell bodies of neurones earlier in the pathway. However, this is unlikely since the baclofen-induced depression of the e.p.s.p. is still observed in the presence of a concentration of bath-applied barium seen to block the post-synaptic response. Barium itself resulted in an increase in the activity of all neurones studied, an observation which may be explained in more than one way. Firstly, since barium is taken up into the neurone through calcium channels, it may be leading to transmitter release. This is unlikely as barium can, but does not consistently, substitute for calcium in transmitter release (Miledi, 1966). Secondly, barium-blockade of potassium channels results in depolarization of the neuronal membrane with depolarization of terminals leading to calcium entry and transmitter release. In either case, the involvement of terminal calcium channels is implicated and supported experimentally by the blockade of barium-induced activity seen on application of a high concentration of magnesium ions which act as a competitive antagonist for calcium at a variety of chemical synapses (Katz, 1969). The observation that baclofen can also depress barium-induced activity implies that it too may act at these sites, but could only be confirmed if intracellular recording from nerve terminals was possible in this preparation.

(−)-Baclofen is an extremely potent anti-spastic agent which, unlike other drugs of this class, depresses monosynaptic as well as polysynaptic extensor reflex transmission in spinal cord (Fehr & Bein, 1974). It has no effect on transmitter release from motoneurone terminals (Glavinovic, 1979) implying a central site of action but has not been found to affect transmitter release from excitatory spinal interneurones, cholinergic motor axon collaterals or from descending fibres in the dorsolateral funiculus (Curtis *et al.*, 1981; Curtis & Malik, 1985). Since (−)-baclofen invariably hyperpolarizes neurones in spinal dorsal horn, this may account for its potent depression of spinal reflex transmission. Although two mechanisms of action of (−)-baclofen on dorsal horn neurones can be distinguished in this spinal cord slice preparation, it is possible that the postsynaptic activation of a potassium current is functionally the more important.

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Mechanism of dexamphetamine-induced mydriasis in the anaesthetized rat

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1 The effect of intravenous administration of dexamphetamine ((+)-Amp) on rat pupil diameter was investigated. In all experiments, the vagosympathetic trunks were sectioned bilaterally at the cervical level.

2 In rats anaesthetized with urethane, (+)-Amp (0.1–0.3 mg kg^{-1}, i.v.) produced a dose-related increase in pupil size. The mydriatic effects of (+)-Amp were evident immediately after administration.

3 Pretreatment with the α_2-adrenoceptor antagonists yohimbine (1.5 mg kg^{-1} i.v.) or idazoxan (0.5 mg kg^{-1}, i.v.) blocked the pupillary response to (+)-Amp. Yohimbine caused about a 30 fold shift to the right in the dose-response curve whereas idazoxan almost completely abolished the mydriatic response to (+)-Amp.

4 In contrast, pretreatment with the α_1-adrenoceptor antagonist phenoxybenzamine (2 mg kg^{-1}, i.v.), failed to alter significantly the pupillary response to (+)-Amp.

5 Depletion of central nervous system (CNS) monoamines with reserpine (5 mg kg^{-1}, i.p.) and α-methyl-*p*-tyrosine (2 × 300 mg kg^{-1}, i.p.) prevented the pupillary response to (+)-Amp.

6 The mydriatic effect of (+)-Amp was present only in preparations that had intact parasympathetic neural tone to the iris. Central preganglionic denervation of the oculomotor nerve abolished the mydriatic response of (+)-Amp.

7 These results indicate the (+)-Amp acts in the CNS to produce mydriasis in the anaesthetized rat by stimulating CNS postsynaptic α_2-adrenoceptors, findings that are consistent with the hypothesis that (+)-Amp acts predominantly as an indirect sympathomimetic agent to release endogenous stores of a monoaminergic neurotransmitter (perhaps noradrenaline).

Introduction

Amphetamine produces mydriasis in many species including man, cats, dogs and rats (Marley, 1961; Martin *et al.*, 1971; Koss, 1980b; Klemfuss & Adler, 1986). Controvery still exists, however, regarding the precise mechanism by which amphetamine exerts its pupillary effects. Several studies indicate that amphetamine may produce mydriasis, in part, by a catecholamine releasing action at the level of the iris radial muscle in anaesthetized cats (Marley, 1961) and conscious rats (Schipper *et al.*, 1979). In contrast, other studies in anaesthetized cats (Koss, 1980b) and conscious rats (Klemfuss & Seiden, 1980) indicate that the primary mode of action is to elicit CNS inhibition of parasympathetic pupilloconstrictor tone to the iris sphincter muscle.

The present study was undertaken to elucidate the mechanism by which dexamphetamine ((+)-Amp) produces mydriasis in the anaesthetized, sympathectomized rat. Experiments were designed to determine the site of action and receptor types involved. Toward this end, graded pupillary responses to (+)-Amp were observed in anaesthetized rats under the following experimental conditions: (1) pretreatment with α_1- or α_2-adrenoceptor selective antagonists; (2) depletion of CNS monoamines to determine if effects are mediated by an indirect or direct sympathomimetic action; (3) preparations in which one oculomotor nerve was denervated to determine if the effect is mediated by a central or peripheral action. In these studies we observed that (+)-Amp produced a mydriasis of central origin mediated by activation of postsynaptic α_2-adrenoceptors.

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Methods

General

Rats of either sex were anaesthetized with urethane ($2.0\,g\,kg^{-1}$, i.p.). The dose of anaesthetic was reduced by 50% in monoamine depleted experiments. In all animals, the vagosympathetic nerve trunks were sectioned bilaterally at the cervical level. The rats were then intubated, placed on a respirator and kept warm with a heating pad. A femoral vein and artery were cannulated for i.v. drug administration and cardiovascular parameter recordings. Blood pressure and heart rate were monitored with a Statham P23Dd pressure transducer and recorded on a Grass 7B polygraph. The rats were then positioned in a stereotaxic apparatus and paralyzed with gallamine triethiodide ($2–5\,mg\,kg^{-1}$, i.v.). Pupillary responses were measured with a ruler (0.1 mm graduations) and a binocular microscope. All observations were done under green light to minimize light-induced reflex, pupillary constriction and to add contrast to the iris.

Monoamine depletion

Some rats were depleted of CNS monoamines by intraperitoneal injections of reserpine ($5\,mg\,kg^{-1}$) and two doses of tyrosine hydroxylase inhibitor α-methyl-*p*-tyrosine ($300\,mg\,kg^{-1}$). The dose of reserpine and one dose of α-methyl-*p*-tyrosine were administered 20 h before the experiments. The second dose of α-methyl-*p*-tyrosine was administered 2 h before the experiment.

This combined pretreatment regimen with reserpine and α-methyl-*p*-tyrosine has been reported by a number of investigators to lower CNS noradrenaline, adrenaline, dopamine and 5-hydroxytryptamine levels by at least 95% (Haeusler, 1974; Koss & Christensen, 1979; Hey *et al.*, 1985).

Oculomotor nerve lesions

In experiments in which parasympathetic tone to the iris was eliminated, the intersection of the coronal sutures, bregma, was used as a reference point for determination of electrolytic lesion coordinates (oculomotor nerve). A coaxial electrode was introduced to the appropriate stereotaxic coordinates by a dorsal approach as designated by Pellegrino *et al.* (1979) using a coordinate correction to adjust for varying weights (Whishaw *et al.*, 1977). A Grass S88 stimulator, and constant current unit (model CCU1A) was used to deliver 1.5 mA of d.c. current for a period of 15 s to the designated area. Complete unilateral oculomotor denervation was verified by maximal pupillary dilatation of approximately 5.0 mm and the abolition of the light-evoked constrictor responses. One or two drops of 0.5% physostigmine (w/v) was then applied topically to the denervated eye to produce miosis of less than 1.0 mm. Maximal pupillary constriction with physostigmine was observed within 15–30 min.

Statistics

The evaluations of experimental results were done with the use of two-way analysis of variance (ANOVA). The test of significance for the experimental findings was at the $P < 0.05$ level.

Drugs

The following drugs were dissolved in physiological sodium chloride solution: idazoxan hydrochloride (Reckitt and Colman); (±)-adrenaline hydrochloride (Parke-Davis); physostigmine salicylate (Sigma); α-methyl-*p*-tyrosine methylester hydrochloride (Sigma); reserpine as Serpasil (Ciba-Geigy); phenoxybenzamine hydrochloride (Smith, Kline and French); and dexamphetamine sulphate (National Institute of Drug Abuse). Yohimbine hydrochloride (Aldrich) was dissolved in distilled water.

Results

Effects of intravenous administration of dexamphetamine on pupil diameter

Control responses Intravenous cumulative doses of dexamphetamine ((+)-Amp) ($0.1–3.0\,mg\,kg^{-1}$) were administered to five urethane-anaesthetized rats in which the cervical vagosympathetic nerve trunks had been sectioned. As illustrated in Figure 1, (+)-Amp produced a dose-dependent mydriasis in these preparations. The maximal response was usually attained within 3–5 min and remained unchanged until the next dose was injected. Subsequent administration of yohimbine ($1.5\,mg\,kg^{-1}$ i.v.) partially reversed the dexamphetamine-induced mydriasis (data not shown).

Pretreatment with α_2-adrenoceptor antagonists These experiments were performed to determine whether (+)-Amp-induced mydriasis was mediated by CNS α_2-adrenoceptors. Pretreatment with yohimbine (1.5 mg kg, i.v.) or idazoxan ($0.5\,mg\,kg^{-1}$, i.v.) 15 min before the i.v. administration of (+)-Amp effectively blocked the dexamphetamine-induced mydriasis (Figure 1). It is of interest that both yohimbine and idazoxan produced a small but statistically significant miosis as compared to the control untreated pupil. The control (pre-amphetamine, pre-α_2-antagonist) pupil diameter was 0.98 ± 0.20 mm com-

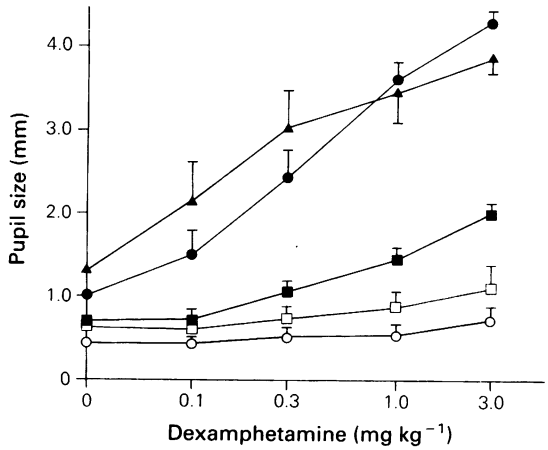


Figure 1 Pupillary responses to graded i.v. doses of dexamphetamine (0.1–3.0 mg kg^{-1}) in five groups of anaesthetized rats: (1) (●) control; (2) (▲) pretreated with phenoxybenzamine (2.0 mg kg^{-1}, i.v.); (3) (■) pretreated with yohimbine (1.5 mg kg^{-1}, i.v.); (4) (□) pretreated with idazoxan (0.5 mg kg^{-1}, i.v.); (5) (○) monoamine depleted. Note that monoamine depletion as well as yohimbine and idazoxan pretreatment antagonized the mydriatic action of amphetamine. Values are means with s.e.mean shown by vertical bars ($n = 5 - 6$) for each group. Both α_2-adrenoceptor pretreatment curves and the monoamine depletion curve are significantly different from the control responses; $P < 0.05$. Control and phenoxybenzamine pretreated curves are not statistically different.

pared with 0.70 ± 0.10 mm in the yohimbine pretreated rats and 0.60 ± 0.07 mm in the idazoxan pretreated group. Four of the five yohimbine pretreated rats received an additional higher dose of (+)-Amp (10 mg kg^{-1}, i.v.). At 10 mg kg^{-1} i.v. the (+)-Amp-induced mydriasis was 2.80 ± 0.24 mm in the yohimbine group and 1.50 ± 0.27 mm for the idazoxan group. The shift to the right in the pupillary response curve seen with yohimbine was about 30 fold, and about 100 fold for idazoxan.

Pretreatment with phenoxybenzamine These experiments were performed to determine whether (+)-Amp-induced mydriasis might be mediated, in part, by activation of peripheral α_1-adrenoceptors located on the iris. Pretreatment with phenoxybenzamine (2.0 mg kg^{-1}, i.v.) 30 min before administration of (+)-Amp failed to block its pupillary response (Figure 1). The 30 min delay between phenoxybenzamine administration and the first dose of (+)-Amp allowed for adequate distribution and irreversible binding of phenoxybenzamine to the peripheral α_1-adrenoceptors of the iris dilator muscle. As can be seen in Figure 1, phenoxybenzamine produced a small mydriasis in the naive (pre-amphetamine) pupil. The pupil diameter in this group ($n = 6$) was 1.30 ± 0.20 mm as compared with 0.98 ± 0.20 mm of the control pupils. With low doses of (+)-Amp (0.1–0.3 mg kg^{-1}) the phenoxybenzamine-pretreated rats showed an increase of the mydriatic response as compared with the control curve. In contrast, at the higher doses of (+)-Amp (1.0–3.0 mg kg^{-1}) the mydriatic response was slightly lower than the control pupillary response to (+)-Amp.

Monoamine depletion In order to determine whether (+)-Amp-induced mydriasis is mediated by an indirect sympathomimetic effect (release of an endogenous monoamine), five rats were depleted of their endogenous stores of monoamines (Figure 1) and pupillary responses tested with cumulative doses of (+)-Amp (0.1–3.0 mg kg^{-1}). In these preparations the mydriatic response to (+)-Amp was completely blocked. These findings suggest that the pupillary effects of (+)-Amp are mediated by the release of an endogenous monoaminergic neurotransmitter. There was no evidence for a direct adrenoceptor activation of the iris dilator even at the highest dose of amphetamine (3.0 mg kg^{-1}).

Unilateral oculomotor denervation

This series of experiments were performed to determine if oculomotor innervation of the eye was required for the mydriatic effects of (+)-Amp, thus confirming a centrally-mediated pupillary action of (+)-Amp. Activation of iris radial muscle α-adrenoceptors would elicit mydriasis in both eyes, whereas if a CNS parasympathetic neural effect was involved only the innervated eye would dilate in response to i.v. (+)-Amp.

In a separate group of rats intracranial (preganglionic) lesion of one efferent oculomotor nerve was made. The subsequently dilated pupil was constricted with several drops of physostigmine (0.5% w/v). Pupillary constriction to less than 1.0 mm occurred within 15–30 min. Intravenous administration of (+)-Amp (0.1–3.0 mg kg^{-1}) caused a dose-related mydriasis only in the eye with intact parasympathetic tone (Figure 2a). Although the contralateral totally denervated eye did not respond to (+)-Amp, pupillary dilatation could still be evoked in both the denervated and innervated eyes following i.v. administration of the direct acting sympathomimetic, adrenaline (Figure 2b).

Discussion

The present findings demonstrate that (+)-Amp acts to produce mydriasis in the anaesthetized rat by

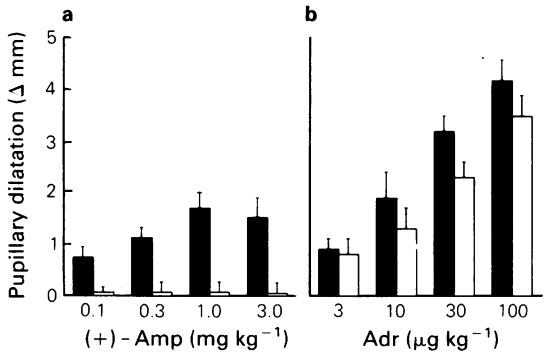


Figure 2 Effects of unilateral oculomotor nerve lesion on the pupillary responses to indirect and direct sympathomimetic agents. One or two drops of 0.5% physostigmine (w/v) was applied to the denervated eye to produce miosis similar to the intact eye (pupil size < 1.0 mm). Solid columns: intact eye; open columns: denervated eye. In (a) the responses to graded doses of dexamphetamine ((+)-Amp) (0.1–3.0 mg kg^{-1}, i.v.) on the intact and denervated eye are shown. Note that dexamphetamine only dilated the eye with the oculomotor nerve intact. In (b) the responses to graded dose of adrenaline (Adr) (3–100 μg kg^{-1}, i.v.) on both intact and denervated eyes. Adrenaline elicited a mydriatic response in both eyes. Each panel represents an independent set of rats. Values represent means with s.e.mean shown by vertical bar ($n = 5$). The differences in the pupillary responses to dexamphetamine between the intact and denervated eye shown in (a) are statistically significant, $P < 0.01$. The differences in the responses to i.v. adrenaline between the intact and denervated eyes are not statistically significant.

inhibiting parasympathetic pupillo-constrictor tone by activation of CNS α_2-adrenoceptors. Several studies in different species are in agreement with the present findings. Sharpe *et al.* (1977) reported that (+)-Amp (1.0 mg kg^{-1}, i.v.) produces pupillary dilatation in conscious, sympathectomized dogs. They concluded that amphetamine acts centrally to inhibit pupilloconstrictor tone as phenoxybenzamine only partially blocked the (+)-Amp mydriasis at a dose that completely blocked the effects of intravenously administered adrenaline and noradrenaline. In a recent study, Koss (1980b) showed that i.c.v. (+)-Amp produces a yohimbine-sensitive, dose-dependent mydriasis by inhibition of pupilloconstrictor neural tone in anaesthetized cats. Also in support of our conclusions, Klemfuss & Seiden (1980) found that single-dose administration of (+)-Amp in anaesthetized rats produces a mydriatic response that is not altered by sympathetic denervation nor adrenalectomy.

A considerable body of evidence has accumulated supporting the hypothesis that activation of central postsynaptic α_2-adrenoceptors produces withdrawal of parasympathetic pupilloconstrictor tone and mydriasis in rats and cats. Studies with the α_2-adrenoceptor agonists clonidine, UK-14,304, guanoxabenz, xylazine, guanabenz and several analogues of clonidine have been shown to produce a yohimbine-sensitive mydriasis (Gherezghiher & Koss, 1979; Koss & Christensen, 1979; Hsu *et al.*, 1981; Koss, 1981; 1983; Berridge *et al.*, 1983; Hey *et al.*, 1985). A similar yohimbine-sensitive mydriatic effect, with about a 2 h delay in attaining the maximal response, has been observed with α-methyldopa after conversion in the brain to the active metabolite α-methylnoradrenaline (Koss, 1980a; Gherezghiher *et al.*, 1982; 1983). Evidence for a central parasympathetic-inhibitory action of α_2-adrenoceptor agonists has also been provided by the finding that removal of CNS parasympathetic neural tone prevents drug-induced mydriasis. In pithed rats, Berridge *et al.* (1983) showed that guanoxabenz did not cause dilatation of the physostigmine-constricted pupil. Similarly, Koss & San (1976) demonstrated, in cats, that unilateral parasympathectomy prevented the mydriatic action of clonidine. These support the concept that α_2-adrenoceptor agonists act centrally to inhibit parasympathetic tone to the iris causing mydriasis.

In contrast to the present results, Klemfuss & Adler (1986) recently proposed that part of the mydriatic effect of (+)-Amp in the rat was due to activation of iris α_1-adrenoceptors. There are several possible explanations that may explain the apparent discrepancy between this study and our results. A primary difference between the two studies concerns the use of different experimental conditions. In the Klemfuss & Adler (1986) study, (+)-Amp was administered topically or s.c. as compared to an i.v. route in the present study. This difference may account for the greater mydriatic response to (+)-Amp in our study. The mydriatic response to 1.0 mg kg^{-1} (+)-Amp in the present study was 2.2 mm as compared with 0.6 mm in the Klemfuss & Adler (1986) study. A second difference was their use of conscious, unrestrained rats. It is possible that the degree of sympathetic activation is greater in the conscious rat as opposed to the anaesthetized rat. This potential increase in sympathetic tone to the iris, in turn, could account for the α_1-adrenoceptor-mediated component of the mydriatic response with (+)-Amp reported in their study. Thirdly, there is a critical difference in the dose of α_1-adrenoceptor antagonist phenoxybenzamine used to prevent the pupillary effects of (+)-Amp. The dose of phenoxybenzamine used in their study (10 mg kg^{-1}) was five times greater than in the present study. Although phenoxybenzamine is more selective for α_1- than α_2-adrenoceptors it is likely

that at higher doses it has central α_2-adrenoceptor blocking effects. In our study phenoxybenzamine ($2\,mg\,kg^{-1}$) did not affect the mydriatic response to a range of (+)-Amp doses. The dose of phenoxybenzamine used in the present study, however, has been shown to block the peripherally-mediated mydriasis produced by activation of iris α_1-adrenoceptors with adrenaline (Gherezghiher & Koss, 1979).

The functional integrity of the oculomotor-denervated iris was tested in the present study. Our observations indicate that the denervated physostigmine-treated iris and the innervated eye show similar mydriatic responses to systemic adrenaline (Figure 2). These findings confirm earlier studies demonstrating the pharmacological viability of the parasympathectomized iris done by Ury & Gellhorn (1939) and Koss & Rieger (1976). In studies with cats, they demonstrated that the mydriatic response to systemic adrenaline is similar in both the innervated and denervated physostigmine-treated iris. Mydriasis elicited by activation of peripheral α-adrenoceptors with α_1-agonists is blocked by the α_1-adrenoceptor antagonist phenoxybenzamine (Gherezghiher & Koss, 1979; Koss, 1980b) but has been shown to be insensitive to the α_2-adrenoceptor antagonist yohimbine (Gherezghiher & Koss, 1979; Koss, 1980a,b). These findings are consistent with the earlier report by Yonkman *et al.* (1944) who found that doses of yohimbine as high as $28\,mg\,kg^{-1}$ were required to antagonize the mydriatic responses due to sympathetic nerve stimulation.

In summary, (+)-Amp produces a dose-dependent, α_2-adrenoceptor antagonist sensitive mydriasis in the anaesthetized rat by a CNS action. These results indicate that (+)-Amp acts as an indirect sympathomimetic agent to release an endogenous monoamine (perhaps noradrenaline) to produce mydriasis by acting on CNS postsynaptic α_2-adrenoceptors. The present findings are consistent with the hypothesis that, as seen in the cat, (+)-Amp produces mydriasis in this species primarily by means of CNS α_2-adrenoceptor parasympatho-inhibitory action without a significant peripheral α_1-adrenoceptor component to its pupillary effect.

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β-adrenoceptor-mediated facilitation of [^{3}H]-noradrenaline release from the intramural nerves of bovine mesenteric lymphatic vessels

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1 Isolated segments of bovine mesenteric lymphatic vessels were loaded with [^{3}H]-noradrenaline and its efflux in response to field stimulation at 2 Hz (0.3 ms pulses, 1 min train) examined.

2 Isoprenaline (10^{-6} M) increased evoked fractional ^{3}H efflux (^{3}H released as a percentage of total tissue ^{3}H at the onset of stimulation) from its control value of 0.67 $\pm$ 0.09 to 1.2 $\pm$ 0.18% (s.e.mean; $n = 5$).

3 Propranolol (10^{-6} M) alone had no effect on evoked fractional ^{3}H efflux but blocked the increase in transmitter overflow induced by isoprenaline (10^{-6} M).

4 In the presence of the α-adrenoceptor antagonist phentolamine (10^{-6} M) the facilitatory effect of isoprenaline (10^{-6} M) on transmitter efflux was enhanced. In 5 experiments isoprenaline increased evoked fractional ^{3}H efflux from its control value of 1.07 $\pm$ 0.17 to 2.5 $\pm$ 0.37% when phentolamine was present.

5 Adrenaline (10^{-8} M) increased evoked fractional ^{3}H efflux from its control value of 0.76 $\pm$ 0.09 to 1.13 $\pm$ 0.17% ($n = 7$).

6 These findings are consistent with the presence of presynaptic β-adrenoceptors in this preparation. There is no evidence that these receptors play any role in feedback regulation of transmitter release.

Introduction

Lymphatic vessels show regular spontaneous contractions which are capable of propelling fluid in both isolated cannulated preparations and *in vivo* (Hall *et al.*, 1965; Campbell & Heath, 1973; McHale & Roddie, 1976; Olszweski & Engeset, 1985; McGeown *et al.*, 1987a). Histochemical, pharmacological and electrophysiological evidence suggests that these vessels are innervated by adrenergic nerves which can modulate both the frequency and force of spontaneous contraction and thus lymph flow. For example, isolated bovine mesenteric lymphatics respond to field stimulation by an increase in the frequency and force of their spontaneous contraction. This excitatory effect is mediated via α-adrenoceptors and is converted to a β-adrenoceptor inhibitory response after α-adrenoceptor blockade with phentolamine or phenoxybenzamine (McHale *et al.*, 1980; Allen & McHale, 1986). Further, *in vivo* studies on the sheep suggest that sympathetic stimulation increases lymph flow as a consequence of a direct effect on lymphatic pumping (McGeown *et al.*, 1987b).

There is now a considerable body of evidence which supports the postulate that total transmitter efflux from sympathetic nerves is not simply under the control of the rate of discharge from the CNS. For example, the nerve terminals may possess an array of receptor types sensitive to neurally liberated or circulating substances. Stimulation of these receptors can modify the amount of transmitter released per impulse (for reviews see: Westfall, 1977; Langer, 1981). The presence of these receptors may give the

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nerve terminals which possess them a means for local regulation of transmitter release and thus the postsynaptic response.

Transmitter release from adrenergic neurones may be examined by preloading terminals with [^{3}H]-noradrenaline and monitoring its efflux in response to field stimulation (Su & Bevan, 1970). Despite their relatively sparse innervation when compared to accompanying arteries or veins (Alessandrini *et al.*, 1981) this technique has been applied successfully to isolated lympahtic vessels (Allen *et al.*, 1988). In the latter study the α-adrenoceptor agonists clonidine, xylazine and noradrenaline were shown to depress field evoked release of labelled transmitter while the α-adrenoceptor antagonists phentolamine, yohimbine and rauwolscine increased field stimulated release. These findings were interpreted as evidence for the existence of presynaptic α-adrenoceptors to which the endogenous transmitter could bind and limit further noradrenaline release.

In addition to α-adrenoceptor-mediated negative feedback control of transmitter release, evidence for the existence of presynaptic β-adrenoceptors has also been obtained in a number of preparations (for review see: Majewski, 1983). It has been suggested that in those terminals possessing β-adrenoceptors the endogenous transmitter may bind to this receptor and provide positive (facilitatory) control for the release process (Adler-Graschinsky & Langer, 1975). It was the purpose of this investigation to examine the innervation to mesenteric lymphatic vessels for the presence of β-adrenoceptor-mediated facilitatory control of transmitter release.

Methods

Techniques for the collection and isolation of bovine mesenteric lymphatic vessels have been described in detail previously (McHale & Roddie, 1976; Allen *et al.*, 1983). Vessel segments approximately 3 cm in length and 2 mm in external diameter were sutured at both ends and mounted in a water-jacketed organ bath (1 ml capacity) maintained at 37°C. Krebs solution (in mM: NaCl 120, KCl 4.7, $NaHCO_3$ 25, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5 and glucose 5.55) gassed with 5% CO_2 in O_2 was perfused through the organ bath at a rate of 1 ml min^{-1} by a Gilson Miniplus II flow inducer. The lower end of the vessel was fixed and the upper end attached to an isometric force transducer (Gould UC3) the output of which was displayed on a Gould 8000S chart recorder. Initially the lymphatics were placed under approximately 2 mN tension and left to equilibrate for at least 45 min. After the onset of spontaneous activity the vessels were superfused for 45 min with oxygenated Krebs solution containing [^{3}H]-noradrenaline (50 μCi ml^{-1}) and ascorbic acid (10 mM). Following this period of incubation the vessels were washed by continuous perfusion with Krebs solution for a 2 h period.

Platinum ring electrodes positioned at the top and bottom of the organ bath were connected to a Grass S88 pulse generator for field stimulation of the preparation and the standard stimulus consisted of trains of pulses of 0.3 ms duration at 2 Hz and 40 V (nominal) applied for 1 min. Previous work has shown pulses of this duration to stimulate selectively intramural nerves in isolated lymphatic vessels (McHale *et al.*, 1980; Allen & McHale, 1986). Following incubation with [^{3}H]-noradrenaline all preparations were stimulated initially at 4 Hz for 1 min to displace extraneuronal transmitter which may have been released upon contraction of the vessel. Under these conditions subsequent field stimulation-evoked ^{3}H-efflux reflects endogenous transmitter release (Allen *et al.*, 1988).

Unless otherwise stated cocaine (10^{-6} M) and normetanephrine (10^{-6} M) were added to the perfusing solution at this time to block neuronal and extraneuronal uptake of noradrenaline and remained present for the rest of the experiment.

Drugs and radiochemicals

The drugs used and their sources were as follows: cocaine hydrochloride (Sigma); isoprenaline sulphate (Saventrine, Pharmax); propranolol hydrochloride (Inderal, ICI); adrenaline ((−)-epinephrine, Sigma); phentolamine mesylate (Rogitine, CIBA); normetanephrine hydrochloride (Sigma); (−)-noradrenaline bitartrate (Levophed, Winthrop) and the radioisotope (−)-[7-8-^{3}H]-noradrenaline (Amersham, specific activity 30–50 Ci mmol^{-1}). All drugs were present in the perfusate for approximately 15 min before their effect on evoked ^{3}H overflow was examined.

Efflux of [^{3}H]-*noradrenaline*

Tritium efflux from the preparation was determined by counting the perfusate collected over a 1 or 2 min period i.e. 1 or 2 ml. To the fraction was added aqueous counting scintillant (Flouran-HV, BDH; 7 ml to a 1 ml sample) and the samples were counted on an LKB Betarack Liquid Spectrometer with automatic channels ratio to determine efficiency. At the end of each experiment the vessels were removed from the organ bath and placed in scintillant for a 48 h period. At the end of this time vessels had disintegrated and total tissue ^{3}H was determined.

Fractional ^{3}H efflux represents tritium efflux expressed as a percentage of total ^{3}H in the tissue at

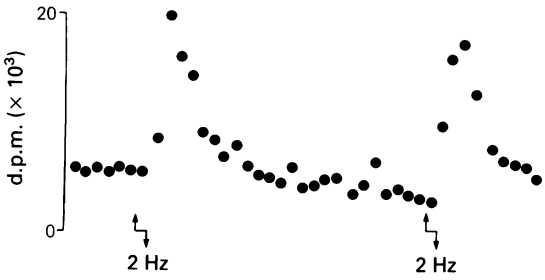


Figure 1 Tritium efflux (in d.p.m.) from a single lymphatic vessel in response to field stimulation on two consecutive occasions (S1 and S2). Each point represents efflux over a 1 min period.

the time of stimulation (Farnebo & Hamberger, 1971) calculated as:-

$$\frac{\text{evoked rise, above baseline, in efflux of } ^3\text{H}}{\text{total tissue } ^3\text{H at the onset of stimulation.}} \times 100$$

Mean data are expressed together with s.e.mean. Where indicated the statistical test applied was a Student's t test for matched pairs. P values of <0.05 were considered significant.

Results

Field stimulation applied to lymphatic vessels on two occasions (S1 and S2) with a 25 min interval between trains evoked ^{3}H efflux (d.p.m.) to a similar extent on each occasion (Figure 1). Results from 5 such experiments showed no significant difference in fractional ^{3}H efflux between S1 ($0.72 \pm 0.07\%$) and S2 ($0.76 \pm 0.11\%$) with an S2/S1 ratio of 1.05.

Isoprenaline

The β-adrenoceptor agonist isoprenaline (10^{-6} M) enhanced stimulation-induced overflow of ^{3}H without affecting basal ^{3}H efflux (d.p.m. Figure 2). Figure 2b summarizes the results from 5 such experiments and shows isoprenaline to increase significantly ($P < 0.05$) field-stimulated fractional ^{3}H efflux (S2/S1 = 1.8).

In 6 experiments control (S1) evoked fractional ^{3}H efflux in the presence of propranolol (10^{-6} M) was $0.73 \pm 0.1\%$ and after the inclusion of isoprenaline (S2) was not significantly different at $0.72 \pm 0.08\%$.

The α-adrenoceptor antagonist phentolamine is known to increase evoked ^{3}H overflow by blocking presynaptic α-adrenoceptors (Allen *et al.*, 1988). In

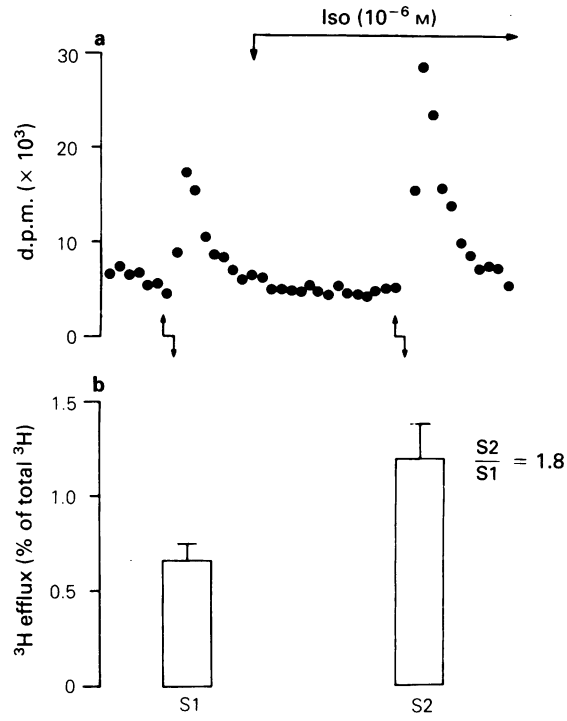


Figure 2 (a) ^{3}H efflux (in d.p.m.) from a single lymphatic vessel: the vessel was stimulated on two consecutive occasions (S1 and S2) as before. Each point represents efflux over a 1 min period. Periods of stimulation are indicated below the graph. At the time indicated above the graph, isoprenaline (Iso; 10^{-6} M) was added and remained for the rest of the experiment. In (b) mean fractional ^{3}H effluxes from 5 similar experiments are presented. Vertical lines represent s.e.mean.

the present study, phentolamine (10^{-6} M) potentiated the effect of isoprenaline on field stimulated release of ^{3}H. In 5 experiments in which phentolamine was present, isoprenaline increased evoked fractional ^{3}H efflux from its control (S1) value of $1.07 \pm 0.17\%$ to $2.5 \pm 0.37\%$ (S2/S1 = 2.34).

Propranolol

The β-adrenoceptor antagonist propranolol (10^{-6} M) did not affect field evoked fractional ^{3}H efflux. In 5 experiments, fractional ^{3}H efflux was $0.56 \pm 0.07\%$ before (S1) propranolol and was not significantly altered ($0.52 \pm 0.08\%$) after (S2) drug addition (S2/S1 = 0.93). Similarly, propranolol (10^{-6} M) was without effect on evoked fractional ^{3}H release after α-adrenoceptor blockade with phentolamine

(10^{-6} M). In 4 experiments, evoked fractional ^{3}H efflux in the presence of phentolamine was $1.56 \pm 0.28\%$ and after the inclusion of propranolol was not significantly different at $1.4 \pm 0.25\%$ (S2/S1 = 0.90).

Adrenaline

Adrenaline (10^{-8} M), like isoprenaline, potentiated the evoked release of ^{3}H. In 7 experiments fractional ^{3}H efflux to field stimulation was $0.76 \pm 0.09\%$ before drug addition (S1) and was increased significantly to $1.13 \pm 0.1\%$ 15 min after (S2) the addition of adrenaline (S2/S1 = 1.49).

Discussion

The selective β-adrenoceptor agonist isoprenaline (10^{-6} M) increased field stimulation-evoked fractional ^{3}H efflux from isolated mesenteric lymphatic vessels by approximately 80%; an effect abolished by the β-adrenoceptor antagonist propranolol (10^{-6} M). This observation suggests that the sympathetic nerve fibres to these vessels are endowed with facilitatory β-adrenoceptors.

Exogenously applied β-adrenoceptor agonists have been shown to increase evoked release of [^{3}H]-noradrenaline in many preparations including guinea-pig atria (Adler-Graschinsky & Langer, 1975) and vasa deferentia (Stjarne, 1975); cat nictitating membrane and aortic strips (Langer *et al.*, 1975); human omental blood vessels (Stjarne & Brundin, 1975) and oviduct (Hedqvist & Moawad, 1975), mouse atria and rabbit pulmonary artery (Johnston & Majewski, 1986). However, an effect of β-adrenoceptor agonists on transmitter release is not always easily demonstrated. Starke *et al.* (1975) and Endo *et al.* (1977) failed to observe an effect of isoprenaline on evoked [^{3}H]-noradrenaline release in the rabbit pulmonary artery. Similarly, early work with mouse atria suggested that prejunctional β-adrenoceptors were absent from this tissue (Farnebo & Hamberger, 1974). Nevertheless, in a more recent study of both these tissues (Johnston & Majewski, 1986) isoprenaline was shown to be effective in enhancing transmitter release when the α-adrenoceptor antagonist phentolamine was present.

The ability to demonstrate a presynaptic effect of isoprenaline only in the presence of an appropriate α-adrenoceptor antagonist has been reported for a number of different preparations including rabbit ear artery (Majewski & Rand 1981) and rat portal vein (Dahlof *et al.*, 1978). Moreover, in the rat atria the facilitatory action of isoprenaline on endogenous noradrenaline release is inhibited by exogenous noradrenaline and enhanced after α-adrenoceptor blockade by phentolamine (Majewski & Rand, 1981). In agreement with this, Kalsner (1982) found noradrenaline prevented isoprenaline increasing transmitter release from the guinea-pig atria. The explanation for these findings is still a matter of debate although Majewski & Rand (1981) have proposed that activation of the α-adrenoceptor-mediated inhibitory feedback loop normally predominates over any β-adrenoceptor-mediated facilitation of transmitter release. Such an explanation is not consistent with all the available evidence (Johnston & Majewski, 1986) but interaction between these two opposing systems might explain the failure of isoprenaline to enhance noradrenaline release, at least in some preparations. It would also help to explain the finding of the present study that phentolamine was effective in potentiating the action of isoprenaline on evoked overflow of radio-labelled noradrenaline.

If presynaptic β-adrenoceptors form part of an ongoing positive feedback loop it might be expected that the introduction of a β-adrenoceptor antagonist, such as propranolol, would result in a depression of evoked transmitter release. In some studies this has been found to be the case. Nevertheless, the operation of on-going β-facilitation of transmitter release is not always easily demonstrated and there are many studies reporting the failure of β-adrenoceptor antagonists to alter noradrenaline release (see Majewski, 1983); such was the case in the current investigation. Propranolol (10^{-6} M) did not alter evoked release of [^{3}H]-noradrenaline even after blockade of prejunctional α-adrenoceptors with phentolamine. This would imply the absence of any functional β-adrenoceptor-mediated feedback, at least under the conditions of the present experiments. However, Adler-Graschinsky & Carrara (1982) have demonstrated that the neuronal uptake blocker desipramine can prevent propranolol decreasing evoked noradrenaline release in guinea-pig isolated atria. It was suggested that the latter finding might help to explain the failure of several authors to observe a decrease in the overflow of noradrenaline with β-adrenoceptor antagonists, since in all of these studies neuronal uptake blockers were employed. This might also explain the findings of the present work. However, in a more recent examination of prejunctional β-adrenoceptors in the rabbit pulmonary artery and mouse atria, in which neuronal uptake blockers were excluded, no effect of propranolol on transmitter release could be demonstrated (Johnston & Majewski, 1986). It seems that neuronally released noradrenaline does not normally activate the prejunctional β-adrenoceptor.

The absence of a physiological role for noradrenaline in activating the presynaptic β-adrenoceptor does not exclude a functional role for β-facilitation of transmitter release. Adrenaline is known to enhance noradrenaline release in a number of different preparations and to do so even when noradrenaline is without effect (see Majewski, 1983). In our preparation, a low concentration of adrenaline (10^{-8} M) significantly enhanced transmitter release. Thus, it may be that adrenaline is the normal physiological activator of the prejunctional β-adrenoceptors.

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Impaired mitochondrial oxidative energy metabolism following paracetamol-induced hepatotoxicity in the rat

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1 Effects of paracetamol treatment *in vivo* at subtoxic (375 mg kg^{-1} body weight) and toxic (750 mg kg^{-1} body weight) doses on energy metabolism in rat liver mitochondria were examined.

2 Paracetamol treatment resulted in a significant loss in body weights without affecting the liver protein contents. Toxic doses, however, resulted in 21% decrease in the yield of mitochondrial proteins.

3 Subtoxic doses of paracetamol did not, in general, affect the respiratory parameters in the liver mitochondria except in the case of succinate where both the state 3 respiration and the ADP-phosphorylation rates increased by 28%.

4 Toxic doses of paracetamol caused 25 to 47% decrease in the state 3 respiration rates depending on the substrate used. ADP/O ratios also decreased significantly with pyruvate + malate and succinate as the substrates. Consequently, ADP-phosphorylation was impaired significantly from 20 to 63%.

5 Subtoxic doses of paracetamol resulted in increased contents of cytochrome $c + c_1$ while the toxic doses caused lowering of the cytochromes aa_3 and b contents.

6 Glutamate and succinate dehydrogenase activities decreased in both the experimental groups while Mg^{2+}-ATPase activity was impaired only after toxic dose-treatment.

7 The results show that toxic doses of paracetamol result in impaired energy coupling in the liver mitochondria. Effects of subtoxic doses were also demonstrable in terms of impaired dehydrogenases activities.

Introduction

Actaminophen (N-acetyl-*p*-aminophenol, 4-hydroxyacetanilide), commonly known as paracetamol, is a widely used analgesic and antipyretic drug (Mitchell *et al.*, 1973; Hinson *et al.*, 1981; Breen *et al.*, 1982; McClain, 1982). In the U.S.A., the sale of this drug constitutes about 25% of the total analgesic sale over-the-counter (McClain, 1982). When used at normal therapeutic dose levels, it is considered to be a safe and an effective analgesic drug; however, at high doses this drug produces acute hepatotoxic and nephrotoxic effects, both in experimental animals and in man (Mitchell *et al.*, 1973; Dixon *et al.*, 1975; Hinson *et al.*, 1981; Dixon, 1984). Massive hepatic centrilobular necrosis and acute renal failure are reported following paracetamol overdoses (Hinson *et al.*, 1981; Cobden *et al.*, 1982; Newton *et al.*, 1983; Dixon, 1984). Self-poisoning with paracetamol has been a major problem in the past few years in that paracetamol overdose has become a common means of attempted suicide; cause of death was often acute hepatic necrosis (McClain, 1982).

Paracetamol toxicity is believed to result from interaction of paracetamol and/or its metabolites formed by cytochrome P-450 system, with cellular processes (Hinson *et al.*, 1981; Dixon, 1984). Histochemical studies have shown that liver necrosis is associated with structural damage to subcellular components including mitochondria in man and in experimental animals (Dixon, 1984). Consequently, the serum transaminase levels were also found to be elevated (Dixon, 1984). Swelling of mitochondria,

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disruption of cristae and transient inconsistent increase in succinate dehydrogenase activity at 6 h followed by a uniform loss in this enzyme activity have also been reported, based on histological and histochemical studies (Dixon *et al.*, 1975; Dixon, 1984). However, there are few reports demonstrating the *in vivo* effects of paracetamol toxicity on cellular processes at biochemical levels.

In view of the observations that paracetamol toxicity causes structural alterations in mitochondria (Dixon, 1984), it is possible that the toxic effects could be traced to the functional attributes of these organelles. We have, therefore, carried out studies to examine the effects of paracetamol-induced hepatoxicity on mitochondrial functions.

Methods

Animals

Male albino rats of Wistar strain weighing between 250–260 g were used. The average weights of the animals in the control and the experimental groups were matched within 5 g at the start of the experiment. The animals were fasted overnight and were given paracetamol injections next morning (Walker *et al.*, 1982). Saturated solutions of paracetamol (35 mg ml^{-1}) were prepared in warm (45–50°C) saline (0.9% w/v) and animals were injected intraperitoneally (i.p.) with a dose of 750 mg kg^{-1} body weight (Hinson *et al.*, 1981). This dose which is known to result in massive hepatic necrosis is hereafter referred to as 'toxic' dose. A second group of animals was injected with half-saturated solutions of paracetamol (17.5 mg ml^{-1} of saline at 45–50°C) at a dose of 375 mg kg^{-1} body weight. This dose is hereafter referred to as a 'subtoxic' dose. The control animals received an equivalent volume of warm saline. The reason for employing two doses was that increase in the liver necrosis with graded subtoxic doses of paracetamol has been described by Hinson *et al.* (1981). After the injections, the animals were returned to their respective cages and had free access to food and water. The animals were killed after 24 h of paracetamol administration for further studies since maximum liver necrosis is reported to be seen at this time interval (Dixon, 1984).

Isolation of mitochondria

The animals were killed by decapitation and their livers were quickly removed, weighed and 12% (w/v) homogenates were made in chilled (0–4°C) 0.25 M sucrose with a Potter-Elvehjeim type glass-teflon homogenizer. Measured aliquots of homogenates (70 ml) were taken for isolation of mitochondria essentially according to the procedure described previously (Satav & Katyare, 1982). The mitochondria were washed once and the pellets from 1 g tissue were finally suspended in 1.0 ml of 0.25 M sucrose. All operations were carried out at 0–4°C.

Aliquots of homogenates and mitochondria were saved for protein measurements and paracetamol estimation.

Oxidative phosphorylation

Measurements of oxidative phosphorylation were carried out at 25°C with a Clark-type oxygen electrode as described earlier (Chance & Williams, 1955; Satav & Katyare, 1982; Katyare & Rajan, 1988) in the respiration medium (total volume: 1.3 ml) consisting of 225 mM sucrose, 10 mM potassium phosphate buffer, pH 7.4, 10 mM Tris-HCl buffer, pH 7.4, 5 mM $MgCl_2$ and 2–3 mg of mitochondrial proteins. Final concentrations of substrates were 10 mM; pyruvate was supplemented with 1 mM L-malate and 0.1 mM TMPD was employed in conjunction with ascorbate. Rotenone (1.0 μM) was used in studies with succinate and ascorbate + TMPD. The state 3 respiration rates (initiated by addition of 150 nmol of ADP in 15–20 μl volume) and the state 4 respiration rates (after the depletion of added ADP) were recorded. Calculations of ADP/O ratios (mol ADP phosphorylated per atom of O_2 consumed) and ADP-phosphorylation rates were as described earlier (Chance & Williams, 1955; Katyare *et al.*, 1971; Ferreira & Gil, 1984).

Mitochondrial cytochromes

For determination of cytochrome contents, mitochondrial suspensions in phosphate-buffered isolation medium were solubilized with Triton X-100 at a final concentration of 3–4 mg protein ml^{-1} and the difference spectra of dithionite-reduced minus ferricyanide-oxidized cytochromes were recorded in a Hitachi model 150-20 double-beam spectrophotometer. Cytochrome contents were calculated by using the wavelength pairs and the extinction coefficients as described earlier (Satav & Katyare, 1982).

Enzyme assays

ATPase: ATPase activities were measured in a medium (final volume: 1.0 ml) consisting of 50 mM Tris-HCl buffer, pH 7.4, 75 mM KCl and 0.4 mM EDTA; 6.0 mM $MgCl_2$ and/or 0.1 mM DNP were included wherever indicated (Satav & Katyare, 1982). After preincubating 200–250 μg of mitochon-

Table 1 Effects of paracetamol treatment on body and liver weights

	Animals		
		Paracetamol treated	
Parameter	*Control* (9)	*Subtoxic dose* (11)	*Toxic dose* (11)
Body weight (g)	265.6 ± 12.4	226.8 ± 6.0*	226.9 ± 3.9**
Liver weight:			
(g)	13.0 ± 0.53	12.0 ± 0.58 NS	11.3 ± 0.32*
% body weight	4.93 ± 0.20	5.20 ± 0.18 NS	4.93 ± 0.11 NS

Male Wistar rats weighing between 250–260 g were injected (i.p.) with paracetamol at a dose of 375 mg kg^{-1} (subtoxic) or 750 mg kg^{-1} (toxic) body weights as described in 'Methods'. Control animals received only equivalent volumes of saline vehicle. Animals were killed 24 h later for isolation of mitochondria as given in the text. Results are given as mean ± s.e.mean of the number of observations indicated in parentheses.
* $P < 0.02$, ** $P < 0.01$, NS not significant.

drial proteins in the reaction medium at 25°C for 2 min, the reaction was started by adding 6.0 mM ATP and carried out for 15 min. At the end of the incubation period, the reaction was terminated by adding 0.1 ml of 10% (w/v) SDS and the inorganic phosphate liberated was estimated according to the method of Fiske & Subba Row (1925).

Dehydrogenases: For estimation of dehydrogenase activities, mitochondria at a concentration of 10–12 mg ml^{-1} in 250 mM sucrose containing 10 mM Tris-HCl, pH 7.4 were sonicated for 2 min (10 s sonication followed by 10 s rest interval) at 20 kHz in a 'Vibro-cell' ultrasonic disintegrator (Sonics and Materials Inc. CT, U.S.A.), temperature being maintained at 0–4°C during sonication. Glutamate dehydrogenase (Leighton *et al.*, 1968). β-hydroxybutyrate dehydrogenase (Miyahara *et al.*, 1981), malate dehydrogenase (Ochoa, 1955) and succinate dehydrogenase (Caplan & Greenawalt, 1968) were determined in sonicated mitochondria.

Paracetamol estimation

Paracetamol estimations in serum, homogenate and isolated mitochondria were carried out according to the procedure of Swanson & Walters (1982). Briefly, samples were deproteinized with freshly prepared *p*-diazobenzene sulphonic acid and released paracetamol was extracted in ethyl acetate which was estimated under alkaline conditions using Folin-Ciocalteau's reagent (Meola, 1978). Separate recovery experiments indicated that the extent of recovery of paracetamol in these experiments was from 90–102% (data not given).

Protein estimations were carried out according to Lowry *et al.* (1951) with crystalline bovine serum albumin used as the standard.

Chemicals

Paracetamol (A.R.) was purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Sodium salts of L-glutamic acid, β-hydroxybutyric acid, L-malic acid, pyruvic acid, ascorbic acid, succinic acid, and ADP, ATP, rotenone, Triton X-100 and sodium dodecyl sulphate (SDS) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. 2,4-Dinitrophenol (DNP) and N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD) were from British Drug Houses, Poole, Dorset, U.K. All other chemicals were of analytical-reagent grade.

Results

The results in Table 1 summarize the effects of paracetamol treatment on body and liver weights. It can be noted that treatment with both 'toxic' and 'subtoxic' doses of paracetamol resulted in decrease in the body weights. However, the liver weights decreased by 13% in only those animals given toxic doses of the drug (Table 1). The total protein content in the liver tissue was not significantly affected by paracetamol treatment (Table 2). However, interestingly, the yield of mitochondrial proteins expressed either as mg g^{-1} tissue or as % of liver proteins decreased in the animals receiving toxic doses of the drug (Table 2).

The data on oxidative energy metabolism in rat liver mitochondria as affected by paracetamol treatment are given in Table 3. It can be seen that the treatment with subtoxic doses of paracetamol did not significantly affect the state 3 and state 4 respiration rates nor did it have any effects on the ADP/O ratios and the ADP-phosphorylation rates with any of the substrates tested, except in the case of succinate where both the state 3 respiration rate and

Table 2 Effect of paracetamol treatment on liver protein content and mitochondrial yield

	Animals		
		Paracetamol-treated	
Parameter	*Control* (12)	*Subtoxic dose* (11)	*Toxic dose* (11)
Liver protein content, (mg g^{-1} tissue)	184.61 ± 4.87	181.11 ± 6.19 NS	176.77 ± 6.86 NS
Yield of mitochondrial protein:			
(mg g^{-1} tissue)	18.95 ± 1.05	18.37 ± 1.09 NS	14.60 ± 1.39*
% liver protein	10.46 ± 0.78	10.36 ± 0.83 NS	8.27 ± 0.67*

Experimental details are as given in Table 1 and in 'Methods'. Results are given as mean ± s.e.mean of the number of observations given in parentheses.
* $P < 0.05$, NS not signficant.

the ADP-phosphorylation rate increased by 28% (Table 3).

By contrast, when the animals were treated with toxic doses of paracetamol (Table 3), the state 3 respiration rates decreased with all the substrates used (from 25 to 47%); the highest decrease being seen with glutamate and pyruvate + malate. The state 4 respiration rates were generally unchanged for glutamate, β-hydroxybutyrate and succinate but increased with pyruvate + malate and decreased for ascorbate + TMPD. The ADP/O ratios decreased by 19% and 27% for succinate and pyruvate + malate respectively. The ADP-phosphorylation rates registered a decrease corresponding to the lowered state 3 respiration rates in the case of glutamate, β-hydroxybutyrate and ascorbate + TMPD; these values for pyruvate + malate and succinate decreased to an even greater extent because of the impairments in the ADP/O ratios referred to above (Table 3).

With a view to understanding the lesion underlying the impairment in the coupled (state 3) respir-

Table 3 Effects of paracetamol treatment on oxidative energy metabolism in rat liver mitochondria

Substrate	*Treatment*	*ADP/O ratio*	*Respiration rate* (nmol O_2 mg protein min^{-1}) +ADP	−ADP	*ADP-phosphorylation rate* (nmol ATP formed mg^{-1} protein min^{-1})
Glutamate	Control (16)	2.28 ± 0.10	38.7 ± 1.6	2.5 ± 0.5	177.2 ± 10.7
	Subtoxic dose (12)	2.30 ± 0.11 NS	41.7 ± 2.0 NS	3.7 ± 0.8 NS	192.6 ± 15.3 NS
	Toxic dose (12)	2.03 ± 0.20 NS	21.6 ± 2.5††	3.8 ± 1.2 NS	89.3 ± 15.2††
β-Hydroxybutyrate	Control (14)	2.39 ± 0.10	37.8 ± 2.8	2.4 ± 0.6	178.3 ± 12.1
	Subtoxic dose (12)	2.32 ± 0.11 NS	43.1 ± 2.6 NS	3.2 ± 0.8 NS	196.9 ± 9.5 NS
	Toxic dose (12)	2.42 ± 0.13 NS	28.9 ± 2.9*	3.4 ± 0.6 NS	143.7 ± 17.8 NS
Pyruvate + malate	Control (12)	2.46 ± 0.17	24.7 ± 1.3	2.3 ± 0.4	120.9 ± 9.5
	Subtoxic dose (14)	2.52 ± 0.14 NS	25.7 ± 1.1 NS	5.0 ± 1.5 NS	127.4 ± 7.2 NS
	Toxic dose (12)	1.80 ± 0.17**	13.2 ± 1.0††	5.4 ± 1.4*	45.1 ± 6.3††
Succinate	Control (12)	1.48 ± 0.08	70.3 ± 3.1	8.7 ± 2.3	202.8 ± 8.7
	Subtoxic dose (12)	1.46 ± 0.08 NS	90.0 ± 3.6††	10.6 ± 2.3 NS	261.1 ± 15.2***
	Toxic dose (12)	1.20 ± 0.10*	48.9 ± 4.8†	10.7 ± 2.1 NS	123.3 ± 19.1††
Ascorbate + TMPD	Control (12)	0.43 ± 0.01	41.3 ± 3.0	19.4 ± 1.6	36.1 ± 2.9
	Subtoxic dose (12)	0.44 ± 0.01 NS	46.4 ± 2.3 NS	23.9 ± 2.2 NS	40.8 ± 2.5 NS
	Toxic dose (11)	0.42 ± 0.02 NS	30.8 ± 2.0**	15.1 ± 1.2*	26.0 ± 2.4***

Animals were given subtoxic or toxic doses of paracetamol as described in the text. Other experimental details are as described in Table 1 and 'Methods'. Results are given as mean ± s.e.mean of the number of observations indicated in parentheses.
* $P < 0.05$; ** $P < 0.02$, *** $P < 0.01$, † $P < 0.002$, †† $P < 0.001$, NS not significant.

Table 4 Effects of paracetamol treatment on the dehydrogenase activities in rat liver mitochondria

Dehydrogenases	*Control*	*Paracetamol-treated* *Subtoxic dose*	*Toxic dose*
Glutamate dehydrogenase	182.2 ± 11.3 (11)	142.6 ± 7.4* (9)	142.7 ± 9.1* (10)
β-Hydroxybutyrate dehydrogenase	510.7 ± 19.7 (13)	525.2 ± 29.7 (12) NS	516.0 ± 28.2 (18) NS
Malate dehydrogenase	3512.3 ± 209.2 (9)	3654.5 ± 209.5 (10) NS	3405.4 ± 216.8 (14) NS
Succinate dehydrogenase	113.0 ± 8.2 (13)	81.3 ± 6.2* (13)	82.5 ± 6.2* (17)

Dehydrogenases activities were determined in sonicated mitochondria as described in 'Methods'. Other experimental details are as given in Table 1 and in the text. Dehydrogenases activities are expressed as nmol substrate transformed mg^{-1} protein min^{-1}, except for succinate dehydrogenase which is expressed as μmol substrate transformed mg^{-1} protein min^{-1}. Results are given as mean ± s.e.mean of the number of observations indicated in parentheses.
* $P < 0.01$, NS not significant.

ation rates, we extended our observations to examine the possible functional alterations/defects in the mitochondrial electron transport chain components. From the data in Table 4 it can be noted that paracetamol treatment did not significantly affect either β-hydroxybutyrate dehydrogenase or malate dehydrogenase activities. The glutamate and succinate dehydrogenase activities were lowered under these conditions (Table 4). Interestingly, both toxic and subtoxic doses of the paracetamol brought about reduction in the activities of these enzymes to the same extent (24 to 28% decrease). Thus even with subtoxic doses, the paracetamol-induced lesion was already apparent at the primary dehydrogenase level although this was not manifested in terms of coupled state 3 respiration rates (Table 3).

The examination of intramitochondrial cytochromes content (Table 5) revealed that treatment with subtoxic doses of paracetamol resulted in a 13% increase in the cytochrome $c + c_1$ content. By contrast, treatment with toxic doses of paracetamol brought about 11% and 20% decrease respectively in the contents of cytochrome aa_3 and b without affecting the cytochrome $c + c_1$ content (Table 5).

The data in Table 6 show that the mitochondria from the control animals were characterized by low basal ATPase activity obtained in the absence of both Mg^{2+} and DNP. This activity was not stimulated by Mg^{2+} alone but could be fully evoked by DNP or DNP + Mg^{2+}, thus emphasizing the intactness of the mitochondrial membranes (Katyare *et al.*, 1970). This was also reflected in terms of high ratio of DNP/Mg^{2+} ATPase activities (Table 6). A similar trend was noted also for mitochondria from animals receiving subtoxic doses of paracetamol. However, when the animals were given toxic doses of paracetamol, this apparently caused damage to the mitochondrial membranes as evident from a greater stimulation of basal ATPase activity by Mg^{2+}. At the same time DNP and DNP + Mg^{2+} failed to evoke fully the ATPase activity. Consequently, the ratio of DNP to Mg^{2+}-ATPase activity was low (Table 6).

Experiments to estimate paracetamol levels in serum, tissue homogenates and isolated mitochondria revealed the presence of detectable levels of the drug only in the tissue homogenates (4.6 ± 1.3 and 4.8 ± 1.2 μg g^{-1} liver respectively in the two groups).

Table 5 Effect of paracetamol treatment on cytochrome content in rat liver mitochondria

	Cytochrome content (nmol mg^{-1} protein)		
Cytochrome	*Control* (12)	*Paracetamol-treated* *Subtoxic dose* (10)	*Toxic dose* (16)
aa_3	0.131 ± 0.006	0.138 ± 0.010 NS	0.116 ± 0.004*
b	0.236 ± 0.006	0.257 ± 0.013 NS	0.188 ± 0.012**
$c + c_1$	0.434 ± 0.014	0.490 ± 0.021*	0.410 ± 0.013 NS

Intramitochondrial cytochrome contents were calculated from the difference spectra of dithionite-reduced and ferricyanide-oxidized mitochondria solubilized with freshly prepared Triton X-100 as described in 'Methods'. Results are given as mean ± s.e.mean of the number of observations indicated in parentheses.
* $P < 0.05$, ** $P < 0.02$, NS not significant.

Table 6 Effect of paracetamol treatment on ATPase activities in rat liver mitochondria

	ATPase activity (μmol Pi, mg^{-1} protein h^{-1})		
		Paracetamol-treated	
Additions	*Control* (12)	*Subtoxic dose* (12)	*Toxic dose* (14)
None	1.33 ± 0.23	1.09 ± 0.25 NS	1.28 ± 0.15 NS
+ Mg^{2+}	1.66 ± 0.15	1.87 ± 0.30 NS	2.50 ± 0.31*
+ DNP	13.89 ± 0.74	15.93 ± 0.95 NS	13.16 ± 1.23 NS
+ DNP + Mg^{2+}	16.21 ± 0.65	15.90 ± 1.10 NS	13.24 ± 0.73**
$\frac{DNP}{Mg^{+}}$ activity ratio	8.4	8.8	5.3

ATPase activity in mitochondria was measured as described in the 'Methods'. Results are given as mean ± s.e.mean of the number of observations given in parentheses.
* $P < 0.05$, ** $P < 0.01$, NS not significant.

No detectable levels of paracetamol were found either in the serum or mitochondria (data not given).

Discussion

The present studies have clearly demonstrated that subtoxic doses of paracetamol did not affect any of the respiratory parameters except in the case of succinate where a stimulatory effect was seen (Table 3). This may perhaps correlate with an increased content of cytochrome c + c_1 in mitochondria from this group of animals (Table 5). The regulatory role of cytochrome c in succinate oxidation has previously been demonstrated by us (Katyare *et al.*, 1970). Interestingly, paracetamol-induced lesions could be seen even in the subtoxic group in terms of impaired glutamate and succinate dehydrogenase activities (Table 4). However, the subtoxic dose did not affect the liver protein content and the mitochondrial protein content, though this treatment had a definite adverse effect on the body weights (Tables 1 and 2). Toxic doses of paracetamol, on the other hand, caused impairments in active state 3 respiration rates from 25 to 47% depending on the substrate used, besides lowering the ADP/O ratios with succinate and pyruvate + malate. Consequently, the rate of ATP synthesis (ADP-phosphorylation) decreased from 20 to 63%. The observed decrease in the respiration rates correlates well with the decreased contents of cytochrome aa_3 and b. The decrease in glutamate and succinate dehydrogenase levels may also be responsible for the lowering of respiration rates but their contribution may be less important when viewed in the context of the results in the subtoxic group (Table 3) and the decreased cytochrome aa_3 and b contents would seem to play a major role. The structural damage inflicted on mitochondrial membrane integrity can also be inferred from the Mg^{2+}-ATPase activity and decreased mitochondrial yield. These results have thus demonstrated, for the first time the effects of paracetamol toxicity at the level of organelle dysfunction. Disruption of the mitochondrial cristae and the transient increase in the succinate dehydrogenase activity followed by a decrease have been demonstrated by earlier workers with histological and histochemical techniques (Dixon *et al.*, 1975; Dixon, 1984). The results of our present studies corroborate these reported observations. Our findings on damage to mitochondrial membrane structure in terms of ATPase activity also correlate well with the reported elevated levels of serum transaminase activities (Dixon, 1984).

In our other studies we have also observed that the kinetic properties of membrane-bound enzyme systems such as succinoxidase, NADH oxidase and ATPase were significantly altered by both subtoxic and toxic doses of paracetamol (Satav & Katyare, unpublished observations).

In separate experiments, levels of paracetamol in serum, whole liver homogenate and isolated mitochondria were estimated with a view to examining whether the observed effects on oxidative energy metabolism in mitochondria (Tables 3 to 6) are direct effects of paracetamol resulting from the accumulation of the drug in these subcellular organelles. As is to be expected, there were no detectable levels of paracetamol in the serum since most of the drug is known to be detoxified within 4–6 h of ingestion (Green & Fisher, 1981). While the tissue homogenates contained barely detectable concentrations of paracetamol, the mitochondria were totally devoid of it. These results therefore clearly indicate that the observed effects are not due to accumulation of the drug by mitochondria but may be a consequence of initial toxic insult to the hepatocytes. In this connec-

tion it is of interest to note that only very high (6–10 mM) concentrations of paracetamol could inhibit respiration in isolated kidney tubules or kidney mitochondria (Porter & Dawson, 1979). It is also likely that paracetamol may be altering intracellular Ca^{2+} homeostatis in the hepatocytes and thereby impairing the mitochondrial functions (Schanne *et al.*, 1979). However, this possibility has not been examined in the present studies.

Paracetamol toxicity is believed to be mediated by adduct formation of its metabolites with subcellular components, mostly proteins (Hinson *et al.*, 1981; Dixon, 1984). It seems likely that the observed effects on mitochondrial functions may result from the modifications in respiratory chain components because of such an adduct formation. It is also possible that impairment in mitochondrial functions may have occurred much earlier i.e. within hours of paracetamol treatment. We have, however, not examined this possibility and restricted our studies only to 24 h post-paracetamol treatment. Nevertheless, the results of the present studies have clearly shown that significant effects of paracetamol persist even 24 h after its ingestion and the overall effect is the impaired rate of ATP synthesis.

Mitochondria are the major site of ATP synthesis in cellular metabolism and cellular synthetic processes are known to be energy-dependent (Lehninger, 1982). While paracetamol is known to deplete the cellular glutathione levels (Hinson *et al.*, 1983), impairments in ATP synthesis will lead to a further set-back in recovery from the necrosis.

In conclusion, the results of the present studies have shown for the first time that paracetamol toxicity can lead to dysfunction in mitochondrial energy-linked functions. As far as we are aware, such *in vivo* effects at the organelle level have not been described earlier.

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Effects of pertussis toxin on the behavioural and ECoG spectrum changes induced by clonidine and yohimbine after their microinfusion into the locus coeruleus

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1 Pertussis toxin, a substance which interferes selectively with receptor-mediated signal transduction mechanisms, was injected into the locus coeruleus of rats 1, 2, 3, 6 or 10 days before the microinjection of clonidine or yohimbine into the same site.

2 Clonidine produced in control rats typical behavioural sedation and/or sleep and ECoG synchronization while yohimbine produced behavioural arousal and ECoG desynchronization.

3 The behavioural and ECoG effects of both compounds were blocked in animals pretreated with pertussis toxin. This activity was more marked from 2 to 6 days after pertussis toxin pretreatment and was restored 10 days after toxin administration. In addition, the behavioural and ECoG slow-wave sleep observed after intraperitoneal administration of clonidine ($0.2\,\mu mol\,kg^{-1}$) was significantly reduced by prior (3 days) microinfusion of pertussis toxin into the locus coeruleus.

4 These results are consistent with the hypothesis that the behavioural and ECoG effects of clonidine and yohimbine are mediated via a guanine regulatory protein which is affected by pertussis toxin.

Introduction

It is widely accepted that the molecular events subsequent to α_2-adrenoceptor stimulation involve the inhibition of adenylate cyclase via the inhibitory guanosine 5'-triphosphate (GTP) binding regulatory protein (Gi) in a variety of tissues and cells (see Aghajanian & Wang, 1986). Recently the availability of agents such as cholera and pertussis toxin (PTx), which interfere selectively with receptor-mediated signal transduction processes (Bokoch *et al.*, 1983; Abood *et al.*, 1985; Ohta *et al.*, 1985), has been used as a valid tool to improve our knowledge of cellular events leading to the pharmacological action of compounds acting on α_2-adrenoceptors. In particular, PTx has been shown to prevent receptor-mediated adenylate cyclase inhibition by adenosine 5'-diphosphate (ADP) ribosylation of a nucleotide binding protein (Gi) in the cell membrane (Hazeki & Ui, 1981; Jakobs *et al.*, 1984).

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In previous experiments we have shown that clonidine microinfused directly into the rat locus coeruleus (LC) at extremely low doses produces dose-dependent behavioural sedation and/or sleep accompanied by a significant increase in total voltage power with a predominant increase in the lower frequency bands (De Sarro *et al.*, 1987).

In the present study we planned to ascertain whether the intracerebral injection of PTx was able to affect the behavioural and electrocortical spectrum power changes induced by selective α_2-adrenoceptor agonists, i.e. clonidine or antagonists, i.e. yohimbine, after their infusion into the locus coeruleus of the rat. This could give some indication as to whether the α_2-adrenoceptors located on the noradrenergic neurones in the locus coeruleus are linked in a negative manner to adenylate cyclase.

Methods

Male Sprague Dawley rats (50–70 days old; 200 ± 20.2 g) were purchased from Charles River,

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(Calco, Como, Italy) and housed in stable conditions of humidity (60 ± 5%) and temperature (22 ± 2°C). They were fed with standard diet and had water *ad libitum*. Animals were maintained on a 12 h light and 12 h dark cycle (lights on 06 h 00 min–18 h 00 min, off 18 h 00 min–06 h 00 min).

Rats were stereotaxically implanted under choral hydrate anaesthesia (400 mg kg^{-1} i.p.; Carlo Erba, Milan) with stainless steel guide cannula (25 gauge), according to the atlas coordinates of Paxinos & Watson (1982), to permit drug microinfusion into the locus coeruleus.

After surgery a minimum of 24 h was allowed before experiments were carried out. All experiments were performed beginning at approximately 10 h 00 min. Freely moving rats were microinjected via an injector cannula (28 gauge) which extended to approximately 2 mm below the tip of the guide cannula.

Electrocortical (ECoG) activity was recorded via 4 chronically and symmetrically implanted steel screw electrodes inserted onto each fronto-parietal cortex (±2 mm behind the bregma and ±2 mm lateral to the midline) by a Stoelting stereotaxic frame. The ground electrode was implanted epidurally over the nasal bone. All electrodes and the injection guide cannula were anchored to the skull by acrylic dental cement. Electrocortical activity was recorded by means of an 8 channel EEG machine (OTE BIOMEDICA, Florence). For statistical purposes, the quantification of total voltage power (0–16 Hz) and of single frequency bands (0–3; 3–6; 6–12; 12–16 Hz) was carried out by using a Berg-Fourier analyser (OTE BIOMEDICA) according to De Sarro *et al.* (1987). ECoG power spectra were obtained by averaging spectra derived from 5 min ECoG epochs and the integrated energy signals were expressed as $\mu V^2 s^{-1}$; the time constant (0.03 s) was short enough to reduce the number of artefacts (HF cut-off = 5.3 Hz).

The behavioural and electrocortical spectrum power effects were studied in rats randomly divided into two groups. The first group was treated with PTx, while the second group received the vehicle in which PTx was dissolved (67 mM sodium phosphate buffer pH 7.4). The experiments with clonidine and yohimbine were carried out 1, 2, 3, 6 and 10 days after the administration of PTx or vehicle.

In addition, another 12 rats were randomly separated into two groups, the first group receiving PTx, the second treated with vehicle. In this case clonidine was administered intraperitoneally 3 days after the administration of PTx or vehicle.

The animals, placed individually in transparent cages (35 × 35 × 25 cm), were allowed 60 min before drug administration to acclimatize to the new environment. The behavioural changes and their onset and duration were recorded after drug injection. Each animal was used only once.

Post-mortem histological examination confirmed the location of the guide cannula. Only animals in which the location of the injection site was confirmed histologically were used in the analysis of behavioural and ECoG data.

To quantify changes of total voltage power and of preselected bands of frequency induced by clonidine or yohimbine in rats pretreated with PTx or vehicle, the area (expressed in mm^2) under the curve corresponding to plotted total voltage values during 30 min periods after each compound was integrated by means of a Commodore computer and the percentage changes of the integrated area in comparison to the same interval area during the pretreatment period were calculated according to the 'trapezoidal rule' (Tallarida & Murray, 1981). To reduce inter-animal variation of baseline electrocortical activity and of single frequency bands, the percentage changes following drug treatment were compared to the values of the corresponding period before treatment by use of analysis of variance (ANOVA) followed, where appropriate, by analysis with Student's *t* test. The number of experiments are shown in parentheses.

Drugs

Purified pertussis toxin (gift from Dr E. Relyveld, Institut Pasteur, Garches, France) or vehicle were injected into the locus coeruleus in a volume of a 0.5 µl the day of stereotaxic implantation of cannula and electrodes.

Clonidine hydrochloride (Boehringer-Ingelheim, Germany) and yohimbine hydrochloride (Sigma St. Louis, MO, U.S.A.) were infused into the locus coeruleus by using a 5 µl Hamilton syringe, connected by a teflon tube to an injector cannula. The drug was infused in a volume of 0.5 µl at a rate of 0.2 µl min^{-1} and the cannula left *in situ* for a further 1 min.

Results

Clonidine and yohimbine effects in control rats

The behavioural and ECoG spectrum power changes following focal microinfusion of clonidine (0.56 and 1.2 nmol) or yohimbine (1.3 and 2.6 nmol) into the LC were similar to those already obtained (De Sarro *et al.*, 1987). In particular, clonidine induced typical sedation and/or sleep and ECoG synchronization with a periodic increase in total voltage power, predominantly in the 0–3, 3–6 and 9–12 Hz frequency bands, lasting from 125 to

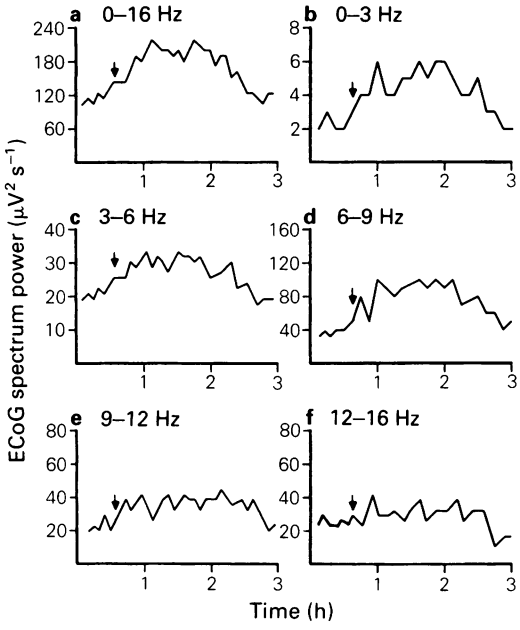


Figure 1 Effects of a single microinjection into the locus coeruleus of clonidine (0.56 nmol at arrow) on electrocortical spectrum power. Note the significant increase in (a) total and (b) 0–3, (c) 3–6, (d) 6–9 and (e) 9–12 Hz voltage power. The slight increase in (f) 12–16 Hz band was not statistically significant.

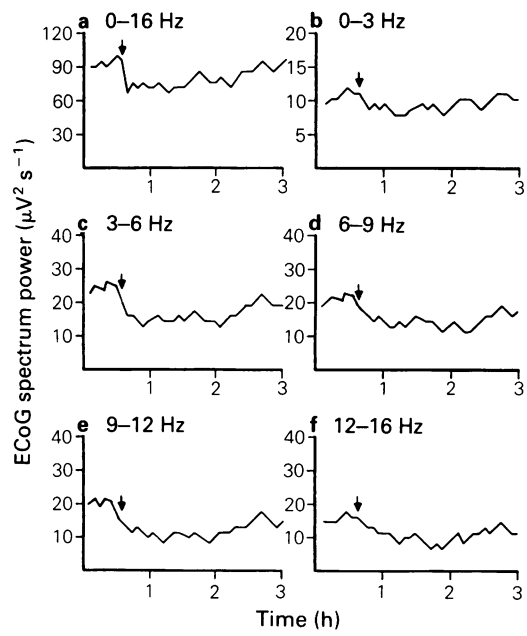


Figure 2 Effects of a single microinjection into the locus coeruleus of yohimbine (2.6 nmol at arrow) on electrocortical spectrum power. Note the significant fall in (a) total and in (c) 3–6, (d) 6–9 and (e) 9–12 Hz voltage power.

220 min according to the dose. In contrast, yohimbine produced behavioural arousal, an increase in locomotor and exploratory activity and ECoG desynchronization, which were associated with a significant decrease in total voltage power and that in the 3–6, 6–9 and sometimes in the 9–12 Hz frequency bands lasting from 60 to 160 min depending on the dose. Figures 1 and 2 show some typical examples of the ECoG changes which usually occur following clonidine and yohimbine infusion into the LC.

Clonidine and yohimbine effects in PTx pretreated rats

Preliminary experiments have indicated that PTx (0.34, 0.68 and 3.4 μg) was only minimally effective within the day of injection, while at 1, 2, 3, 6 days after the administration of PTx (0.34, 0.68 and 3.4 μg) the rats ($n = 6$ for each group) usually displayed 'hyperalgesia' (i.e. they squealed when handled or touched) and showed an alert state after sound or touch stimuli. However, no significant changes in the ECoG spectrum were observed.

Pretreatment with PTx (0.34 μg in 0.5 μl) produced a gradual decrease of the responsiveness of the LC neurones to clonidine (Figure 3) and yohimbine (Figure 4).

No significant changes of the effects of clonidine (0.56 and 1.2 nmol) and yohimbine (1.3 and 2.6 nmol) were observed, in comparison to vehicle-treated animals, 1 day after PTx treatment. In contrast, 2, 3 and 6 days after toxin treatment the behavioural and ECoG spectrum power effects induced by clonidine and yohimbine were reduced, abolished or reversed in a dose- and time-dependent manner (Tables 1 and 2; Figures 3 and 4). In particular, a tendency to reduce significantly or modify the responsiveness of the LC neurones to the action of clonidine and yohimbine was observed 2 days after PTx and it was more markedly reduced or almost absent 6 days after the pretreatment with PTx. However, the clonidine ECoG synchronizing response and yohimbine desynchronizing effect were restored 10 days after PTx treatment ($n = 8$ animals).

Intraperitoneal administration of clonidine (0.2 μmol kg^{-1}) also induced, in rats injected into the locus coeruleus with vehicle, behavioural and electrocortical soporific effects with an increase in total voltage power and that in the 0–3, 3–6, 6–9 and 9–12 Hz frequency bands, whereas rats pretreated (3 days before) with PTx showed a slight and short

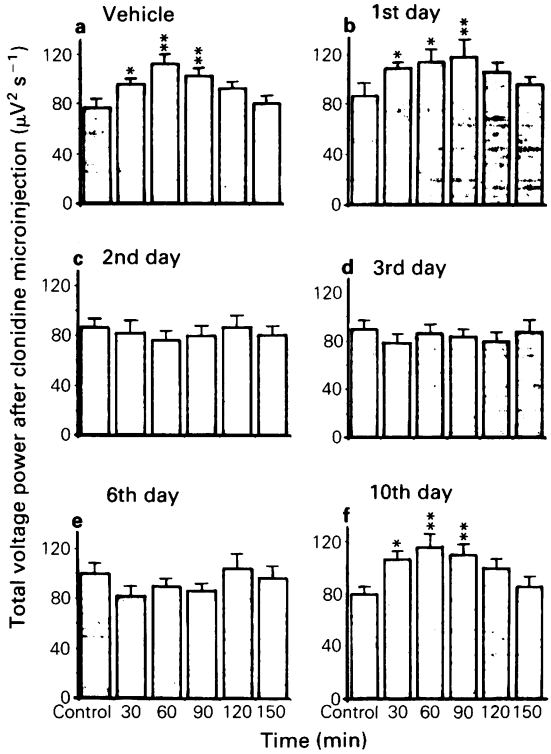


Figure 3 Time course of the effects of an infusion into the locus coeruleus of clonidine (0.56 nmol in 0.5 μl) on the ECoG total voltage power at various times after pretreatment, into the same brain site, with pertussis toxin (PTx) (0.34 μg in 0.5 μl). Each column represents the mean (bars show s.e.mean) of at least 6 observations. Groups of (a) vehicle- and (b–f) PTx-treated rats were previously analysed by use of ANOVA followed, where appropriate, by paired Student's *t* test. Significance of the differences of total voltage power between pretreatment period and post-drug periods are denoted: * $P < 0.05$ and ** $P < 0.01$.

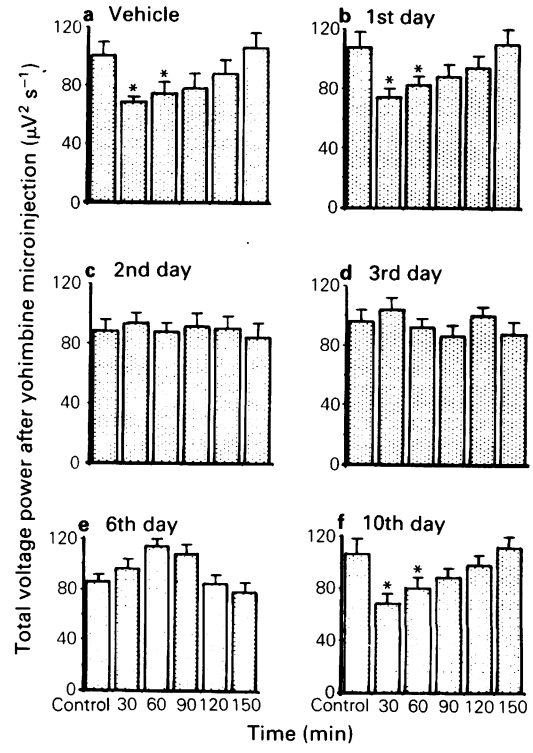


Figure 4 Time course of the effects of an infusion into the locus coeruleus of yohimbine (2.6 nmol in 0.5 μl) on the ECoG total voltage power at various times after pretreatment, into the same brain site, with pertussis toxin (PTx) (0.34 μg in 0.5 μl). Each column represents the mean (bars show s.e.mean), of at least 6 observations. Groups of (a) vehicle- and (b–f) PTx-treated rats were previously analysed by use of ANOVA followed, where appropriate, by paired Student's *t* test. Significance of the differences of total voltage power between pretreatment period and post-drug periods are denoted: * $P < 0.05$.

lasting behavioural sedation without an increase in total voltage power and that in preselected frequency bands.

Discussion

The signal translation of α_2-adrenoceptor occupation into a specific cell response negatively influences adenylate cyclase via the inhibitory binding regulatory protein (Gi) in a variety of tissues (Jakobs *et al.*, 1976; Aktories *et al.*, 1980; Yamazaky *et al.*, 1982; Kurose *et al.*, 1983). Pertussis toxin (islet-activating protein) is well known to prevent receptor-mediated adenylate cyclase inhibition by ADP-ribosylation of a nucleotide-binding protein (Gi) in the cell membrane (Hazeki & Ui, 1981; Jakobs *et al.*, 1984). In addition, it has been demonstrated that Gi regulates the binding affinity of the agonist and antagonist to α_2-adrenoceptors in cerebral cortical membranes (Nomura *et al.*, 1985).

The present data clearly show that PTx injected into the LC of the rat significantly reduced or abolished the effects of clonidine and yohimbine, two specific compounds acting on α_2-adrenoceptors. Our study also showed that behavioural and ECoG soporific effects induced by intraperitoneal administration of clonidine were markedly decreased in rats pretreated with PTx. This strongly supports our hypothesis that the locus coeruleus plays a major

Table 1 Effects of clonidine (0.56 and 1.2 nmol) injected into the locus coeruleus from control (vehicle) and pertussis toxin (PTx)-pretreated rats

Drug pretreatment	*Time* (days)	*Clonidine* (nmol)	*% increase of baseline total voltage power 1 h after clonidine*	*2 h after clonidine*	*Number of animals*
Vehicle	1	0.56	+40.7 ± 4.1	+33.6 ± 4.5	8
Vehicle	1	1.2	+68.2 ± 6.8	+57.4 ± 5.7	8
PTx	1	0.56	+35.4 ± 3.9	+21.7 ± 3.2	8
PTx	1	1.2	+57.6 ± 6.6	+39.3 ± 4.1	8
Vehicle	2	0.56	+40.4 ± 4.2	+31.9 ± 4.0	6
PTx	2	0.56	−3.5 ± 2.2**	−1.7 ± 1.9**	6
Vehicle	3	0.56	+41.6 ± 4.6	+32.3 ± 3.7	6
PTx	3	0.56	−2.8 ± 2.4**	−2.7 ± 1.6**	6
Vehicle	6	0.56	+39.7 ± 3.9	+32.2 ± 4.6	8
Vehicle	6	1.2	+66.3 ± 7.1	+58.6 ± 6.1	6
PTx	6	0.56	−3.9 ± 2.0**	−2.3 ± 1.7**	8
PTx	6	1.2	+3.5 ± 2.7**	+4.4 ± 2.2**	6
Vehicle	10	0.56	+40.4 ± 4.3	+32.1 ± 4.3	8
Vehicle	10	1.2	+67.2 ± 6.7	+56.4 ± 6.4	8
PTx	10	0.56	+41.2 ± 3.8	+31.9 ± 4.1	8
PTx	10	1.2	+68.1 ± 6.4	+56.5 ± 6.2	8

Pertussis toxin (0.34 μg) or vehicle (phosphate buffer 67 mM) was injected into the locus coeruleus 1, 2, 3, 6 and 10 days before testing. The results are presented as mean values of the % increase of baseline total voltage power 1 h and 2 h after clondine in comparison to control period in PTx- and vehicle-treated rats. Significant differences relative to concurrent vehicle and PTx-groups are denoted: **$P < 0.01$ (unpaired Student's t test).

role in the soporific effects of clonidine (De Sarro *et al.*, 1987).

The antagonistic effects of PTx required some time to take effect, since 24 h after PTx pretreatment the ECoG changes induced by clonidine or yohimbine were still present whereas they were significantly reduced 2, 3 and 6 days after PTx pretreatment. This phenomenon may reflect the slow penetration of the toxin molecules into the cell membrane before reaching their ultimate site of action on intracellular membrane surface, and/or a lag period between the activation of PTx and the induction of its biochemical effects on Gi as previously suggested (Katada & Ui, 1982; Lujian *et al.*, 1984; Fujita *et al.*, 1985; Parenti *et al.*, 1986). Some authors have shown that in radioligand binding studies PTx reduces the affinity of α_2-adrenoceptor agonists, probably as a consequence of uncoupling Gi from the receptor (Boyer *et*

Table 2 Effects of yohimbine (1.3 and 2.6 nmol) injected into the locus coeruleus from control (vehicle) and pertussis toxin (PTx) pretreated rats

Drug pretreatment	*Time* (days)	*Yohimbine* (nmol)	*% decrease of baseline total voltage power 1 h after yohimbine*	*2 h after yohimbine*	*Number of animals*
Vehicle	1	1.3	−27.4 ± 2.4	−2.6 ± 2.0	8
Vehicle	1	2.6	−39.2 ± 3.6	−21.4 ± 2.2	8
PTx	1	1.3	−26.4 ± 2.9	−2.7 ± 2.2	8
PTx	1	2.6	−38.6 ± 3.8	−20.3 ± 2.6	8
Vehicle	2	1.3	−26.9 ± 2.5	−2.4 ± 2.3	6
PTx	2	1.3	+2.5 ± 1.3**	−2.9 ± 1.8**	6
Vehicle	3	1.3	−27.6 ± 2.3	−2.3 ± 1.9	6
PTx	3	1.3	+3.7 ± 1.5**	+2.4 ± 1.8**	6
Vehicle	6	1.3	−26.7 ± 2.4	−2.2 ± 1.7	8
PTx	6	1.3	+13.3 ± 3.4**	+9.6 ± 1.6**	6
Vehicle	10	1.3	−27.2 ± 2.6	−2.5 ± 1.7	6
PTx	10	1.3	−28.3 ± 3.7	−3.1 ± 2.1	6

Pertussis toxin (0.34 μg) or vehicle (phosphate buffer 67 mM) was injected into the locus coeruleus 1, 2, 3, 6 and 10 days before testing. The results are presented as mean values of the % increase of baseline total voltage power 1 h and 2 h after yohimbine in comparison to control period in PTx- and vehicle-treated rats. Significant differences between the concurrent vehicle and PTx-groups are denoted: **$P < 0.01$ (unpaired Student's t test).

al., 1982; Kurose et al., 1983; Garcia-Sainz et al., 1984; Nomura et al., 1985). Both the inhibition of the GTPase and/or the uncoupling of Gi from the receptor with the subsequent reduction of receptor affinity should decrease the effects of clonidine and yohimbine in PTx-treated rats. Pretreatment with PTx was also able to prevent the hyperpolarizing effects of the microiontophoretic application of clonidine on LC neurones (Aghajanian & Wang, 1986). The latter and our data suggest that the regulatory Gi protein, the multicomponent adenylate cyclase system or other biochemical events associated with G proteins, might be primarily involved in the genesis of the behavioural and ECoG spectrum power effects elicited at the level of the locus coeruleus by drugs acting on α_2-adrenoceptors.

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Inhibition of Ca-spikes in rat preganglionic cervical sympathetic nerves by sympathomimetic amines

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1 Propagated Ca-spikes were recorded from isolated cervical sympathetic nerve trunks of the rat when bathed in a solution containing 5 mM Ca^{2+}, 0.5 or 1 μM tetrodotoxin (to block Na currents) and 1 mM 4-aminopyridine (to reduce K currents).

2 Spikes persisted when external Ca^{2+} was replaced with Sr^{2+} or Ba^{2+}, but were blocked by the addition of the following inorganic Ca-channel blockers (in descending order of potency): $Cd^{2+} > La^{3+} > Ni^{2+} > Co^{2+} > Mn^{2+} > Mg^{2+}$.

3 Ca-spike amplitude was reduced by up to 90% by (−)-noradrenaline (IC_{50} 1.5 μM). The following sympathomimetic amines imitated this effect (in descending order of potency): clonidine ⩾ (−)-adrenaline ⩾ [(−)-noradrenaline] ⩾ dopamine > (−)-phenylephrine ⩾ (±)-amidephrine.

4 Ca-spike inhibition by (−)-noradrenaline was antagonized by phentolamine (pA_2 6.5). Yohimbine was about 10 times weaker than phentolamine; (±)-propranolol (1 μM) and prazosin (10 μM) had no clear effect.

5 (−)-Noradrenaline reduced the amplitude of the compound action potential recorded from the superior cervical sympathetic ganglion following supramaximal preganglionic trunk stimulation when recorded in normal Krebs solution and hyperpolarized the ganglion with respect to the postganglionic trunk. Depression of the transmitted ganglionic action potential was antagonized by phentolamine (5 μM) but not by yohimbine (1 μM); in contrast 1 μM yohimbine completely prevented the ganglionic hyperpolarization. (−)-Noradrenaline did not hyperpolarize the preganglionic cervical sympathetic nerve trunk under these recording conditions.

6 It is suggested that inhibition of transmitter release from sympathetic preganglionic fibres produced by noradrenaline results from a depression of the voltage-gated Ca current in the fibres and/or their terminals, and that this action is mediated by an α-adrenoceptor which does not fully conform to either α_1 or α_2 subtypes.

Introduction

Catecholamines inhibit transmitter release from cervical sympathetic preganglionic nerves (see, for examples, Birks & MacIntosh, 1961; Christ & Nishi, 1971a,b; Dawes & Vizi, 1973; Dun & Nishi, 1974; Dun & Karczmar, 1977; Brown & Caulfield, 1981). The mechanism responsible for this is not yet clear. One possibility is that they inhibit voltage-gated Ca currents in the preganglionic nerve fibres or their terminals, since inhibition of these currents has been detected in postganglionic sympathetic neurones (Minota & Koketsu, 1977; Horn & MacAfee, 1980; Koketsu & Akasu, 1982; Galvan & Adams, 1982; Lipscombe & Tsien, 1987) and in some other peripheral neurones (e.g., sensory ganglion cells: Dunlap & Fischbach, 1978; 1981; Forscher & Oxford, 1985; neuroblastoma hybrid cells: Docherty & McFadzean, 1987).

It is difficult to test this in sympathetic fibres since the normal conducted action potential does not show a component which can clearly be attributed to a Ca current. However, we have found that a propagated Ca-spike can be generated in rat preganglionic cervical sympathetic fibres when the Na current is blocked with tetrodotoxin and K-currents

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diminished with 4-aminopyridine (cf. Kuan et al., 1985). We have therefore used this preparation to assess the possible effect of noradrenaline and other sympathomimetic amines on the presynaptic Ca-current. Some of our results have been published in abstract form (Elliott, 1986).

Methods

Preganglionic cervical sympathetic nerves, with or without attached superior cervical ganglia, were excised from male Wistar rats (200–400 g weight) anaesthetized with urethane ($1.5\,g\,kg^{-1}$). Their connective tissue sheaths were stripped off and the preparations were maintained in Krebs solution bubbled with 95% O_2 plus 5% CO_2 (see Brown & Caulfield, 1979, for composition).

To record compound action potentials from the preganglionic trunk, the preparation was mounted horizontally in a three-chambered 'perspex' bath as described by Brown & Marsh (1978). The chambers were insulated with silicon grease and filled with oxygenated Krebs solution. The centre chamber was superfused with oxygenated Krebs solution at $5\,ml\,min^{-1}$, at ambient temperature (18–22°C). Drugs were applied to the centre chamber by superfusion at known concentrations. The preganglionic nerve was stimulated between the proximal and centre chambers through twin Pt wires, the centre chamber being grounded. The normal parameters were 20 V, 1 ms, 0.2 Hz. Evoked compound action potentials were recorded differentially between the centre and distal chambers with Ag/AgCl/agar-saline electrodes. In experiments where transmission through the superior cervical ganglion was measured, the ganglion was placed in the centre chamber and evoked potentials recorded with respect to the postganglionic trunk in the distal chamber. Evoked potentials were monitored on an oscilloscope, photographed on a Medelec u.v. recorder and amplitude recorded and displayed on a peak-height detector (Courtice, 1977). To record Ca-spikes, the normal Krebs solution was replaced by a HEPES-buffered solution containing 0.5–1 μM tetrodotoxin (TTX), 1 mM 4-aminopyridine (4-AP) and 5 mM $CaCl_2$, and the stimulus frequency reduced to 0.017 Hz (see Results). Drug-induced spike depression was analysed by fitting a logistic curve to the experimental data (Parker & Waud, 1971).

Solutions

Normal Krebs solution contained the following (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.18, $MgSO_4 \cdot 7H_2O$ 1.18, D-glucose 11. The pH was maintained at pH 7.4 by continuously gassing the solution with 95% O_2: 5% CO_2. To record Ca-spikes the following solution was used (mM): NaCl 118, KCl 6, $CaCl_2$ 5, $MgCl_2$ 1.2, D-glucose 11, HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid, Sigma) 10, 0.5–1 μM TTX, 1 mM 4-AP. The pH was adjusted to pH 7.4 with 1 M NaOH and the solution continuously bubbled with 100% O_2. In experiments involving catecholamines, 0.5 mM ascorbic acid was added to prevent oxidation. Na-free Tris solution had the following composition (mM): Tris(trihydroxymethylaminomethane)-base 143, KCl 4.8, $CaCl_2$ 5, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2 and D-glucose 11; titrated to pH 7.4 with HCl, and gassed with 100% O_2.

Drugs

The following drugs were obtained from commercial sources: tetrodotoxin (TTX), 4-aminopyridine (4-AP), tetraethylammonium chloride (TEA), (−)-noradrenaline bitartrate, (−)-adrenaline bitartrate, dopamine HCl, (−)-phenylephrine HCl, adenosine, (±)-propranolol HCl, yohimbine HCl (Sigma), urethane (Fisons). Amidephrine mesylate was supplied by Mead Johnson, clonidine HCl by Boehringer Ingelheim, phentolamine methanesulphonate by CIBA and prazosin HCl by Pfizer.

$BaCl_2$, $SrCl_2$, $CdCl_2$, $LaCl_3$, $NiCl_2$, $CoCl_2$, $MnCl_2$ and the salts used to make normal Krebs solution were all AnalaR grade reagents from BDH.

Agonist drugs were dissolved in Krebs solution or the HEPES buffered solution to give stock solutions of 10 mM. The antagonists were dissolved in distilled water to give stock solutions of 1 mM or dissolved directly in the appropriate recording medium. The inorganic calcium antagonists were dissolved in distilled water to give stock solutions of 10 mM–1 M.

Results

Ca-spikes

The normal compound action potential recorded from the cervical sympathetic trunk was rapidly and completely blocked by 1 μM tetrodotoxin (TTX: Figure 1) or by Na-free Tris-solution (see Methods), indicating that it was generated by a conventional TTX-sensitive Na-current. However, on adding to the HEPES-buffered Krebs solution a further 2.5 mM Ca^{2+} (bringing the total concentration to 5 mM) and 1 mM 4-aminopyridine (4-AP) (Kuan et al., 1985) a large, slowly-conducted compound action potential of about 50 to 100 ms duration could be evoked (Figure 1). This tended to run down quite quickly ($t_{1/2}$ = 5–10 min) at high ($\geq$ 0.2 Hz) stimulus

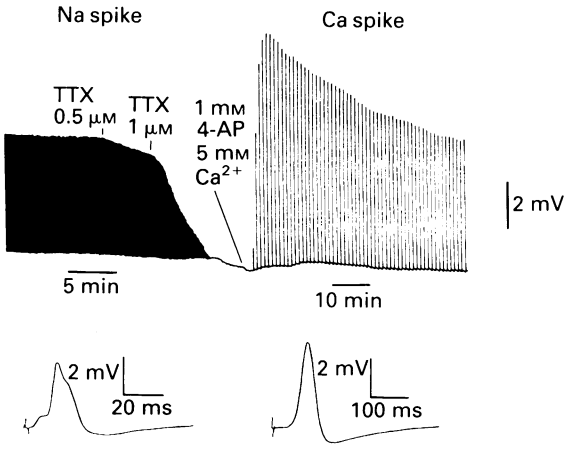


Figure 1 Evoked compound action potentials recorded from an isolated preganglionic cervical sympathetic nerve trunk preparation of the rat bathed in normal Krebs solution ('Na spike') and then after adding tetrodotoxin (TTX, 0.5 and 1 μM) and replacing the normal Krebs solution with a HEPES-buffered Krebs solution containing 1 μM TTX, 1 mM 4-aminopyridine (4-AP) and 5 mM $CaCl_2$ ('Ca spike'). (See text.) The stimulus frequency was 0.2 Hz in normal Krebs solution and 0.017 Hz (1 min^{-1}) in the modified solution. Upper trace: continuous display of peak action potential amplitude, recorded with a peak height detector (Courtice, 1977); note the change of recorder speed. Lower records: single evoked action potentials in the two solutions recorded on an oscilloscope; note the change in time-base.

rates but run-down could be slowed by using a stimulus rate of 1 min^{-1} (0.017 Hz). The properties of the spikes recorded in normal Krebs solution and in this modified solution (hereafter referred to as 'Na-spikes' and 'Ca-spikes' respectively, see Discussion) are summarized in Table 1.

Conducted spikes could also be recorded in TTX/4-AP solution in the presence of 5 mM Sr^{2+} or Ba^{2+} (Figure 2). Spikes were broader in these solutions, particularly so in Ba^{2+} solution where they lasted several hundred ms. This accords with previous reports that Sr^{2+} and Ba^{2+} act as charge carriers for voltage-activated Ca-currents (see, for examples, Hagiwara & Byerly, 1981). The prolonged and larger spike in Ba^{2+} solution may reflect additional block of residual K-currents (e.g., Werman & Grundfest, 1961; Arhem, 1980).

Ca-spikes evoked by 5 mM external Ca^{2+} were completely blocked by a number of divalent cations previously reported to block voltage-activated Ca-currents (Hagiwara & Byerly, 1981) (Figure 3). In order of descending potency, these were: $Cd^{2+} > La^{3+} > Ni^{2+} > Co^{2+} > Mn^{2+} > Mg^{2+}$ (see Table 2).

Table 2 Potencies of inorganic calcium channel antagonists on the Ca-spike

	ID_{50} (mean ± s.e.mean)	n
Cd^{2+}	3.0 ± 0.67 μM	3
La^{3+}	10.23 ± 3.23 μM	4
Ni^{2+}	54.67 ± 19.9 μM	3
Co^{2+}	0.51 ± 0.01 mM	3
Mn^{2+}	1.07 ± 0.16 mM	4
Mg^{2+}	15.7 ± 1.86 mM	3

Action of sympathomimetic amines

A range of sympathomimetic amines was tested for their ability to reduce the amplitude of the Ca-spike. Representative effects are illustrated in Figure 4 and pooled dose-response curves from a number of preparations shown in Figure 5. A fresh preparation was used for each drug, because of the tendency of the Ca-spike to run down with time; with this caveat, effects of all drugs tested were readily reversible on washing. Table 3 lists the maximum depression attained and mean IC_{50} values for 6 amines in descending order of potency (clonidine > (−)-adrenaline > (−)-noradrenaline > dopamine > (−)-phenylephrine > amidephrine).

Table 1 Differences between Na- and Ca-spikes in rat preganglionic cervical sympathetic nerves

	Amplitude (mV)	*Delay* (ms)	*Duration* (ms)	*After-hyperpolarization* (mV)
Na-spike	6.8 ± 0.46	17.1 ± 0.48	24.4 ± 0.83	—
Ca-spike	8.8 ± 0.58	90.2 ± 2.4	72.8 ±.1.96	1.76 ± 0.16

Comparison of Na- and Ca-spikes from 26 separate experiments. Values are mean ± s.e.mean. The amplitude of the Ca-spike was measured after the initial run-down (see text). The delay is an indication of the conduction velocity: it was measured from the stimulus artifact to the peak of the spike. The duration of the spike was measured at baseline potential. The peak amplitude of the after-hyperpolarization following the spike was also measured. For details of the recording conditions see Figure 1.

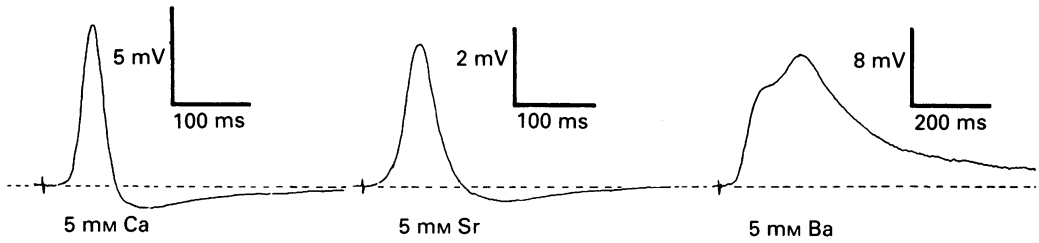


Figure 2 Representative compound action potentials recorded from three different nerve trunk preparations bathed in HEPES-buffered Krebs solution containing 1 μM tetrodotoxin, 1 mM 4-aminopyridine and 5 mM of either Ca^{2+}, Sr^{2+} or Ba^{2+} as indicated. Note the slower time base in Ba^{2+} solution.

At sufficiently high concentration (10 to 30 μM) the more potent group of agonists (clonidine, adrenaline and noradrenaline) produced up to 90% inhibition of the Ca-spike. The effect of clonidine was noticeably more slow to reverse on washing than that of adrenaline or noradrenaline (see Figure 4a). The dose-response curves for dopamine and phenylephrine were rather shallow (slopes 0.54 and 0.81 respec-

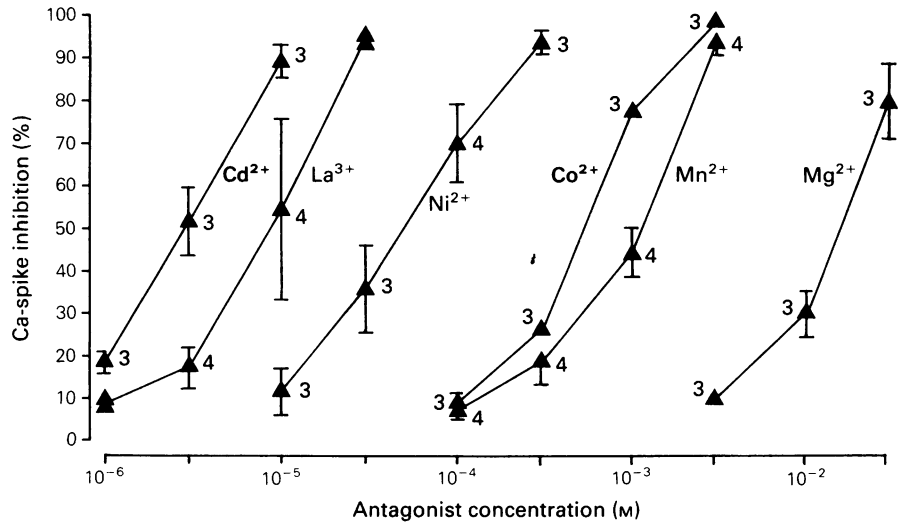


Figure 3 Depression of the cervical sympathetic Ca-spike by some inorganic Ca-channel blockers. Action potentials were recorded in HEPES-buffered Krebs solution containing 0.5 or 1 μM tetrodotoxin, 1 mM 4-aminopyridine and 5 mM Ca^{2+} (see Figure 1) and increasing concentrations of the antagonists applied cumulatively. Graphs show mean % reduction of peak amplitude (bars show s.e.mean). The numbers beside the points give the number of preparations used.

Table 3 Pooled data for agonist inhibition of Ca-spikes in rat preganglionic cervical sympathetic nerves

Agonist	*Maximum depression* (%)	IC_{50} (μM)	*Slope factor*	n
Clonidine	76.1 ± 4.9	0.44 ± 0.11	0.739 ± 0.246	5
(−)-Adrenaline	92.6 ± 12.1	1.3 ± 0.74	0.633 ± 0.315	7
(−)-Noradrenaline	88.8 ± 5.6	1.52 ± 0.32	1.057 ± 0.315	4
Dopamine	66.9 ± 6.8	45.9 ± 23.6	0.541 ± 0.258	3
(−)-Phenylephrine	61.6 ± 5.7	154 ± 50.4	0.814 ± 0.401	3
Amidephrine	<50	$>10^{-2}$ M	—	2
Adenosine	38.5 ± 1.2	3.1 ± 0.5	0.654 ± 0.150	3

The dose-response curves were analyzed by the method of Parker & Waud (1971). Numbers are means ± s.e.mean.

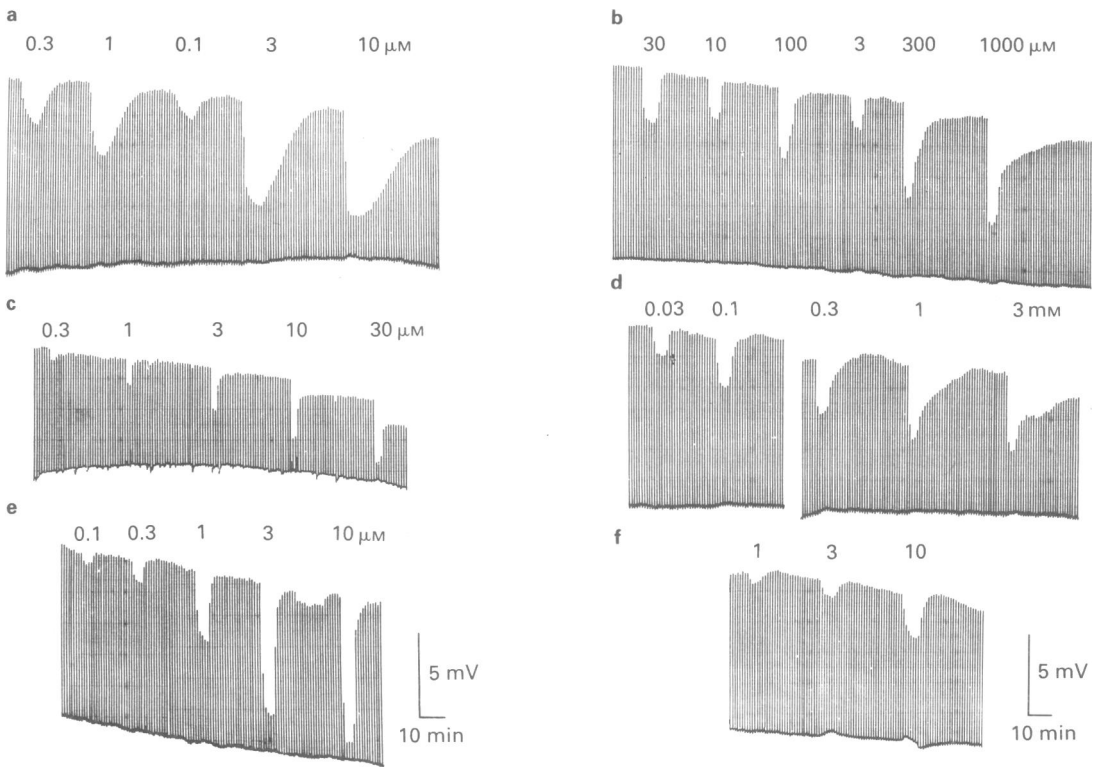


Figure 4 Examples of the inhibition of the Ca-spike (see Figure 1, legend) produced by increasing concentrations of some sympathomimetic amines: (a) clonidine, (b) dopamine, (c) (−)-adrenaline, (d) phenylephrine, (e) (−)-noradrenaline and (f) amidephrine. Each record is from a different experiment.

tively, see Table 3) and block was incomplete at 1 mM or more. Amidephrine was very weak, 10 mM being required for appreciable (up to 30%) block.

Notwithstanding the presence of 4-AP, it seemed likely that some residual K-currents contributed to the repolarization of the Ca-spike, as suggested by the effect of Ba described above. In this case, the inhibition of the Ca-spike produced by the catecholamines might in part have been due to an increased K-conductance, some evidence for which has been previously detected in the postganglionic neurones of this preparation (Brown & Caulfield, 1981). To obviate this, K-currents were further blocked by addition of tetraethylammonium (TEA, 10 mM) or Ba^{2+} (1 or 5 mM). Under these conditions, Ca-spikes were augmented and prolonged and a second, slower component of the waveform became apparent (Figure 6). Noradrenaline still reduced the peak amplitude of the spike, albeit by less than in 4-aminopyridine solution alone, and eliminated the second spike component. Quantitative assessment of noradrenaline action in TEA or Ba solution was precluded by the faster run-down of these spikes than of those obtained in 4-aminopyridine solution.

Antagonists Several antagonists were tested against the inhibitory action of (−)-noradrenaline. Phentolamine antagonized the effect of noradrenaline at concentrations of 1 to 10 μM (Figure 7a); at 100 μM phentolamine produced some additional depression of the Ca-spike itself. An attempt was made to estimate a pA_2 from a Schild plot (Arunlakshana & Schild, 1959) using concentrations of phentolamine up to 10 μM (Figure 8). The slope was less than unity; with this constraint, the apparent pA_2 was 6.47 ± 0.07 ($n = 3$).

The α_2-adrenoceptor antagonist yohimbine had no antagonistic action at 1 μM but reduced the effect of 10 μM noradrenaline by about 35% at 10 μM and by 60% at 100 μM (Figure 7b; $n = 3$); since phentolamine produced 60 to 70% reduction of the response to 10 μM noradrenaline at 10 μM, yohimbine appeared to be about 10 times weaker than phentolamine. The α_1-adrenoceptor antagonist prazosin had

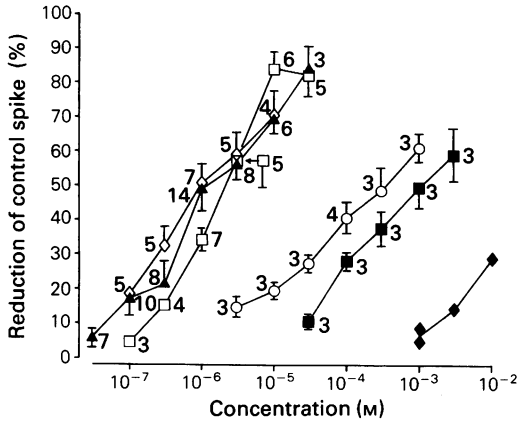


Figure 5 Mean percentage reduction of the Ca-spike produced by clonidine (◇), (−)-adrenaline (▲), (−)-noradrenaline (□), dopamine (○), phenylephrine (■) and amidephrine (◆). Bars are s.e.mean; numbers give number of preparations tested at that concentration, each drug being tested on a different preparation.

no clear effect at concentrations up to 100 μM (Figure 7c) and the β-adrenoceptor antagonist (±)-propranol had no effect up to 1 μM (not illustrated).

To control for non-specific effects, the action of these antagonists on responses to adenosine was also determined. Adenosine inhibits Ca-currents in sympathetic and sensory neurones (see Henon & McAfee, 1983; Dolphin *et al.*, 1986; MacDonald *et al.*, 1986) and, in the present experiments, reduced the amplitude of the Ca-spikes with an IC_{50} of 3.1 μM (Table 3). However, none of the adrenoceptor antagonists affected inhibitory responses to 10 μM adenosine (Figure 7).

Effects on transmission

When the superior cervical ganglion preparation was bathed in normal Krebs solution, application of 10 μM noradrenaline reduced the amplitude of the ganglionic action potential evoked by single supramaximal preganglionic stimuli by 20 to 30% (Figure 9). This effect was accompanied by a small hyperpo-

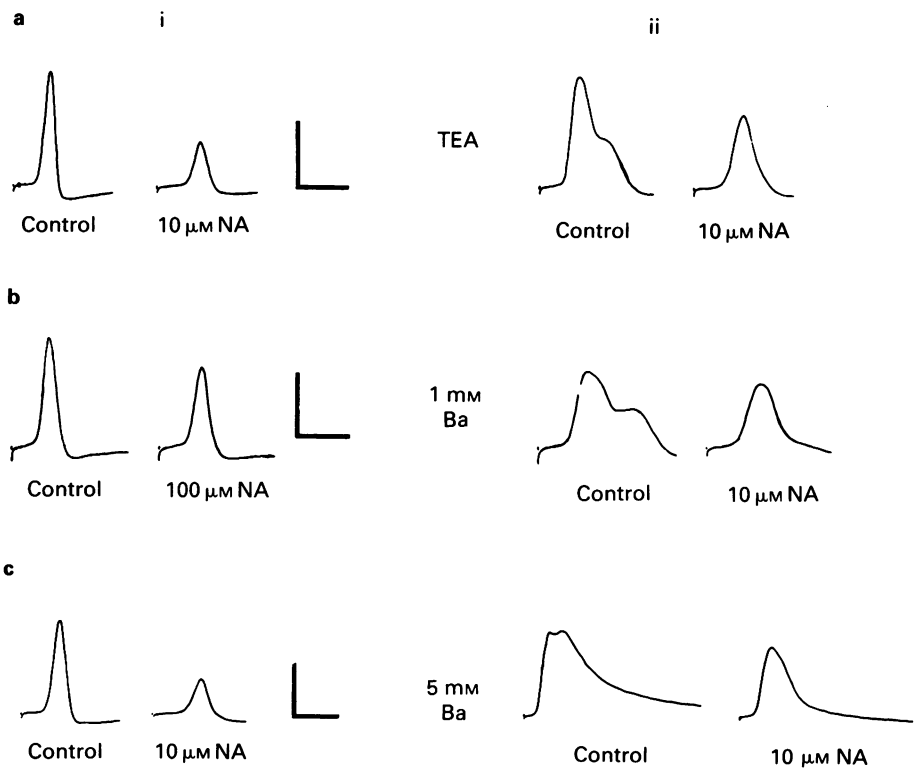


Figure 6 Comparison of the effects of noradrenaline (NA, 10 μM) on Ca-spikes recorded using the solution in Figure 1 (i) and then (ii) on (a) adding 10 mM tetraethylammonium (TEA), (b) adding 1 mM Ba^{2+} or (c) replacing 5 mM Ca^{2+} with 5 mM Ba^{2+}. Calibration: 5 mV and 100 ms (500 ms for row c ii).

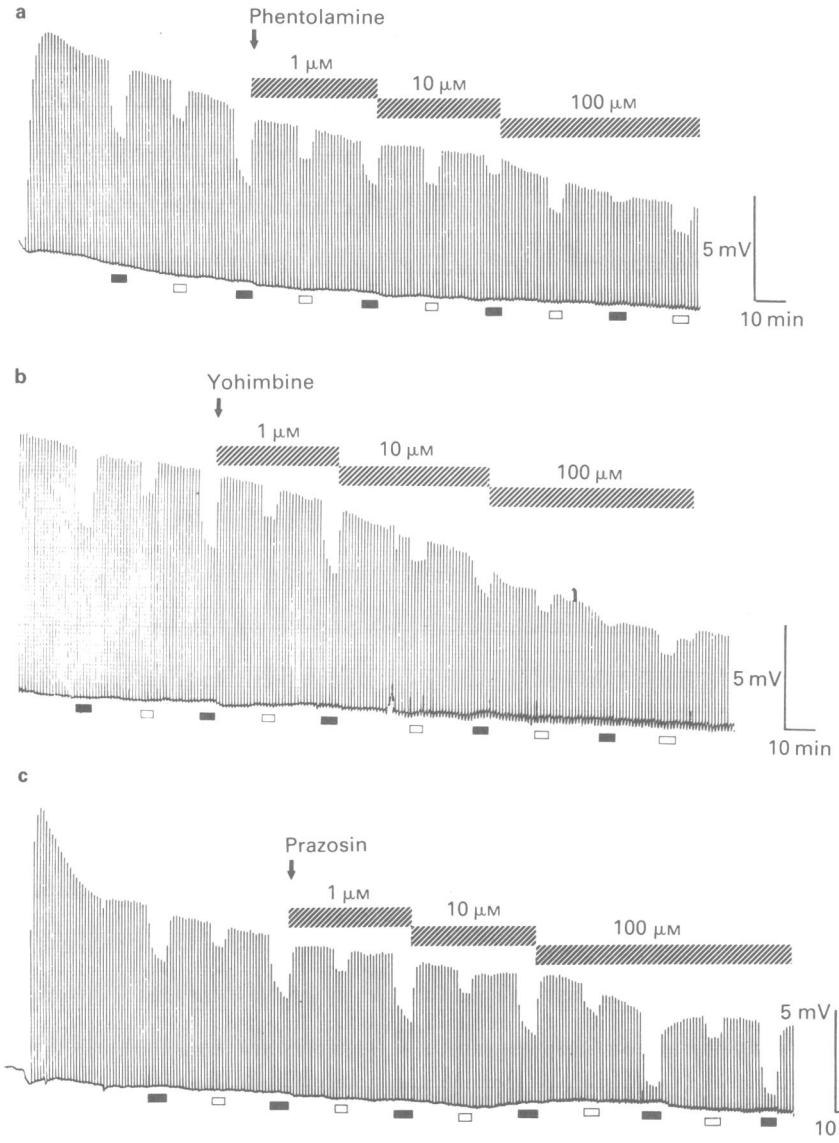


Figure 7 Effect of increasing concentrations of (a) phentolamine, (b) yohimbine and (c) prazosin on the inhibition of Ca-spikes (recorded as in Figure 1) produced by 10 μM (−)-noradrenaline (solid rectangles below trace) and 10 μM adenosine (open rectangles below trace). (Three experiments).

larization of the ganglion, as previously reported (Brown & Caulfield, 1979; see also below). Phentolamine (5 μM) completely antagonized the inhibition of transmission even though, at this concentration, the ganglion hyperpolarization was only slightly reduced. In contrast, 1 μM yohimbine very effectively suppressed the hyperpolarization as noted previously (Brown & Caulfield, 1979), but only partly antagonized the inhibition of transmission, even though it produced some depression of transmission itself (Figure 9b); the residual effect of noradrenaline on transmission was then completely blocked by further addition of 5 μM phentolamine.

The hyperpolarizing action of noradrenaline on the superior cervical ganglion persisted in the presence of 1 mM 4-aminopyridine (i.e. under conditions

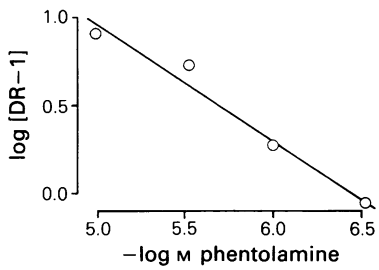


Figure 8 Schild plot of log (dose-ratio − 1) against log [antagonist concentration] for the antagonism of the inhibitory effect of (−)-noradrenaline on the Ca-spike by phentolamine. The graph shows pooled data from 3 experiments using 2-point dose-response curves for (−)-noradrenaline in the absence and presence of 4 concentrations of phentolamine. Least-squares regression analysis yielded a slope of -0.65 ± 0.077 and an intercept ('pA_2') of 6.47 ± 0.067.

in which Ca-spikes were recorded: Figure 10a). In contrast, noradrenaline (10 μM) did not hyperpolarize the preganglionic cervical sympathetic trunk but instead produced a small ($\leqslant 100\,\mu$V) depolarization which also persisted in 1 mM 4-aminopyridine solution (Figure 10b; $n = 3$). This action was shared by clonidine (10 μM) and persisted in the presence of 1 μM (±)-propranolol which inhibited the preganglionic depolarization of isoprenaline (see Brown *et al.*, 1982). In the same preparations adenosine produced a very small hyperpolarization ($\leqslant 50\,\mu$V) while γ-aminobutyric acid depolarized the fibres as reported previously (Brown & Marsh, 1978).

Discussion

The main points established in these experiments are that, under conditions where the normal Na-spike is blocked with tetrodotoxin and K-currents reduced with 4-aminopyridine, propagated Ca-spikes can be recorded from preganglionic cervical sympathetic nerves, and that these can be blocked by sympathomimetic amines.

The principal evidence that the propagated spikes are true Ca-spikes, rather than TTX-resistant Na-spikes, is that spikes can be recorded with Ca^{2+}, Sr^{2+} or Ba^{2+} as charge-carriers, and that the spikes are blocked by micromolar concentrations of divalent cations known to block Ca-channels such as Cd^{2+}, La^{3+}, Ni^{2+} and Co^{2+}. This accords with the properties of Ca-spikes recorded from a variety of nerve cell somata (see Hagiwara & Byerly, 1981).

Following strong suppression of K-currents with additional TEA or Ba^{2+}, the Ca-spike showed two components (see Figure 6). At least two explanations for this are possible. Firstly, the preganglionic cervical sympathetic trunk in the rat contains more than one population of fibres as determined by their conduction velocities and fibre diameters (Dunant, 1967). It is therefore possible that the balance of inward Ca-current and outward K-currents in the different fibre groups varies, such that the K-currents of the slower-conducting group requires greater suppression before the Ca-current becomes net inward and hence capable of producing a regenerative Ca-spike. A second possibility is that, as in postganglionic sympathetic neurones (Lipscombe & Tsien, 1987), there may be more than one kinetically-distinct type of Ca-current in individual preganglionic axons, of different magnitudes such that their expression as regenerative currents depends on the degree of K-current suppression. This interpretation has been offered for the appearance of a second, slower component of inward current in mouse motor nerve terminal bundles in the presence of high concentrations of tetraethylammonium and 3,4-diaminopyridine (Penner & Dreyer, 1986). This view stemmed from a large differential sensitivity of the two components to both inorganic and organic Ca-channel blocking agents. We have not compared the sensitivities of the two Ca-spikes in the sympathetic fibres to a variety of Ca-channel blocking agents, so cannot discriminate between these two possibilities.

Some indication that preganglionic cervical sympathetic nerves are potentially capable of generating Ca-spikes has previously been obtained by Greengard & Straub (1959), who reported that long-lasting action potentials could be recorded from desheathed rabbit cervical sympathetic nerves on replacing Locke solution with an isotonic Ba^{2+} solution. Bowers (1985) has also reported that TTX-resistant spikes can be recorded in bullfrog autonomic fibres; however, unlike the spikes recorded in the present study, these were attributed to entry of Na^+ through TTX-resistant Na-channels, since they were lost in Na-free solution, required high concentrations (100 to 300 μM) of Cd^{2+} ions for block and were not blocked by Co^{2+} or Ni^{2+} at concentrations up to 5 mM.

The inhibitory effect of the sympathomimetic amines might be attributable to a direct effect on the inward Ca-current or to an increase in K-current or leak current, thereby raising the threshold for regenerative Ca-entry. Both effects have been reported in other preparations. A direct effect on the inward Ca-current under conditions where K-currents have been eliminated has been recorded in sympathetic neurones (Galvan & Adams, 1982; Koketsu & Akasu, 1982; Lipscombe & Tsien, 1987), sensory neurones (Dunlap & Fischbach, 1981; Forscher & Oxford, 1985) and neuroblastoma hybrid cells (Docherty & McFadzean, 1987). On the other hand, increases in K-conductance induced by catecholamines have been detected in a variety of neurones

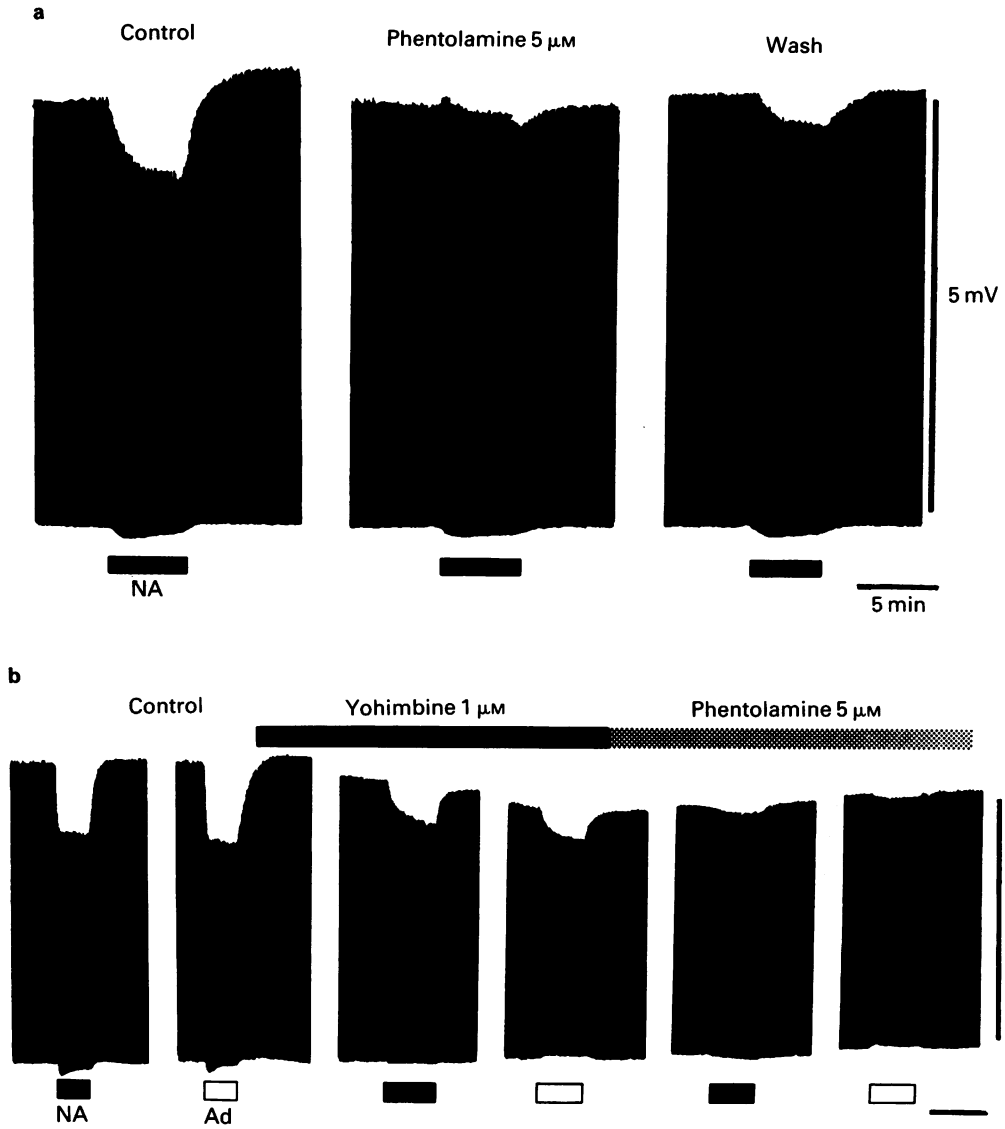


Figure 9 Effects of (a) phentolamine (5 μM) and (b) yohimbine (1 μM) followed by 5 μM phentolamine on the inhibition of the transmitted compound action potential produced by noradrenaline (10 μM; NA) and adrenaline (1 μM; Ad). Records show the amplitude of the compound action potential recorded from the superior cervical ganglion with respect to the postganglionic trunk following single supramaximal preganglionic stimuli at 0.2 Hz. Changes in baseline reflect ganglionic hyperpolarizations (see Brown & Caulfield, 1979).

including sympathetic neurones (Brown & Caulfield, 1979 and unpublished), parasympathetic neurones of the myenteric and submucous plexus (Morita & North, 1981; North & Suprenant, 1985) and cat urinary bladder (Akasu *et al.*, 1986), spinal substantia gelatinosa neurones (North & Yoshimura, 1984) and brainstem locus coeruleus neurones (Williams *et al.*, 1984).

The evidence available suggests that the inhibition of the cervical sympathetic Ca-spike most probably results primarily from a direct action on the Ca-current, for the following reasons. (i) An increased

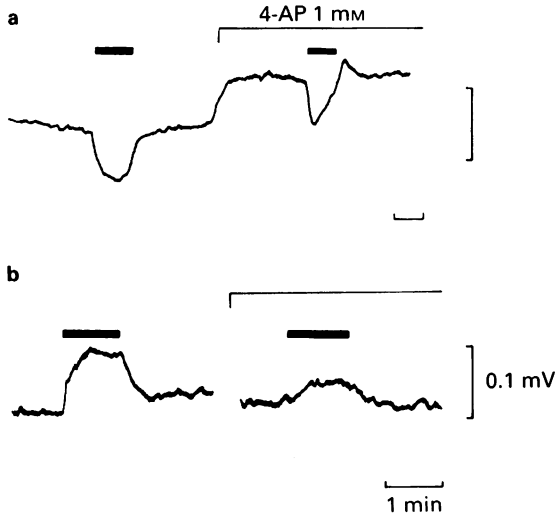


Figure 10 Extracellular d.c. potentials recorded (a) from the superior cervical ganglion with reference to the postganglionic trunk and (b) from the preganglionic trunk with reference to an adjacent segment of the same trunk (see Methods) following 1 min applications of 10 μM (−)-noradrenaline (at bars) before and after adding 1 mM 4-aminopyridine (4-AP) to the perfusion fluid. Two preparations; hyperpolarization downwards. 4-AP depolarized the preganglionic trunk by 0.6 mV; this was backed off during the break in the two records in (b). The preparations were superfused with normal Krebs solution (see Methods). Note that noradrenaline hyperpolarized the ganglion but depolarized the preganglionic trunk. (The reduced depolarization produced by noradrenaline in 4-aminopyridine solution in b may have been due to the large depolarization induced by the 4-aminopyridine itself).

K-conductance would hyperpolarize the preganglionic fibres. No such hyperpolarization could be detected under the same recording conditions where a clear hyperpolarization of the ganglion cells was observed (cf. Brown & Caulfield, 1979). (ii) The Ca-spike was still depressed when residual K-currents retained in 4-AP solution were further blocked with Ba^{2+} ions and/or tetraethylammonium. Although the depression of the peak Ba-spike by noradrenaline was about half that of the Ca-spike, this may be due to the fact that the larger and more prolonged Ba-spike allowed a larger fraction of the Ca-channels to open, so that effects of noradrenaline were not compared at the same current densities; further, the late component of the Ba spike (see above) was annulled by noradrenaline.

The receptors responsible for the inhibition of the Ca-spike appear to be α-adrenoceptors in view of the effectiveness of phentolamine and the ineffectiveness of propranolol as antagonists. The high potency of clonidine (Starke *et al.*, 1975), coupled with the weak effect of amidephrine (Butler & Jenkinson, 1978) suggests an α_2-receptor. This would accord with the inability of prazosin, an α_1-adrenoceptor antagonist (Cambridge *et al.*, 1977; Doxey *et al.*, 1977), to block the inhibitory effect of noradrenaline. However, yohimbine, a potent α_2-antagonist on the rat vas deferens (Drew, 1977), also failed to antagonize the effect of noradrenaline at 1 μM. Further, the apparent pA_2 for phentolamine (6.5) was much lower than that in the rat vas deferens (7.5). These weak effects of phentolamine and yohimbine cannot be due to problems of access or other peculiarities of the experimental conditions since both readily block the α_2-mediated hyperpolarization of the ganglion under these conditions (see also Brown & Caulfield, 1979). Thus, the effects of catecholamines on Ca-spikes observed in this study cannot be unequivocally attributed to an interaction with either α_1- or α_2-adrenoceptors. A comparable difficulty in assigning the receptor responsible for inhibition of Ca-spikes to either of these categories has been encountered in tests on sensory neurones (Canfield & Dunlap, 1984), on locus coeruleus neurones (Williams & North, 1985) and on myenteric neurones (Slack, 1986). The receptors encountered in the present study do not appear to correspond to the 'γ-receptors' reported in rat basilar arteries (Hirst *et al.*, 1982) since the latter were activated equally well by noradrenaline and dopamine and were resistant to phentolamine.

It seems reasonable to suppose that the Ca-spikes recorded from these preganglionic trunk fibres are capable of propagating into the terminals, and therefore that sympathomimetic amines are capable of exerting a comparable inhibition of the Ca-current in such terminals. The question then arises whether the well-known inhibition of transmitter release from these fibres (see Introduction) results from this action or from some other effect such as the increase in K-conductance recordable from the postganglionic cells and at many other sites (see above). Three pieces of evidence suggest that Ca-current inhibition might be the primary factor. Firstly (as mentioned above), no hyperpolarization of the preganglionic fibres could be detected under conditions where catecholamines clearly hyperpolarized the ganglion cells, suggesting that catecholamines may not significantly increase the preganglionic K-conductance. (However, it should be noted that noradrenaline can hyperpolarize the cell bodies of preganglionic axons in the cat, as well as reducing the amplitude to the Ca-spike: Yoshimura *et al.*, 1986; 1987). Secondly, the inhibition of orthodromic transmission produced by noradrenaline was fully blocked by a concentration of phentolamine that did not suppress the postgan-

glionic hyperpolarization (Figure 9a); and thirdly, and conversely, yohimbine (at concentrations that did not antagonize Ca-spike inhibition) only partly antagonized the inhibition of transmission yet fully blocked the postganglionic hyperpolarization (Figure 9b).

In conclusion: we have found that sympathomimetic amines strongly and potently inhibit the Ca-spike in the unmyelinated fibres of the rat preganglionic cervical sympathetic nerve by an action on an α-adrenoceptor of uncertain type, and suggest that an equivalent effect on the Ca-current in the terminal of these fibres might contribute to their ability to inhibit transmitter release.

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Release of 5-hydroxytryptamine, dopamine and noradrenaline in the rat vas deferens in the presence of compound 48/80, veratridine or K^+

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1 The release of 5-hydroxytryptamine (5-HT), dopamine and noradrenaline (NA) from the rat isolated vas deferens was estimated by high performance liquid chromatography with electrochemical detection.

2 Compound 48/80, which is known to release 5-HT and histamine from mast cells, induced a concentration-related, rapid overflow of 5-HT from the rat isolated vas deferens, releasing about 50% of the 5-HT present in the tissue at a concentration of 300 $\mu g\,ml^{-1}$. The drug did not induce the release of either dopamine or NA.

3 The depolarizing agents veratridine (10 μM) or K^+ (100 mM) induced the release of 5–10% of the 5-HT present in the vas deferens, but did not evoke the overflow of 5-HT from isolated mast cells.

4 Veratridine induced the release of a greater proportion of the dopamine than of the NA contained in the vas deferens (41.0 $\pm$ 5.5 vs 17.9 $\pm$ 2.0%, after 20 min of exposure to the drug, $n = 6$). In contrast, K^+ evoked the release of both amines equally.

5 It is concluded that in the rat vas deferens 5-HT is stored in mast cells and also in cells responsive to depolarizing stimuli. It is suggested that 5-HT could be a neuromodulator or a neurotransmitter in this organ. Veratridine and K^+ induce the release of dopamine in addition to NA.

Introduction

The rat vas deferens contains 5-hydroxytryptamine (5-HT) and it has been observed that the tissue concentrations of the amine are higher in regions nearer the prostatic gland than the epididymis (Celuch & Sloley, 1988). Moreover, 5-HT seems to be synthesized and deaminated in the rat vas deferens (Fuenmayor *et al.*, 1976; Celuch & Sloley, 1988). The precise location of 5-HT in the rat vas deferens is not known, although it has been suggested that the amine could be stored in mast cells (Fuenmayor *et al.*, 1976). We have examined the ability of compound 48/80 (a stimulant of mast cell secretion), or depolarizing agents (veratridine or K^+) to release 5-HT from the isolated rat vas deferens. The release of dopamine and noradrenaline (NA) by the above mentioned stimuli was also analysed.

Methods

Rat isolated vas deferens

Male Wistar rats of 200–230 g body weight were killed by a blow to the head and exsanguinated. The vasa deferentia were isolated, cleaned of surrounding fat and of loose connective tissue and opened by a longitudinal cut. The blood contained in the main arteries was removed as much as possible by gently squeezing the vas deferens with the fingers while the organ was immersed in Krebs solution. Tissues were attached to a polypropylene cannula which also carried the gassing line and incubated for 30 min in glass test tubes containing 1 ml of Krebs solution at 37°C, bubbled with 95% O_2 plus 5% CO_2, containing pargyline (10 μM) to inhibit monoamine oxidase activity. During this period the vas deferens was transferred to fresh incubation medium every 10 min. A further incubation (5–20 min duration) was performed using 0.5 ml Krebs solution containing compound 48/80, veratridine (10 μM) or K^+ (100 mM). Control experiments were performed by incubating the tissues in Krebs solution with 4.7 μM K^+. The composition of the Krebs solution was (mM): NaCl 118.0, KCl 4.7, $CaCl_2$ 2.6, $MgCl_2$ 1.2, $NaHCO_3$ 25.0, NaH_2PO_4 1.0, glucose 11.1, EDTA 0.004, ascorbic acid 0.055. The K^+-enriched Krebs solution contained 100 mM KCl and 22.6 mM NaCl.

At the end of the experiment the tissues were

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blotted dry, frozen immediately on dry ice and stored in polypropylene microtubes at −70°C until analysis (1–2 days). Six hundred μl of 0.2 N perchloric acid containing 0.1 mM sodium metabisulphite, 0.25 mM EDTA and 0.1 μg ml^{-1} isoprenaline as internal standard were added to each microtube. The samples were homogenized by ultrasonic disruption for 15 s using a Brausonic 1510 ultrasonic probe (400 W setting) and centrifuged for 15 min in an Eppendorf 5412 desk top centrifuge. Aliquots of the supernatants were injected into a high performance liquid chromatograph (h.p.l.c.) equipped with electrochemical detection for determination of 5-HT, dopamine and NA.

To determine the amounts of 5-HT, dopamine and NA released into the incubation medium, the Krebs solution (0.5 ml) corresponding to the last period of incubation was mixed with 100 μl of the 0.2 N perchloric acid solution and centrifuged. Aliquots of the supernatant (100 μl) were injected directly onto the h.p.l.c. column. Chromatograph separations were performed as previously described (Yu *et al.*, 1986) at room temperature on a 250 × 4.6 mm i.d. Ultrasphere I.P. analytical column packed with C-18 spherical-5 μm particles (Beckman, Toronto, Ont.). An SSI precolumn filter (0.5 pm filter elements, Terochem, Rexdale, Ont.) and 30 × 4.6 mm i.d. Brownlee MPLCRP-18 SPHERI-5 guard column (Technical Marketing Associates, Calgary, Alberta) were installed between the Waters WISP 710B automated sample injector and the analytical column. The mobile phase, comprising 75 mM monobasic sodium phosphate, 1 mM sodium octyl sulphate, 500 μM EDTA, 12.5% acetonitrile, with the final pH adjusted to 2.75 with phosphoric acid, was pumped through the column at 1.0 ml min^{-1} using a Waters M45 solvent delivery system (Millipore, Mississauga, Ont.). Before the addition of acetonitrile, the buffer was filtered through a 0.22 μm Millipore filter. Degassing of the mobile phase was accomplished by vacuum. The detector consisted of a BAS Model TL-5A thin-layer amperometric electrode (Mandel, Rockwood, Ont.) which was controlled at 0.93 V vs a Ag-AgCl reference electrode. Signals for the detector were integrated by peak area using a Spectra-Physics SP-4290 integrator (San José, CA).

Rat isolated peritoneal mast cells

Four male Wistar rats (280–320 g body weight) received pargyline 200 mg kg^{-1} (i.p.) and were killed 2 h later by cervical dislocation. Eight ml of 'washing saline solution' were injected intraperitoneally and the abdomen gently massaged for 2 min. The abdominal fluid was collected in ice-cooled polypropylene tubes and centrifuged at 400 *g* for 5 min. The pellet was resuspended in 'washing saline solution', divided into two parts and the centrifugation repeated. One of the resultant pellets was suspended in 6 ml ice-cold 'incubation saline solution' with normal K^+ concentration (2.7 mM) and the other was suspended in 6 ml of K^+-enriched 'incubation saline solution' (K^+ 100 mM). One ml aliquots of the cell suspensions were incubated at 37°C for 15 min. Veratridine (10 μM) or compound 48/80 (1 μg ml^{-1}) was added for the last 10 min of incubation to two of the tubes containing normal K^+-concentration 'incubation saline' while a third one served as control. At the end of the incubation period the samples were cooled in ice and centrifuged at 1000 *g* for 5 min. The pellets and supernatants were processed for determination of 5-HT by h.p.l.c. as previously described for the vas deferens.

The 'washing saline solution' contained (mM): NaCl 137, KCl 2.7, NaH_2PO_4 0.4, HEPES 10, glucose 5.5, bovine serum albumin 0.05%. The 'incubation saline solution' contained, in addition, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (Lohse *et al.*, 1987). When the 'incubation saline solution' contained 100 mM KCl, the concentration of NaCl was lowered to 39.7 mM. All saline solutions contained 0.055 mM ascorbic acid and 0.004 mM EDTA.

Fractional release (%)

The overflow of amines induced by stimulating agents or released spontaneously is expressed as a percentage of the calculated amine content of the vas deferens or peritoneal mast cells at the beginning of the period of incubation. Fractional release (%) = 100 × amine released to the incubation medium ÷ amine released + amine left in the tissue or in the cells at the end of the incubation period.

Drugs

Compound 48/80 (condensation product of N-methyl-*p*-methoxyphenethylamine with formaldehyde), pargyline hydrochloride and veratridine were purchased from Sigma (St. Louis, MO, U.S.A.). Chemicals for h.p.l.c. with the exception of acetonitrile (h.p.l.c. grade, Caledon, Georgetown, Ontario, Canada) and octyl sodium sulphate (Eastman Kodak, Rochester, NY, U.S.A.) were obtained from Sigma (St. Louis, MO, U.S.A.) or were laboratory grade and were used without further purification.

Statistics

Data are expressed as mean values ± s.e.mean. Differences between means were examined by one factor analysis of variance on logarithm transformed

data, followed by Dunnett's test (Sokal & Rohlf, 1981).

Results

The amine concentrations in rat vas deferens before adding stimulating agents to the incubation medium were calculated to be ($n = 132$): 5-HT $162.2 \pm 7.8\,ng\,g^{-1}$; dopamine $293.5 \pm 12.5\,ng\,g^{-1}$; NA $14.16 \pm 0.19\,\mu g\,g^{-1}$ and tissue weight was 38.76 ± 0.72 mg.

Compound 48/80 ($1–300\,\mu g\,ml^{-1}$) induced a concentration-dependent release of 5-HT in the rat isolated vas deferens. At the highest concentration used, the drug released about 50% of the amine present in the tissue (Figure 1). The overflow of 5-HT induced by compound 48/80 ($10\,\mu g\,ml^{-1}$) appeared to be complete after 2 min of exposure of the tissue to the drug, but the releasing effect was observed even at 30 s (Figure 2a). Compound 48/80 did not induce release of NA or dopamine.

Veratridine (10 μM) or K^+ (100 mM) also produced a significant increase in 5-HT overflow. The maximal fractional release (about 10%) was observed after 15–20 min of incubation with either depolarizing agent (Figure 2a). Both veratridine and K^+ induced the release of dopamine and NA. The fractional release of dopamine and NA induced by K^+ were similar (Figure 2b,c). The only exception was that after 20 min of incubation with K^+-enriched medium, the fractional release of dopamine

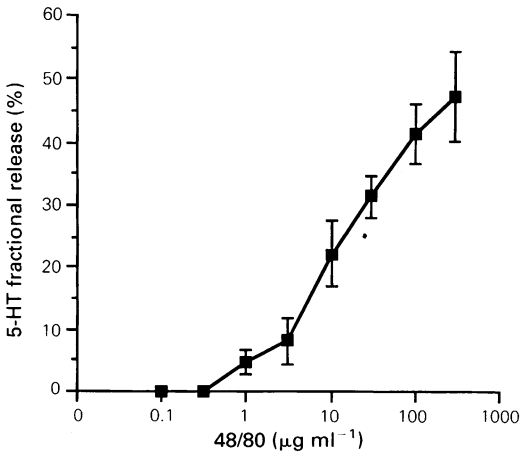


Figure 1 Release of 5-hydroxytryptamine (5-HT) by compound 48/80 in the rat isolated vas deferens. The overflow of 5-HT is expressed as a percentage of the amount of amine present in the tissue at the beginning of the incubation period (5 min). Shown are mean values ($n = 4$) and vertical lines indicate s.e.mean.

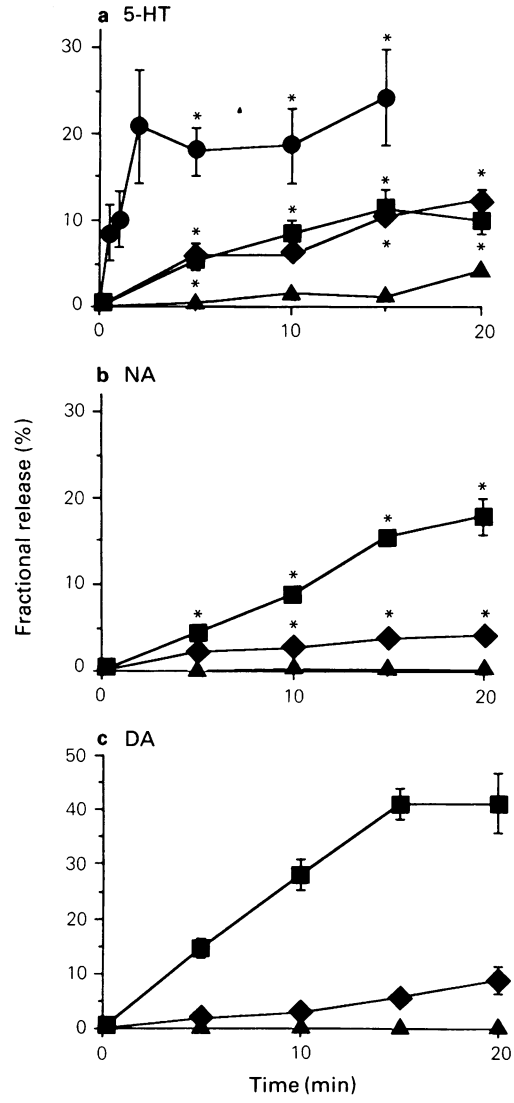


Figure 2 Time-course for the release of (a) 5-hydroxytryptamine (5-HT), (b) noradrenaline (NA) and (c) dopamine (DA), induced by compound 48/80 ($10\,\mu g\,ml^{-1}$, ●), veratridine (10 μM, ■) and potassium (100 mM, ◆), The overflow of amine to the incubation medium is expressed as a percentage of the amount of amine present in the tissue. (▲) Spontaneous fractional release (%) in the absence of stimulating agents. As dopamine and noradrenaline were not released by compound 48/80, these results have been omitted for clarity. Shown are mean values of 6 determinations and vertical lines indicate s.e.mean. * $P < 0.01$ compared to the spontaneous release at the corresponding period of incubation (Dunnett's test). The test was not performed for dopamine values as the spontaneous release of the amine was below the limit of sensitivity of detection.

($8.9 \pm 2.3\%$) was slightly higher than that of NA ($4.3 \pm 0.6\%$) ($n = 6$). In contrast, veratridine released 2–3 times more dopamine than NA in terms of fractional release at all periods of incubation tested (Figure 2b,c). Dopamine was not detected in the incubation medium in the absence of stimulating agents. The spontaneous fractional release of NA ranged from $0.08 \pm 0.04\%$ (5 min, $n = 6$) to $0.23 \pm 0.08\%$ (20 min, $n = 6$).

To investigate whether veratridine or K^+ induced the release of 5-HT from mast cells, isolated peritoneal mast cells were exposed to the depolarizing agents for 10–15 min. Neither veratridine (10 μM) nor K^+ (100 mM) induced the secretion of 5-HT, whereas compound 48/80 (1 $\mu g\,ml^{-1}$) produced a significant 80% depletion of the amine in mast cells.

Discussion

Compound 48/80, which is known to induce the release of 5-HT and histamine from mast cells (Moran *et al.*, 1962; Irman-Florjanc & Erjavec, 1983; Koibuchi *et al.*, 1985), caused a rapid and pronounced overflow of 5-HT from the rat isolated vas deferens (Figures 1 and 2). This finding indicates that a considerable part of the amine in this organ is stored in mast cells, as previously suggested by Fuenmayor *et al.* (1976). Compound 48/80 is, however, less effective in the vas deferens than in isolated mast cells. At 1 $\mu g\,ml^{-1}$, compound 48/80 produces an almost complete depletion of both histamine and 5-HT in isolated mast cells (Carraway *et al.*, 1984; this study), but a concentration 300 times higher released only 50% of the 5-HT content of the rat vas deferens (Figure 2).

Mast cells are not the only source of 5-HT in the rat vas deferens since veratridine and a high concentration of K^+ released 5-hydroxytryptamine from this tissue (Figure 2), but were ineffective in isolated peritoneal mast cells. Contamination with 5-HT from blood platelets is considered unlikely because the volume of blood remaining in the vas deferens was very small. Moreover, the releasing effect of veratridine was still observed in one vas deferens where the blood had been washed out by perfusion with saline (results not shown).

Some possible sources for the 5-HT which was released by either veratridine or K^+ could include 5-hydroxytryptaminergic neurones, chromaffin cells or small intensely fluorescent (SIF) cells. 5-Hydroxytryptaminergic neurones and enterochromaffin cells containing 5-HT are known to exist in the gut (Benditt & Wong, 1957; Penttila, 1966; Robinson & Gershon, 1971; Juorio & Gabella, 1974; Gershon & Sherman, 1982; Holzer & Skofitsch, 1984), and cells emitting a yellow indole fluorescence, morphologically resembling enterochromaffin cells, were observed in human prostatic mucosa (Baumgarten *et al.*, 1968). SIF cells possessing 5-HT have been identified in the superior cervical ganglion of the rat (Eranko & Harkonen, 1965; Verhofstad *et al.*, 1981). Whether the rat vas deferens possesses one or more of these kinds of 5-HT-containing cells is uncertain. An early cytochemical study did not detect endogenous 5-HT in the rat vas deferens (Jaim-Etcheverry & Zieher, 1969), but further investigation is required since the amine has been detected fluorometrically (Fuenmayor *et al.*, 1976) and by h.p.l.c. (present study).

The finding that depolarizing agents release 5-HT suggests that the amine may function as a neurotransmitter or a neuromodulator in the rat vas deferens. This suggestion is supported by pharmacological studies which show that 5-HT induces stimulative and inhibitory effects, mediated by specific 5-HT receptors, on smooth muscle contractility of the rat vas deferens (Kapur & Mottram, 1979; Hay & Wadsworth, 1982; Moritoki *et al.*, 1986a,b). According to our information, however, this is the first study where the release of 5-HT from the rat vas deferens has been determined. It will be of interest to investigate if transmural nerve stimulation induces release of 5-HT as well as veratridine or K^+.

For many years it was thought that the main role of dopamine in sympathetic nerves was as a precursor of NA. However, there is now evidence that sympathetic nerve endings as well as smooth muscle possess dopamine receptors (Enero & Langer, 1975; Simon & Van Maanen, 1976; Tayo, 1979; Ohlstein & Berkowitz, 1985). In addition, the release of dopamine together with NA has been detected in several experimental conditions (Soares-da-Silva, 1987; see also review by Bell, 1988). These findings suggest that dopamine has a further role in sympathetic neurotransmission. In the rat isolated vas deferens veratridine induced the release of a larger proportion of the dopamine than of the NA contained in the tissue. In contrast, depolarization by K^+ induced the release of both amines equally. A tentative explanation is that veratridine and K^+ induced a distinct mobilization of NA-storage vesicles containing different concentrations of dopamine (different proportions of small vs large dense cored vesicles?). According to Klein & Lagercrantz (1981, 1982), the high-affinity uptake of dopamine and the synthesis of NA do not occur in the small dense cored vesicles of sympathetic nerves, but in the large ones. Therefore, it is possible that the content of dopamine in both kinds of vesicles is different. The difference between vereatridine and K^+ with respect to the

fractional release of dopamine and NA in the rat vas deferens could be a consequence of the distinct ionic movements across the cell membrane, caused by either depolarizing agent (Ito *et al.*, 1979; Adler-Graschinsky *et al.*, 1984). The preferential release of dopamine over NA, induced by veratridine (this study) resembles the overflow of these amines during transmural nerve stimulation of the rat and guinea-pig vas deferens (Bell *et al.*, 1984).

In conclusion, the present study shows that in the rat vas deferens, 5-HT is probably stored in mast cells and also in cells responsive to depolarizing stimuli (veratridine or K^+). It is suggested that 5-HT may be a neurotransmitter or a neuromodulator in this organ. Veratridine induces the release of a larger proportion of the dopamine than of the NA present in the tissue, while K^+ induces the release of both amines equally.

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The selective carotid arterial vasoconstrictor action of GR43175 in anaesthetized dogs

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1 GR43175 is a highly selective agonist at 5-HT_1-like receptors in the dog saphenous vein. This study describes the haemodynamic effects of GR43175 in barbitone-anaesthetized dogs.

2 GR43175 (1–1000 $\mu g\,kg^{-1}$, i.v.) produced dose-dependent decreases in carotid arterial blood flow with little or no change in arterial blood pressure. The decrease in blood flow was associated with an increase in carotid arterial vascular resistance. In preliminary studies, the dose of GR43175 producing 50% of the maximum carotid vasoconstrictor response was $39 \pm 8\,\mu g\,kg^{-1}$, i.v.

3 In comparative regional haemodynamic studies, GR43175 (1–1000 $\mu g\,kg^{-1}$, i.v.) had little effect on total peripheral resistance or resistance in the mesenteric, vertebral and coronary arterial vascular beds. Low doses of GR43175 decreased, whilst high doses (100 $\mu g\,kg^{-1}$, i.v. and above) increased femoral arterial vascular resistance. GR43175 (1–1000 $\mu g\,kg^{-1}$, i.v.) had no effect on respiratory inflation pressure. In doses of 100 $\mu g\,kg^{-1}$ i.v. and above, GR43175 caused small decreases in heart rate.

4 The carotid arterial vasoconstrictor action of GR43175 was resistant to antagonism by the 5-HT_2 receptor, 5-HT_3 receptor and α-adrenoceptor blocking drugs, ketanserin, MDL72222 and phentolamine respectively, but could be antagonized by the non-selective 5-HT_1-like receptor blocking drug methiothepin. Methiothepin had no effect on the carotid vasoconstrictor action of the thromboxane A_2 mimetic, U46619.

5 The results demonstrate that GR43175 produces a selective vasoconstriction in the carotid arterial circulation of anaesthetized dogs via activation of 5-HT_1-like receptors, which appear similar to those mediating contraction of the dog isolated saphenous vein.

Introduction

GR43175, (3-[2-dimethylamino]ethyl-N-methyl-1H-indole 5 methane sulphonamide), is a highly selective agonist at 5-HT_1-like receptors mediating contraction of the dog isolated saphenous vein (Humphrey *et al.*, 1987; 1988) and isolated cerebral blood vessels from the dog and primate (Connor *et al.*, 1987). We have previously postulated that the 5-HT_1-like receptor mediating contraction of the dog isolated saphenous vein also mediates vasoconstriction of the carotid arterial circulation (see Apperley *et al.*, 1980; Feniuk *et al.*, 1985). Indeed, methysergide, which acts as a 'partial' agonist at 5-HT_1-like receptors in the dog isolated saphenous vein causes selective vasoconstriction in the carotid arterial circulation of anaesthetized dogs (Saxena, 1974; Apperley *et al.*, 1980). It has been suggested that this action of methysergide could explain its efficacy in the treatment of vascular migraine headache (Curran *et al.*, 1967; Saxena, 1974). However, methysergide is only used prophylactically (Lance, 1973) and, unlike GR43175, is not effective in the acute treatment of migraine (Doenicke *et al.*, 1988). Methysergide also has side effects which preclude its long term use clinically and which may result from the activation of a variety of receptor mechanisms including α-adrenoceptors and histamine receptors (see Curran *et al.*, 1967; Apperley *et al.*, 1976; Gunning *et al.*, 1988). GR43175 is devoid of agonistic activity at α-adrenoceptors and histamine receptors (Humphrey *et al.*, 1988).

In the present study we have investigated the haemodynamic actions of GR43175 in anaesthetized dogs and have compared the effects of a variety of 5-HT receptor blocking drugs on these actions, paying particular attention to the effect of GR43175 on the carotid arterial circulation.

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A preliminary account of these results has been presented to the British Pharmacological Society (Brittain et al., 1987).

Methods

Preliminary studies

Beagle dogs (7–9 kg) of either sex were anaesthetized with barbitone (300 mg kg^{-1}, i.p.) following induction with thiopentone (25 mg kg^{-1}) and pentobarbitone (60 mg) administered intravenously (i.v.). All dogs were intubated with an endotracheal tube and artificially respired with room air using a Palmer ventilation pump at a rate of 20 strokes min^{-1} and a stroke volume of 13–16 ml kg^{-1} which was adjusted to maintain arterial pH, $Paco_2$, and Pao_2 within normal limits. Body temperature was maintained at 39–40°C. Arterial blood pressure was recorded from a cannulated femoral artery with a Bell and Howell pressure transducer (type 4-422-0001) and heart rate derived from the blood pressure signal. Blood flow in the right common carotid artery was recorded by use of a Statham electromagnetic flow probe (2.5 mm) and carotid vascular resistance was derived electronically from the mean arterial pressure and flow signals which were continuously displayed on a Devices M19 chart recorder. Vascular resistance was calculated by dividing mean arterial pressure by the mean carotid arterial flow. Respiratory inflation pressure was monitored from a side branch of the respiratory inflow circuit using a Pye Ether Pressure transducer. Cumulative doses of GR43175 (1–300 or 1000 $\mu g\,kg^{-1}$, i.v.) were administered by bolus injection at 15 min intervals.

Regional haemodynamic studies

In further studies, blood flow in the right common carotid, left femoral and superior mesenteric arteries was recorded. These animals were pretreated with saline 15 min before dosing with GR43175 (1–1000 $\mu g\,kg^{-1}$, i.v., administered cumulatively).

In other experiments, a thoracotomy was performed and animals subjected to positive pressure ventilation (3–4 cmH_2O). Flow probes were placed around either the ascending aorta, the circumflex coronary or the vertebral artery. Aortic flow was assumed to represent cardiac output. Carotid arterial blood flow and vascular resistance was additionally monitored in all experiments as already described. Femoral, mesenteric, total peripheral, coronary or vertebral and carotid arterial vascular resistances were also calculated by dividing the mean aortic pressure by the mean respective blood flows.

Antagonist studies

In view of the long lasting carotid vasoconstrictor action of GR43175 (see below), the effect of antagonists was examined by comparing the actions of GR43175 in the carotid artery bed of saline pretreated dogs with those in dogs that had been pretreated with an antagonist.

Cumulative doses of GR43175 (1–1000 $\mu g\,kg^{-1}$, i.v.) were administered 30 min after pretreatment with a single dose of antagonist or saline and responses measured at the peak change in carotid arterial vascular resistance. Changes in vascular resistance were determined from the basal value immediately prior to dosing with GR43175.

Specificity studies

In these studies, the right cranial thyroid artery was retrogradely cannulated and right common carotid arterial blood flow and vascular resistance calculated as already described. The thromboxane A_2-mimetic U46619 (0.01–3 $\mu g\,kg^{-1}$, i.a.) was administered by bolus retrograde injection into the common carotid artery and vasoconstrictor dose-response curves obtained before and after administration of methiothepin (1 mg kg^{-1}, i.v.). Dose-ratios were calculated from the linear portion of the U46619 dose-response curve.

Statistics

Unless otherwise stated all values shown are mean values ± s.e.mean from n observations.

Regional haemodynamics Vascular resistance before and after each dose of GR43175 was compared for statistical significance by a paired t test. Responses were considered to be significantly different when $P < 0.05$.

Effects of antagonists Carotid arterial vasoconstrictor responses (% increase in vascular resistance) to GR43175 in saline-treated animals and antagonist pretreated animals were compared by an unpaired t test. Responses were considered to be significantly different when $P < 0.05$.

Drugs used

GR43175 succinate, MDL72222 (1αH,3α,5αH-tropan-3yl-3,5 dichlorobenzoate) hydrochloride and U46619 (11, 9 epoxymethano-PGH_2) were synthesized within the Chemistry Department of Glaxo Group Research, Ware. The following compounds were purchased: phentolamine mesylate (Ciba);

methiothepin maleate (Roche). Ketanserin was a gift from Janssen pharmaceuticals.

All drugs with the exception of ketanserin and methiothepin were initially dissolved in distilled water and diluted with 0.9% w/v saline. Ketanserin was initially dissolved in 0.1 M tartaric acid and diluted with 0.9% w/v saline. Methiothepin was dissolved in 10% ethanol in distilled water.

The doses of all drugs refer to the free base.

Results

Preliminary studies of the cardiovascular and respiratory effects of GR43175

The intravenous administration of GR43175 (1–300 or 1000 $\mu g\,kg^{-1}$, i.v.) had little or no effect on arterial blood pressure or tracheal inflation pressure and produced only a small bradycardia at doses of 100 $\mu g\,kg^{-1}$ and above (Table 1). The most prominent effect produced by GR43175 was a selective, dose-dependent, long lasting decrease in carotid arterial blood flow. Since arterial blood pressure was not modified by GR43175, vascular resistance in the carotid arterial circulation increased in a dose-dependent manner. The dose of GR43175 producing 50% of its maximum response in the carotid circulation was $39 \pm 8\,\mu g\,kg^{-1}$ i.v. (values are mean ± s.e.mean from 6 dogs). An experimental recording illustrating these actions of GR43175 is shown in Figure 1. The duration of maximally effective doses of GR43175 in the carotid artery bed exceeded 2 h.

Regional haemodynamics

The effect of GR43175 on total peripheral resistance, vertebral or coronary artery vascular resistance was studied and compared with the effect of GR43175 in the carotid artery bed (Figure 2). GR43175 (1–1000 $\mu g\,kg^{-1}$, i.v.) produced dose-dependent increases in carotid arterial vascular resistance with little or no change in either total peripheral resistance or resistance in the vertebral or coronary arterial vascular beds.

The effects of GR43175 in the femoral arterial circulation were variable. Low doses of GR43175 tended to cause vasodilatation as a consequence of a decrease in femoral vascular resistance, whilst higher doses (100 $\mu g\,kg^{-1}$, i.v. and above) caused vasoconstriction. In the mesenteric arterial bed, GR43175 (1–1000 $\mu g\,kg^{-1}$, i.v.) had no marked effect. The effects of GR43175 in the femoral and mesenteric circulations are compared with those in the carotid bed in the same experiments (Figure 3).

Effect of antagonists on the carotid arterial vasoconstrictor action of GR43175

In view of the long lasting carotid arterial vasoconstrictor action of GR43175, we have compared the carotid vasoconstrictor activity of GR43175 in saline

Table 1 Effect of GR43175 on carotid arterial vascular resistance, diastolic blood pressure, heart rate and tracheal inflation pressure in saline-pretreated anaesthetized dogs

Cumulative dose GR43175 ($\mu g\,kg^{-1}$, i.v.)		% Δ *carotid vascular resistance*	Δ *diastolic blood pressure* (mmHg)	Δ *heart rate* (beats min^{-1})	Δ *respiratory inflation pressure* (mmHg)
1	Peak	−1 ± 4	−7 ± 2	1 ± 2	0
	15 min	2 ± 1	2 ± 1	1 ± 2	0
3	Peak	10 ± 7	−8 ± 2	1 ± 3	0
	15 min	4 ± 3	1 ± 1	0 ± 3	0
10	Peak	23 ± 10	−11 ± 3	−2 ± 4	0
	15 min	17 ± 5	2 ± 1	−2 ± 3	0
30	Peak	67 ± 11	−9 ± 4	−8 ± 3	0
	15 min	43 ± 9	1 ± 2	−6 ± 3	0
100	Peak	127 ± 20	0 ± 6	−16 ± 3	0
	15 min	91 ± 14	5 ± 2	−11 ± 4	0
300	Peak	150 ± 21	3 ± 5	−21 ± 4	0
	15 min	127 ± 26	6 ± 3	−17 ± 5	0

Values are peak changes and changes 15 min after each dose of GR43175. Values stated are mean ± s.e.mean from 6 dogs. Basal values; carotid vascular resistance 0.86 ± 0.08 mmHg min ml^{-1} diastolic blood pressure 105 ± 5 mmHg, heart rate 170 ± 6 b min^{-1} and tracheal inflation pressure 5–10 mmHg (range). In 2 dogs (data not shown) the dose of GR43175 had to be increased to 1000 $\mu g\,kg^{-1}$ in order to produce the maximum carotid vasoconstrictor response. In these animals GR43175 (1000 $\mu g\,kg^{-1}$, i.v.) had no effect on respiratory inflation pressure.

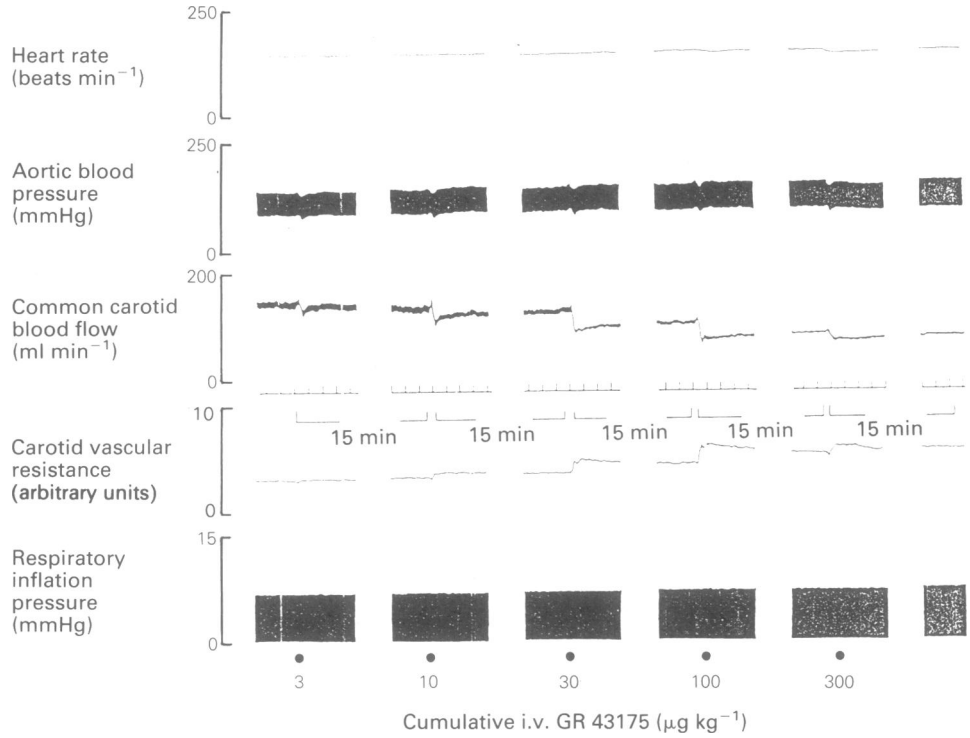


Figure 1 Experimental recording illustrating the effects of cumulatively administered GR43175 in the anaesthetized dog. Note selective vasoconstrictor action in the carotid arterial circulation with little effect on arterial blood pressure, heart rate and tracheal inflation pressure.

pretreated animals with that in animals pretreated with a receptor blocking drug.

Effect of ketanserin Pretreatment of dogs with ketanserin (0.1 mg kg^{-1}, i.v.) caused a small fall in diastolic blood pressure (113 ± 3 mmHg to 93 ± 4 mmHg) and carotid blood flow (149 ± 26 ml min^{-1} to 134 ± 25 ml min^{-1}), although carotid arterial vascular resistance was little changed by ketanserin (0.97 ± 0.17 before ketanserin and 0.92 ± 0.20 mmHg min ml^{-1} after). All values are mean ± s.e.mean from 3 dogs. Ketanserin pretreatment did not attenuate the carotid arterial vasoconstrictor activity of GR43175, indeed the vasoconstrictor action of GR43175 appeared enhanced following ketanserin pretreatment when compared with the saline pretreated dogs (Figure 4).

Effect of MDL72222 Pretreatment of dogs with MDL72222 (1 mg kg^{-1}, i.v.) had little effect on diastolic blood pressure (104 ± 8 mmHg before and 101 ± 8 mmHg after MDL72222) although a small reduction in carotid blood flow was seen (171 ± 16 ml min^{-1} to 159 ± 16 ml min^{-1}). Carotid arterial vascular resistance before and after treatment with MDL72222 was 0.74 ± 0.08 and 0.78 ± 0.10 mmHg min ml^{-1}, respectively. All values are mean ± s.e.mean from 3 dogs. Pretreatment with MDL72222 had no effect on the carotid arterial vasoconstrictor activity of GR43175 (Figure 4).

Effect of phentolamine Pretreatment of dogs with phentolamine (1 mg kg^{-1}, i.v.) reduced diastolic arterial blood pressure (98 ± 4 mmHg to 73 ± 8 mmHg), carotid arterial blood flow (131 ± 14 ml min^{-1} to 116 ± 14 ml min^{-1}) and carotid arterial vascular resistance (0.89 ± 0.08 to 0.76 ± 0.02 mmHg min ml^{-1}). All values are mean ± s.e.mean from 3 dogs. However, phentolamine had little effect on the carotid arterial vasoconstrictor action of GR43175 when compared with its effect in the saline pretreated dogs (Figure 4).

Effect of methiothepin Pretreatment of dogs with methiothepin (1 mg kg^{-1}, i.v.) reduced diastolic blood pressure (108 ± 9 mmHg to 67 ± 4 mmHg) and carotid arterial blood flow (82 ± 9 ml min^{-1} to 50 ± 3 ml min^{-1}), but had little effect on carotid

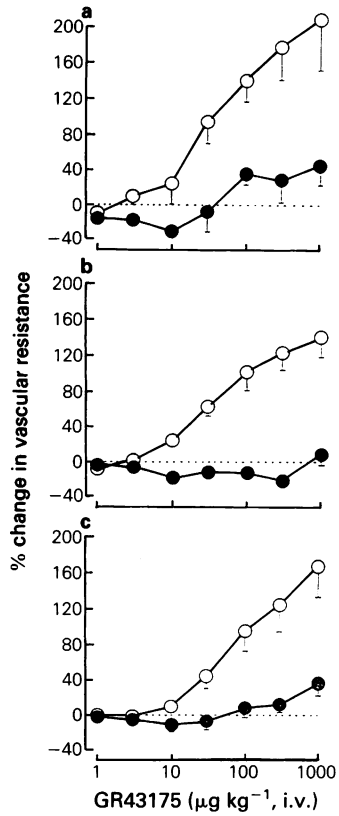


Figure 2 Comparison of the effect of GR43175 on carotid arterial vascular resistance (○) with its effect in other vascular beds (●) in anaesthetized beagle dogs. Values are mean (with s.e.mean shown by vertical bars) from 3 dogs. GR43175 was administered cumulatively and changes were measured from levels before dosing with GR43175. (a) Initial basal carotid (○) and vertebral artery blood (●) flows were 120 ± 14 and $11 \pm 3\,ml\,min^{-1}$ respectively. Initial carotid and vertebral arterial vascular resistances were 0.88 ± 0.06 and $10.81 \pm 2.30\,mmHg\ min\,ml^{-1}$ respectively. Vascular resistance was significantly increased in the carotid artery bed at doses above $30\,\mu g\,kg^{-1}$, i.v. ($P < 0.05$). The effects of GR43175 in the vertebral artery bed were not significant. (b) Initial basal carotid (○) and coronary artery blood (●) flows were 92 ± 10 and $17 \pm 1\,ml\,min^{-1}$ respectively. Initial carotid and coronary arterial vascular resistances were 1.23 ± 0.08 and $6.57 \pm 0.55\,mmHg\ min\,ml^{-1}$ respectively. Vascular resistance was significantly increased in the carotid artery bed at doses above $3\,\mu g\,kg^{-1}$, i.v. ($P < 0.05$). The effects of GR43175 in the coronary artery bed were not significant. (c) Initial basal carotid artery (○) and ascending aortic blood (●) flows were 99 ± 33 and $750 \pm 20\,ml\,min^{-1}$ respectively. Initial carotid and total peripheral arterial vascular resistances were 0.92 ± 0.22 and $0.11 \pm 0.01\,mmHg\ min\,ml^{-1}$ respectively. Vascular resistance was significantly increased in the carotid artery bed at doses above $30\,\mu g\,kg^{-1}$, i.v. ($P < 0.05$). The effects of GR43175 on total peripheral resistance were not significant.

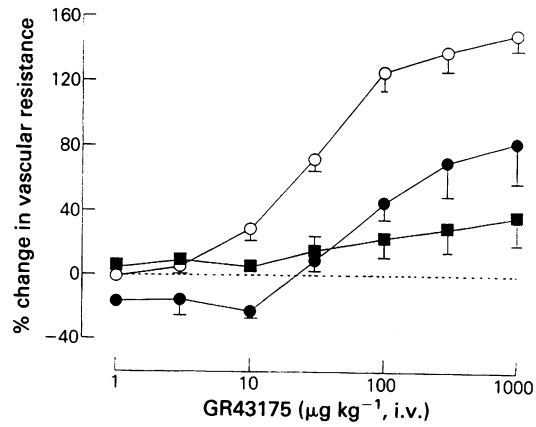


Figure 3 The effect of GR43175 on carotid (○), femoral (●) and mesenteric (■) arterial vascular resistance in anaesthetized beagle dogs. Values are mean (with s.e.mean shown by vertical bars) from 4 dogs. GR43175 was administered cumulatively and changes measured from levels before dosing with GR43175. Initial basal carotid, femoral and mesenteric arterial blood flows were 112 ± 28, 69 ± 4 and $144 \pm 2\,ml\,min^{-1}$, respectively. Initial carotid, femoral and mesenteric arterial vascular resistances were 1.14 ± 0.20, 1.65 ± 0.15 and $0.77 \pm 0.07\,mmHg\ min\,ml^{-1}$ respectively. Vascular resistance in the carotid artery bed was significantly increased at doses above $10\,\mu g\,kg^{-1}$, i.v. ($P < 0.05$). Vascular resistance in the femoral artery bed was significantly reduced at 1, 3 and $10\,\mu g\,kg^{-1}$, i.v. ($P < 0.05$) and increased at doses of $100\,\mu g\,kg^{-1}$, i.v. and above ($P < 0.05$). GR43175 had no significant effect on the mesenteric circulation.

arterial vascular resistance (1.56 ± 0.10 and $1.62 \pm 0.22\,mmHg\ min\,ml^{-1}$ before and after methiothepin treatment respectively). All values are mean ± s.e.mean from 3 dogs. Pretreatment with methiothepin caused a marked attenuation of the carotid arterial vasoconstrictor action of GR43175 when compared with the effect of GR43175 in the saline pretreated animals. This reduction in sensitivity to GR43175 in the carotid artery bed was seen both as an increase in the threshold dose of GR43175 required to cause constriction in the carotid artery bed as well as a reduction in the maximum response (Figure 4).

In three other dogs, the specificity of action of methiothepin was examined by determining its effect upon the carotid vasoconstrictor response to the local administration of the thromboxane A_2-mimetic U46619. Methiothepin ($1\,mg\,kg^{-1}$, i.v.) had no effect

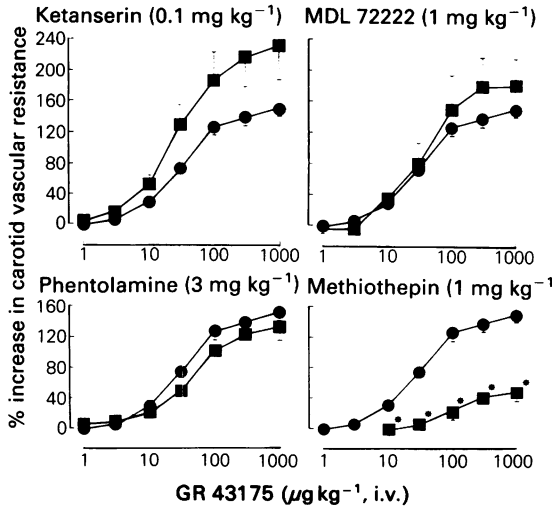


Figure 4 A comparison of the carotid arterial vasoconstrictor activity of intravenously administered GR43175 in saline pretreated anaesthetized dogs (●) and dogs pretreated with a variety of receptor blocking drugs (■). Note the marked attenuation of the carotid arterial vasoconstrictor activity of GR43175 in animals which had been pretreated with methiothepin (1 mg kg^{-1}, i.v.). Values are mean (with s.e.mean shown by vertical bars) from 3 dogs. * Vasoconstrictor responses significantly different from those in saline pretreated animals ($P < 0.05$).

on the carotid vasoconstrictor response to U46619, the U46619 dose-ratios in the presence of methiothepin being 0.70, 0.24 and 1.00.

Discussion

This study is the first detailed description of the haemodynamic actions of the selective 5-HT$_1$-like receptor agonist, GR43175 (Humphrey *et al.*, 1987), a compound which has been shown to be efficacious in the acute treatment of migraine (Doenicke *et al.*, 1988). The aims of the study were two fold. Firstly, to investigate the effect of GR43175 on a variety of different vascular beds and secondly, to characterize the receptor mechanisms involved in its carotid vasoconstrictor action in anaesthetized dogs.

The intravenous administration of GR43175 produced a dose-dependent increase in carotid arterial vascular resistance with little or no change in mesenteric, vertebral, coronary or total peripheral vascular resistance. Furthermore, GR43175 produced little or no change in blood pressure or tracheal inflation pressure. However, high doses of GR43175 (100 μg kg^{-1}, i.v. and above) did produce a small bradycardia which may be centrally mediated (Coote *et al.*, 1987). The most prominent effect of GR43175 was to produce a highly selective vasoconstriction within the carotid arterial circulation, an action which is shared by the antimigraine drugs, ergotamine and methysergide (Saxena, 1974; Saxena & de Vlaam-Schluter, 1974). However ergotamine, unlike GR43175, also causes marked increases in blood pressure, total peripheral, mesenteric, vertebral and coronary vascular resistance (Saxena & de Vlaam-Schluter, 1974). Indeed, the non-selective nature of the vasoconstrictor action of ergotamine probably accounts for many of the unwanted vascular effects associated with ergotamine therapy.

Although GR43175 produced a selective vasoconstriction in the carotid artery bed of anaesthetized dogs, its effects in the femoral circulation were more complex. Low doses of GR43175 tended to cause vasodilatation in the femoral circulation whilst higher doses caused vasoconstriction, which was less than that seen in the carotid artery bed. It seems likely that the overall haemodynamic response to GR43175 in the femoral circulation of the dog depends upon a balance between two opposing actions. We have previously shown that similar changes occur in the femoral circulation of dogs following methysergide administration (Feniuk *et al.*, 1981). Methysergide, like GR43175, is an agonist at 5-HT$_1$-like receptors in the dog isolated saphenous vein, albeit a partial agonist (Apperley *et al.*, 1980; Apperley & Humphrey, 1986; Humphrey *et al.*, 1988). We have shown that the vasomotor actions of methysergide in the femoral circulation are highly dependent upon the inherent degree of sympathetic tone (Feniuk *et al.*, 1981). Vasodilatation appears to be produced by an inhibition of sympathetic neurotransmission and vasoconstriction by a direct effect on the vasculature (Feniuk *et al.*, 1981), the same 5-HT$_1$-like receptor being involved in both actions and existing both pre- and post-junctionally as is the case in the dog isolated saphenous vein (Watts *et al.*, 1981). Furthermore, GR43175 is equiactive at both pre- and post-junctional 5-HT$_1$-like receptors in the dog isolated saphenous vein (Humphrey *et al.*, 1988). It therefore seems likely that the overall haemodynamic effect of GR43175 in the femoral circulation, like that of methysergide, is dependent upon the degree of sympathetic tone. Thus low doses may produce vasodilatation via an inhibition of sympathetic neurotransmission and high doses produce vasoconstriction via a direct effect on the vasculature. This suggests that in the dog at least, 5-HT$_1$-like receptors which mediate vasoconstriction exist not only in the carotid circulation but also in the femoral circulation. This is supported by the observation that 5-HT-induced vasoconstriction of the dog forelimb circulation is not modified by

5-HT_2 receptor blockade (Jandhyala & Kivlighn, 1987), thus by exclusion implicating 5-HT_1-like receptors. Interestingly canine foot pads, like the carotid circulation are known to contain arteriovenous anastomoses or shunt vessels (Delaney & Scarpino, 1973). It may be that 5-HT_1-like receptors, like those in the dog saphenous vein, are mainly localised to shunt vessels.

Saxena *et al.* (1983) have shown that in anaesthetized dogs approximately 40% of carotid arterial blood flow is shunted to the venous circulation by arteriovenous anastomoses. Interestingly, dilatation of arteriovenous shunts has been implicated in the pathogenesis of migraine headache (Heyck, 1969). It is tempting to speculate that the carotid vasoconstrictor action of GR43175 in anaesthetized dogs is due to a decrease in arteriovenous shunt flow. We have recently shown in anaesthetized cats that GR43175 has a marked vasoconstrictor action on arteriovenous shunts (Feniuk *et al.*, 1987) and we are currently examining this action in more detail.

The aim of the second part of this study was to characterize the receptors involved in the carotid arterial vasoconstrictor action of GR43175. We have previously shown that in the dog isolated saphenous vein, the contractile effect of GR43175 is resistant to antagonism by 5-HT_2 and 5-HT_3 receptor blocking drugs, such as ketanserin and MDL72222 but is specifically antagonized by methiothepin, suggesting the activation of 5-HT_1-like receptors (Humphrey *et al.*, 1988). Similar effects were seen in the present study *in vivo*. Neither ketanserin nor MDL72222 attenuated the carotid vasoconstrictor action of GR43175 thereby excluding an action involving 5-HT_2 and 5-HT_3 receptors. Indeed, following ketanserin pretreatment, the carotid vasoconstrictor action of GR43175 appeared enhanced. The mechanism of this enhancement is not known. Nevertheless, GR43175 is devoid of agonist effects in a range of isolated tissue preparations containing 5-HT_2 receptors, including the rabbit aorta, dog femoral artery and pig and dog coronary arteries (Humphrey *et al.*, 1988). An action of GR43175 on α-adrenoceptors can also be excluded since phentolamine had little or no effect on the carotid vasoconstrictor action of GR43175. In marked contrast, following pretreatment with methiothepin, the carotid arterial vasoconstrictor action of GR43175 was markedly attenuated, suggesting an action involving the stimulation of 5-HT_1-like receptors similar to those mediating contraction of the dog isolated saphenous vein (Apperley & Humphrey 1986). Although methiothepin itself had marked cardiovascular effects, its antagonism of the GR43175-induced carotid vasoconstrictor response was specific in nature since it had no effect on the carotid vasoconstrictor response produced by the thromboxane A_2 mimetic, U46619.

In summary, the results from the present study have clearly demonstrated the selective carotid vasoconstrictor action of GR43175, an effect which involves the activation of 5-HT_1-like receptors in the vasculature. Such an action of GR43175 could explain the mechanism by which GR43175 is useful in the treatment of acute migraine headaches (Doenicke *et al.*, 1988).

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Intragastric capsaicin enhances rat gastric acid elimination and mucosal blood flow by afferent nerve stimulation

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1 This study investigated the effects of intragastric capsaicin on acid output, clearance of aniline, potential difference, and morphology of the mucosa in the rat stomach. The experiments were carried out on rats anaesthetized with urethane in which the stomachs were continuously perfused with saline.

2 When the stomach was perfused with normal saline (pH ~6), intragastric capsaicin (32–640 μM) had no effect on the output of titratable acid. In contrast, when acid output was stimulated by pentagastrin or when the stomach was perfused with acid saline (pH 3), capsaicin reduced acid output. Acid loss which occurred during perfusion with saline of pH 2 was not significantly increased by capsaicin. This suggests that capsaicin does not enhance acid back-diffusion but facilitates acid elimination by other means.

3 The gastric clearance of [^{14}C]-aniline, which is an indirect index of gastric mucosal blood flow, was estimated while the stomach was perfused with saline of pH 3. The clearance of aniline rose by 50–60% following intragastric administration of capsaicin (160 μM) whereas the mean arterial blood pressure was increased by about 2.5 mmHg only. Combined pretreatment of the rats with atropine, phentolamine, and propranolol did not alter the effect of capsaicin on the gastric clearance of aniline.

4 The gastric potential difference was not altered by capsaicin (160 μM) administered together with saline of pH 3. This and the finding that there were no signs of mucosal damage by light and scanning electron microscopy indicate that intragastric capsaicin does not irritate the gastric mucosa.

5 The effects of intragastric capsaicin on gastric acid output and aniline clearance and on blood pressure were absent in rats in which capsaicin-sensitive afferent neurones had been ablated by neonatal treatment with a neurotoxic dose of capasicin, which indicates that they result from stimulation of afferent nerve endings in the stomach. It is concluded that facilitation of acid elimination and mucosal blood flow may contribute to the previously reported protective action of capsaicin on the gastric mucosa.

Introduction

Administration of capsaicin into the rat stomach has been found to protect against the formation of gastric mucosal lesions caused by pylorus ligation (Szolcsányi & Barthó, 1981) or ethanol (Holzer & Lippe, 1988). Capsaicin is a selective stimulant of primary afferent neurones with Aδ- and C-fibres (Russell & Burchiel, 1984; Szolcsányi, 1984; Buck & Burks, 1986) and it seems as if the protective effect of capsaicin against ethanol-induced gastric damage is also the result of afferent nerve stimulation since it is absent following ablation of capsaicin-sensitive afferent neurones (Holzer & Lippe, 1988). Further analysis of capsaicin-induced gastric-protection indicated that capsaicin acted by a local mechanism in

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the stomach. As a first step in the analysis of this action, the influence of intragastric capsaicin on gastric acid output and aniline clearance, an indirect measure of mucosal blood flow, was examined in the present study. In addition, an investigation into whether capsaicin-induced changes in acid output and aniline clearance depended on the integrity of the sensory innervation of the stomach was carried out, in order to establish whether changes in these parameters have any bearing on afferent nerve-mediated protection of the gastric mucosa. In view of reports that crude preparations of capsaicin irritate the gastric mucosa (see Limlomwongse *et al.*, 1979; Szolcsányi & Barthó, 1981) the possibility has also to be considered that the gastric-protective influence of capsaicin is analogous to the well-documented protective action of a number of mild irritants (Robert *et al.*, 1983; Takeuchi *et al.*, 1987). Since irritation is associated with histologically visible damage to gastric surface cells and a fall in the gastric potential difference (Whittle, 1977; Takeuchi *et al.*, 1987) the effect of intragastric capsaicin on these indices of gastric barrier disruption was also examined. Some of the present findings have been published in abstract form (Lippe & Holzer, 1987).

Methods

Animals

Adult Sprague-Dawley rats (strain OFA-SD, Forschungsinstitut für Versuchstierzucht, Himberg, Austria) of either sex were used. The weight of the rats at the time of experimentation was 230–320 g. The animals were deprived of food for 20 h before the experiments but allowed free access to tap water.

Sensory denervation

On their second day of life, rats received a subcutaneous injection of capsaicin 0.16 mmol kg^{-1} (Gamse, 1982). This treatment is known to cause a permanent degeneration of unmyelinated afferent neurones (Russell & Burchiel, 1984; Buck & Burks, 1986). Control animals received equal volumes of vehicle (10% ethanol, 10% Tween 80, 80% saline, vol/vol/vol) only. All injections were performed under ether anaesthesia. The rats were then grown to adulthood and used for the experiments at the age of 4–5 months. The effectiveness of the treatment was checked by instilling a drop of a 0.33 mM solution of capsaicin in saline into one eye of the rats and counting the protective wiping movements (Gamse, 1982). This test was carried out one day before experimentation. Untreated or vehicle-treated rats responded instantly with wipings while capsaicin-treated rats did not react (Gamse, 1982). Animals treated with capsaicin that showed any wiping movements were excluded from the study. Whenever a rat had responded with such wipings the afflicted eye was immediately and thoroughly rinsed with water.

Surgical preparation

The rats were anaesthetized by a subcutaneous injection of urethane (1.5 g kg^{-1}). The body temperature was maintained at 36–37°C by means of a rectal thermometer and a heating lamp. A tracheostomy was performed and the trachea cannulated to ensure a patent airway. Blood pressure in a carotid artery was recorded via a cannula filled with heparinized saline and connected to a pressure transducer. A rubber connector positioned 4 cm away from the blood vessel and fitted with a syringe allowed withdrawal of blood samples from the carotid artery. A jugular vein was also cannulated to enable the infusion of pentagastrin or [^{14}C]-aniline at a flow rate of 1.5 ml h^{-1}. The gastric lumen was continuously perfused by the technique described by Main & Whittle (1973). Briefly, a soft catheter (inner diameter 0.8 mm) was inserted into the stomach through an incision in the cervical oesophagus and held in place by a ligature. This catheter was connected to a peristaltic pump, and saline maintained at 37°C was perfused through the gastric lumen at a rate of 0.7–0.8 ml min^{-1}. Gastric outflow was collected by a cannula (inner diameter 3.0 mm) which was inserted into the stomach via an incision in the duodenum and held in place by two ligatures around the duodenum. At the beginning of the experiment the stomach was flushed with 50 ml of saline (37°C) to remove any solid contents. After all surgical preparations had been completed a period of 1 h was allowed for equilibration.

Gastric acid output

Samples of the gastric perfusate were collected at intervals of 10 min (of 30 min in some experiments) and weighed. Their acid content was determined by titration to pH 7.0 with NaOH (0.01–0.001 N) using a digital burette (Brand, Wertheim, F.R.G.) and a digital pH-meter (WPI, New Haven, CT, U.S.A.). Gastric acid output was calculated by subtracting the acid contained in the perfusion medium before entering the stomach from the acid contained in the perfusate. Acid output was expressed as micro-equivalents secreted per minute.

Gastric [^{14}C]-aniline clearance

After the completion of surgery, [^{14}C]-aniline was injected intravenously in a loading dose of 6 $\mu g\,kg^{-1}$ (262 $kBq\,kg^{-1}$) followed by a continuous infusion of 0.1 $\mu g\,kg^{-1}\,min^{-1}$ (4.4 $kBq\,kg^{-1}\,min^{-1}$) to maintain a steady plasma concentration of aniline (Main & Whittle, 1973). The stomach was perfused with saline of pH 3. The estimation of [^{14}C]-aniline in blood and gastric perfusate necessitated the extraction of [^{14}C]-aniline and its separation from more polar metabolites. Blood samples (0.4 ml) were withdrawn 1 h after injection of the loading dose of aniline and at the end of the experiment, diluted to 4 ml with heparinized saline and centrifuged. A 3 ml aliquot of the clear supernatant was adjusted to pH 8, and [^{14}C]-aniline was extracted by adding 3 ml of benzene solvent (1.5% isoamyl alcohol in benzene, vol/vol). After centrifugation, a 2 ml aliquot of the organic phase was re-extracted with 2 ml 0.1 N HCl. Following centrifugation, a 1 ml sample of the acidic phase was added to 4 ml of scintillation fluid (Ready-Solv MP, Beckman, Galway, Ireland), and its radioactivity content determined by scintillation spectometry. Aliquots (3 ml) of the gastric perfusate collected at 10 min intervals were subjected to the same extraction procedure as described above. Clearance was calculated as the ratio of gastric output to blood concentration of [^{14}C]-aniline and expressed as $ml\,min^{-1}$ (Main & Whittle, 1973).

Gastric potential difference

The potential difference across the gastric mucosa was measured with two polyethylene catheters which were filled with saturated KCl in 3% agar (w/w) and which served as electrolyte bridges. One catheter was connected to the gastric outflow cannula, and the other one was connected to the cannula in a carotid artery. The opposite end of each catheter dipped into separate beakers which contained saturated KCl solution and accommodated a Ag/AgCl electrode (Ingold, Steinbach, F.R.G.). The potential difference between the two electrodes was recorded with a digital pH/mV-meter (WPI, New Haven, CT, U.S.A.).

Histology

At the end of a 30-min perfusion of the stomach with saline of pH 3 containing either vehicle or capsaicin (160 μM), the stomachs were rapidly removed, opened along the lesser curvature and rinsed with saline. Pieces from the glandular mucosa of the corpus were dissected and placed in fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer of pH 7.2). For scanning electron microscopy the tissues were left in the fixative for 3 h at room temperature and then postfixed in 2% OsO_4 for 1 h at room temperature. After dehydration in a graded series of ethanol the tissue samples were dried in a critical-point drying apparatus (Balzers CPD 020, Balzers, Liechtenstein) from liquid CO_2, mounted on aluminium stubs, and sputter-coated with gold palladium for examination on a scanning electron microscope (Zeiss DSM 950, Oberkochen, F.R.G.).

For light microscopic examination the tissues were left in the fixative for 3 days at room temperature and, after dehydration in a graded series of ethanol, embedded in Historesin (LKB, Bromma, Sweden). Four-micrometer sections were cut and stained with a mixture of methylene blue-azure II and basic fuchsin.

Substances and solutions

[^{14}C]-aniline hydrogen sulphate (specific activity: 4 $GBq\,mmol^{-1}$) was obtained from Amersham International (Amersham, U.K.), atropine, benzene, ethanol, and isoamyl alcohol were obtained from Merck (Darmstadt, F.R.G.), capsaicin and urethane were purchased from Fluka (Buchs, Switzerland), and Tween 80 was from Merck-Schuchardt (München, F.R.G.). Pentagastrin and propranolol were gifts of ICI (Macclesfield, U.K.), and phentolamine was kindly provided by Ciba-Geigy (Basel, Switzerland).

Atropine, phentolamine, and propranolol were dissolved in saline (0.9% NaCl, wt/wt) and injected intravenously at a volume of $1\,ml\,kg^{-1}$. The dosage of atropine was 0.35 $\mu mol\,kg^{-1}$ (Yokotani & Osumi, 1986), the dosage of phentolamine 3.5 $\mu mol\,kg^{-1}$ (Ruwart *et al.*, 1980), and the dosage of propranolol 1.5 $\mu mol\,kg^{-1}$ (Farmer & Levy, 1968). The solutions of pentagastrin and [^{14}C]-aniline used for intravenous infusion were also prepared with saline. Stock solutions of capsaicin were made up in 10% ethanol, 10% Tween 80, and 80% saline (vol/vol/vol). The stock solutions, or the vehicle, were then diluted 100 fold with saline, and these solutions were used for gastric perfusion. Basal acid output or aniline clearance was determined while vehicle diluted in saline was perfused through the gastric lumen.

Statistics

All data are presented as means $\pm$ s.e.mean. The Mann-Whitney U test (two tailed) was used for statistical analysis of the blood pressure measurements. The acid output and aniline clearance data were statistically evaluated by the Quade test for several

related values (Theodorsson-Norheim, 1987). Probability values $P < 0.05$ were regarded as significant.

Results

Gastric acid output

When the stomachs were perfused with normal saline (pH ~6) the acid output amounted to $0.195 \pm 0.031\ \mu Eq\ min^{-1}$ ($n = 20$) 100 min after the start of perfusion. When the perfusion with normal saline was continued for another 90 min, acid output remained stable during this period ($n = 6$). Administration of capsaicin (160 or 640 μM, $n = 7$ for each concentration) during the period of 100–160 min failed to alter acid output significantly although some tendency towards a decrease was seen in some experiments.

In a second group of experiments (Figure 1), gastric acid output was stimulated by intravenous infusion of a submaximally effective dose of pentagastrin ($0.4\ nmol\ kg^{-1}\ min^{-1}$) (Main & Whittle, 1973). The infusion of pentagastrin was started 60 min after surgery and continued until the end of the experiment. Gastric acid output increased to peak levels within 40 min and then remained constant (Figure 1a). When 60 min after the start of pentagastrin infusion capsaicin was administered intragastrically, pentagastrin-stimulated acid output was reduced. This effect of capsaicin was concentration-related with respect to its onset, magnitude, and duration. At a concentration of 160 μM capsaicin, acid output fell significantly after a latency of 30 min but rapidly recovered following capsaicin withdrawal (Figure 1b). In contrast, with 640 μM capsaicin a significant inhibition of acid output was observed after only 10 min, and the acid output remained depressed even after capsaicin withdrawal and had recovered little within the subsequent 40 min (Figure 1c).

In a third series of experiments the stomachs were perfused with acid saline (pH 3). Also under these conditions acid output remained fairly stable during the experimental period of 190 min (Figure 2a). Addition of capsaicin (32 μM) to the perfusion medium did not significantly reduce acid output although such a tendency was clearly seen ($n = 6$; data not shown). At concentrations of 160–640 μM, capsaicin caused a significant reduction of acid output, and this action was concentration-dependent with regard to its onset, magnitude, and duration. With 160 μM capsaicin, acid output decreased significantly with a lag of 20 min but returned promptly to control values after withdrawal of capsaicin (Figure 2b). When 640 μM capsaicin was administered intragastrically, there was an immediate and significant fall in acid output

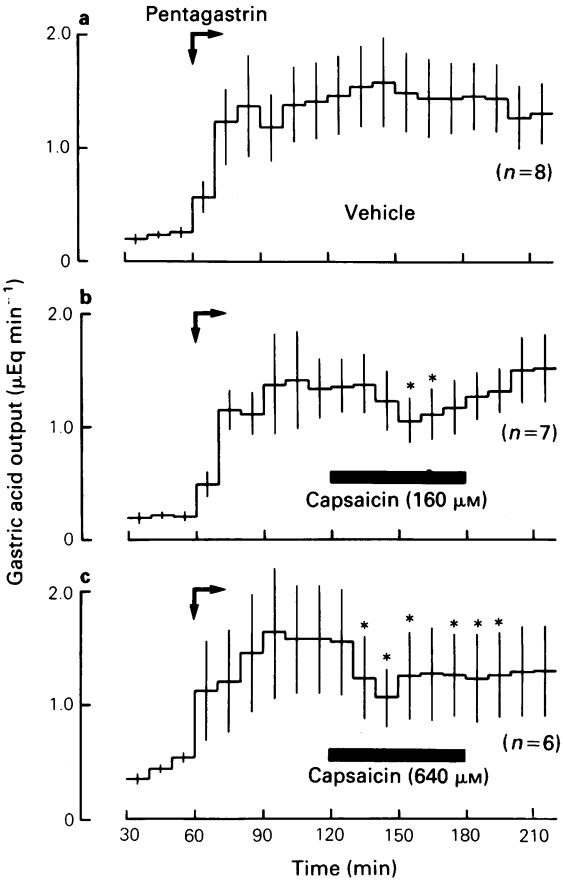


Figure 1 Gastric acid output in urethane-anaesthetized rats with stomachs perfused with normal saline (pH ~6) containing capsaicin (b and c) or an equivalent concentration of its vehicle (a). Pentagastrin ($0.4\ nmol\ kg^{-1}\ min^{-1}$) was administered by intravenous infusion which was started at 60 min and then maintained throughout the experiment. Gastric acid output was calculated by subtracting the acid contained in the perfusion medium before entering the stomach from the acid contained in the perfusate. Abscissa scale: time after completion of surgery. Means of n animals; Vertical lines show s.e.mean; * $P < 0.05$ versus the value obtained immediately before capsaicin administration.

which was sustained. No appreciable recovery of acid output was seen within 30 min following capsaicin withdrawal (Figure 2c).

In a fourth group of experiments, the stomachs were perfused with acid saline adjusted to pH 2. Under these conditions acid output was negative i.e. there was a continuous loss of acid from the gastric perfusate. In the period of 70–100 min after the start of perfusion, acid loss stabilized at a value of $0.191 \pm 0.024\ \mu Eq\ min^{-1}$ ($n = 10$). Capsaicin

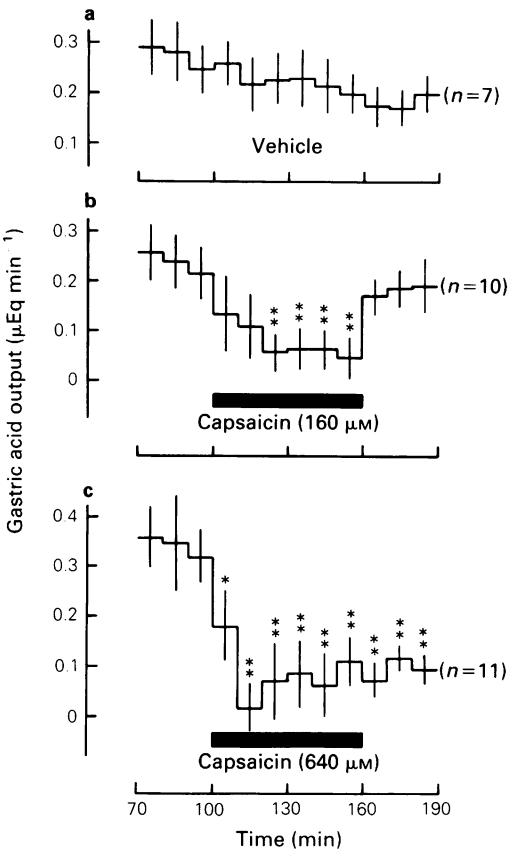


Figure 2 Gastric acid output in urethane-anaesthetized rats with stomachs perfused with acid saline (pH 3) containing capsaicin (b and c) or an equivalent concentration of its vehicle (a). Gastric acid output was calculated by subtracting the acid contained in the perfusion medium before entering the stomach from the acid contained in the perfusate. Abscissa scale: time after completion of surgery. Means of n animals; Vertical lines show s.e.mean. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the value obtained just before capsaicin administration.

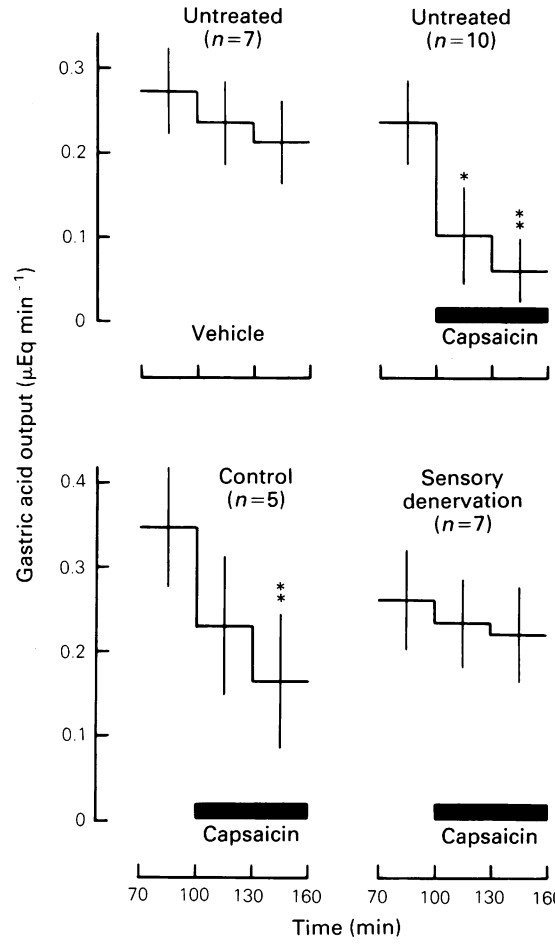


Figure 3 Gastric acid output in urethane-anaesthetized rats with stomachs perfused with acid saline (pH 3) containing capsaicin (160 μM) or an equivalent concentration of its vehicle. The experiments were performed either on untreated rats or on rats treated with capsaicin (0.16 mmol kg^{-1}; sensory denervation) or its vehicle (control) as neonates. Gastric acid output was calculated by subtracting the acid contained in the perfusion medium before entering the stomach from the acid contained in the perfusate. Abscissa scale: time after completion of surgery. Means of n animals; Vertical lines show s.e.mean. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the value obtained just before capsaicin administration.

(160 μM), present in the perfusion medium from 100 min onwards, failed to alter significantly the loss of acid from the perfusate although some tendency towards a transient increase was observed. In the period of 100–130 min, acid loss was 0.258 ± 0.049 μEq min^{-1} ($n = 10$), and in the period of 130–160 min acid loss amounted to 0.187 ± 0.031 μEq min^{-1} ($n = 10$).

In a fifth group of experiments, the effect of intragastric capsaicin (160 μM) on gastric acid output in untreated rats was compared with that in rats which had been treated with a high dose of systemic capsaicin or its vehicle as neonates (Figure 3). The stomachs were again perfused with acid saline (pH 3). Addition of capsaicin to the gastric perfusion medium reduced acid output in untreated rats and in rats treated with the capsaicin vehicle to a similar extent. In contrast, in rats treated with systemic capsaicin as neonates, intragastric capsaicin failed to affect acid output. The basal rate of acid output was

not altered by the neonatal treatment with capsaicin or its vehicle (Figure 3).

Gastric clearance of aniline

The effect of intragastric capsaicin (160 μM) on the clearance of [^{14}C]-aniline into the gastric perfusate was measured in order to obtain an indirect estimate of the effect of capsaicin on gastric mucosal blood flow. In all these experiments acid saline (pH 3) was used for perfusion of the stomach. Addition of capsaicin to the perfusion medium resulted in a prompt rise in the gastric aniline clearance which, after a peak increase by 50–60%, started returning to control values while capsaicin was still present (Figure 4a). This effect of capsaicin remained essentially unchanged when the rats had been treated with a combination of atropine, phentolamine and propranolol 45 min before the administration of capsaicin (Figure 4b). The basal rate of aniline clearance also appeared unaltered by this treatment.

In a second series of experiments the effect of intragastric capsaicin (160 μM) on the gastric clearance of aniline was examined in rats that had been treated with a high dose of systemic capsaicin or its vehicle as neonates (Figure 5). While in vehicle-treated rats intragastric capsaicin enhanced aniline clearance by some 60%, an effect very similar to that observed in untreated rats (compare Figures 4a and 5a), capsaicin failed to change the clearance of aniline in capsaicin-treated rats (Figure 5b). The basal rate of aniline clearance was not altered in capsaicin-treated animals as compared with vehicle-treated rats.

Gastric potential difference

Following a 60-min period of gastric perfusion with saline adjusted to pH 3 the potential difference across the gastric mucosa was -37.1 ± 0.8 mV ($n = 5$). Intragastric administration of 160 μM capsaicin for 30 min did not alter the potential difference which was -37.0 ± 0.8 mV ($n = 5$) 10 min and -36.4 ± 1.0 mV ($n = 5$) 30 min after the start of capsaicin perfusion. The validity of the method was ascertained by showing that perfusion of the stomach with 25% (w/w) ethanol in normal saline led, within 5 min, to a fall of the potential difference from -36.9 ± 2.9 mV to -17.0 ± 2.6 mV ($n = 5$; $P < 0.05$).

Blood pressure

The mean arterial blood pressure was routinely monitored in all experiments of this study. Intragastric capsaicin caused a slight rise in blood pressure in a concentration-dependent manner, reaching a maximal effect ($+2.5 \pm 0.4$ mmHg, $n = 53$; $P < 0.01$) with capsaicin (160 μM). Peak increases in blood pressure were usually observed within 5 min of the start of capsaicin administration; thereafter blood pressure slowly returned to control values. Rats treated with capsaicin as neonates failed to respond to intragastric capsaicin (160 μM) with such a hypertensive reaction ($+0.3 \pm 0.9$ mmHg, $n = 10$) whereas

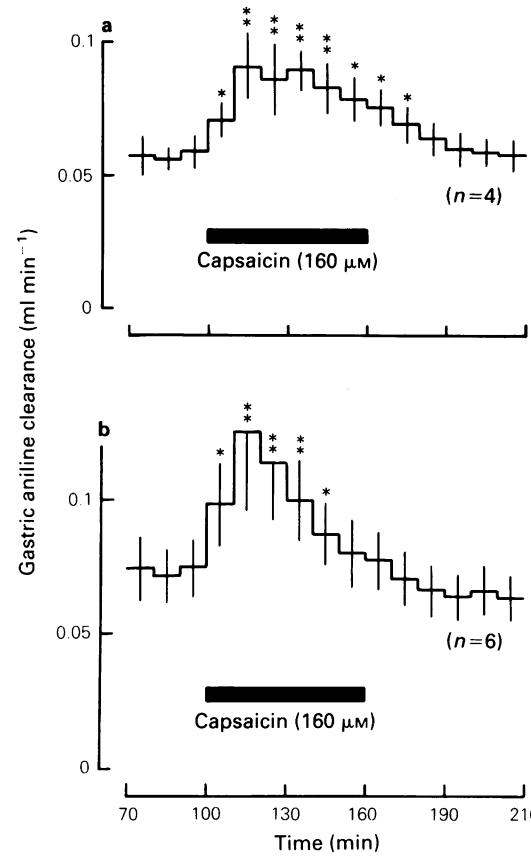


Figure 4 Gastric clearance of [^{14}C]-aniline in urethane-anaesthetized rats with stomachs perfused with acid saline (pH 3) containing capsaicin or an equivalent concentration of its vehicle. (a) Clearance in untreated rats; (b) clearance in rats which had been treated with a combination of atropine (0.35 μmol kg^{-1}), phentolamine (3.5 μmol kg^{-1}) and propranolol (1.5 μmol kg^{-1}) 45 min before capsaicin administration. Abscissa scale: time after completion of surgery. Means of n animals; Vertical lines show s.e.mean. * $P < 0.05$, ** $P < 0.01$ versus the value obtained just before capsaicin administration.

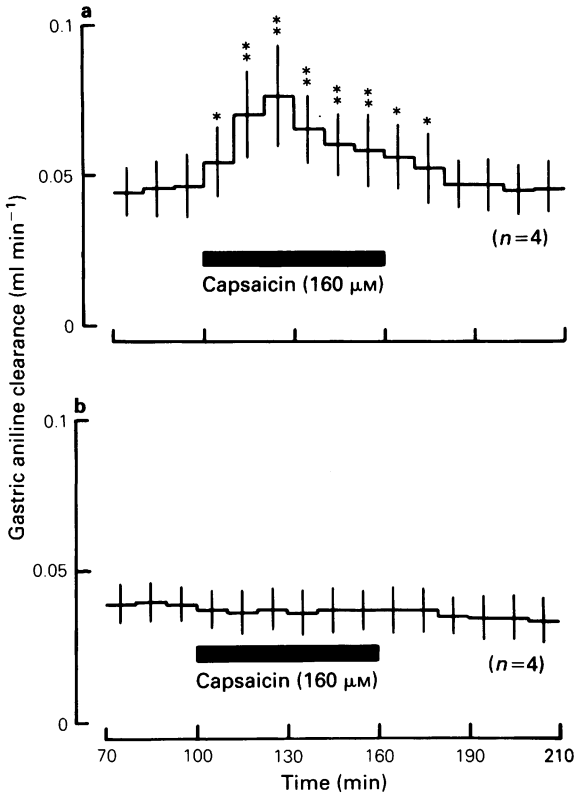


Figure 5 Gastric clearance of [^{14}C]-aniline in urethane-anaesthetized rats with stomachs perfused with acid saline (pH 3) containing capsaicin or an equivalent concentration of its solvent only. Clearance was studied in rats pretreated with 0.16 mmol kg^{-1} capsaicin (b) or its vehicle (a) as neonates. Abscissa scale: time after completion of surgery. Means of n animals; Vertical lines show s.e.mean. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the value obtained just before capsaicin administration.

vehicle-treated rats showed a similar increase in blood pressure to untreated rats ($+1.8 \pm 0.7$ mmHg; $n = 9$; $P < 0.05$). The blood pressure measured just before capsaicin administration did not significantly differ between untreated (88.2 ± 1.1 mmHg, $n = 92$), vehicle-treated (94.3 ± 3.9 mmHg, $n = 9$), and capsaicin-treated (90.9 ± 4.5 mmHg, $n = 10$) animals.

Histology

The glandular mucosa of the stomach was routinely inspected at the end of all experiments described here. No macroscopically visible lesions were observed following perfusion of the stomach with capsaicin. The stomachs from 4 rats, which had been perfused with saline of pH 3 containing 160 μM capsaicin for a period of 30 min, were also examined histologically and compared with stomachs perfused with vehicle-containing saline (pH 3) alone. No damage of the gastric mucosa was detectable in any of the sections examined with the light microscope (Figure 6). Scanning electron microscopy revealed that capsaicin was without injurious effect on the surface epithelium (Figure 6). In no instance did capsaicin disrupt the continuity of the epithelium or cause any overt damage to the surface epithelial

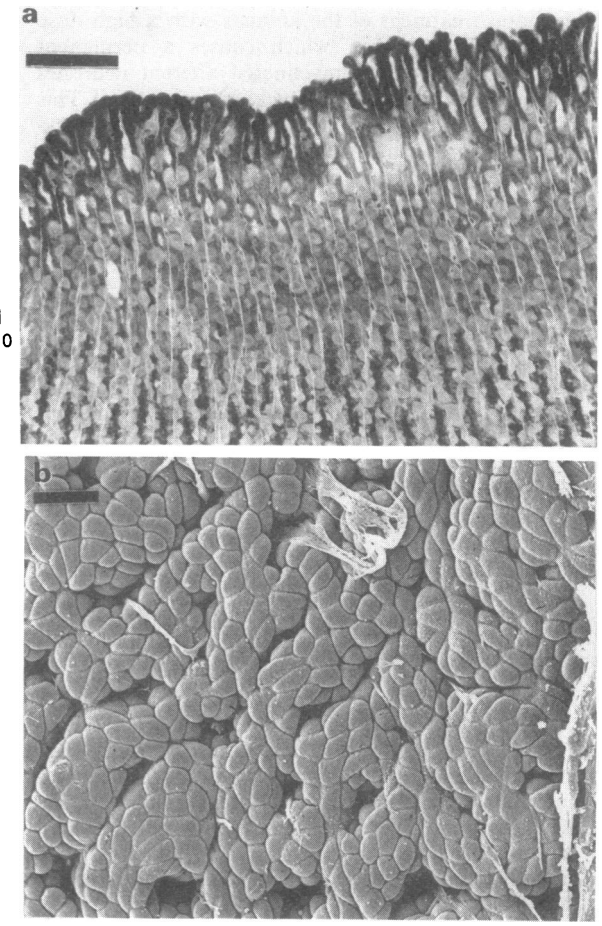


Figure 6 Light (a) and scanning electron (b) microscopic appearance of the mucosa from the rat gastric corpus taken after a 30-min perfusion of the stomach with saline of pH 3 containing 160 μM capsaicin. No differences from control sections were seen. The bar in (a) equals 100 μm, and that in (b) equals 20 μm.

cells. A few cells exhibited apical erosions but this type of injury was also seen in stomachs that had been perfused with saline (pH 3) alone.

Discussion

The present study has shown that intragastric capsaicin, at concentrations identical to those that protect against ethanol-induced haemorrhagic lesions of the gastric mucosa (Holzer & Lippe, 1988), affects gastric acid output, gastric aniline clearance, and blood pressure in the rat. Common to all these actions of intragastric capsaicin is that they are abolished after neonatal treatment of the animals with a high dose of systemic capsaicin which causes a permanent ablation of certain unmyelinated afferent neurones (Russell & Burchiel, 1984; Buck & Burks, 1986). This implies that the acute effects of intra-gastric capsaicin rely on the integrity of the sensory innervation of the stomach or, in other words, probably result from stimulation of afferent nerve endings.

Administration of capsaicin into the stomach reduced acid output only when acid output was stimulated by pentagastrin or when the stomach was perfused with acid saline (pH 3), a procedure which resulted in a similar degree of gastric acidity as that following pentagastrin infusion. Thus it seems as if the ability of capsaicin to reduce acid output depends on the presence of elevated levels of acid but is independent of whether the acid is of endogenous or exogenous origin. A reduction in gastric acidity may be due to a reduction in acid secretion, to an increase in the secretion of neutralizing factors such as bicarbonate (Flemström, 1987; Silen, 1987), or to an increase in other ways of acid disposal including acid back-diffusion (Whittle, 1977; Guth & Leung, 1987). The finding that capsaicin failed to alter acid output when the stomach was perfused with saline of low acidity (pH $\sim$6) may suggest that acid secretion is not inhibited and that hence the reduction of acid output in the presence of elevated levels of acid arises from facilitated elimination of acid. Augmented acid back-diffusion, however, is unlikely to account for capsaicin-induced acid disposal since acid loss at pH 2 was not significantly enhanced by capsaicin. Which other way of acid elimination is actually stimulated by capsaicin remains to be determined.

Ablation of capsaicin-sensitive afferent neurones abolished the effect of intragastric capsaicin on acid output in the presence of elevated acid levels but did not alter basal acid output. This latter observation is in agreement with previous reports (Szolcsányi & Barthó, 1981; Alföldi *et al.*, 1986; Dugani & Glavin, 1986). However, the present results contrast with those of Limlomwongse *et al.* (1979) who found that orally administered capsaicin enhanced pentagastrin-stimulated acid output. It is not unlikely that a number of differences in their experimental model, including the use of pentobarbitone as anaesthetic, the absence of a steady basal acid output, the repeated aspiration of the gastic contents, and different dosages of capsaicin and pentagastrin, could account for the conflicting observations between the two studies.

Consistent, though, with the findings of Limlomwongse *et al.* (1979), who observed that intragastric capsaicin increased the gastric clearance of aminopyrine, is the present result that the gastric clearance of [^{14}C]-aniline was augmented. The gastric clearance of aniline or aminopyrine is thought to provide an indirect estimate of gastric mucosal blood flow, although the validity of this technique is limited inasmuch as the clearance of weak organic bases may also reflect parietal cell secretion of acid and consequently overestimate changes of mucosal blood flow when acid output and mucosal blood flow change in parallel (Müller-Lissner *et al.*, 1981; Leung *et al.*, 1984; Guth & Leung, 1987). Since under the present experimental conditions, acid output decreased while the clearance of aniline increased in response to intragastric capsaicin, the capsaicin-induced increase in mucosal blood flow may have actually been underestimated by the increase in aniline clearance. At any rate, it seems safe to conclude that afferent nerve stimulation with capsaicin results in a pronounced increase in gastric mucosal blood flow although the absolute changes in mucosal blood flow remain to be determined by other techniques. The facilitation of aniline clearance is most likely due to a dilatation of submucosal blood vessels since it appears improbable that the very minor hypertension produced by intragastric capsaicin could to a significant degree account for this effect.

As crude capsaicin has been reported to be a gastric irritant (see Limlomwongse *et al.*, 1979; Szolcsányi & Barthó, 1981) the possibility arises that augmented acid loss is a consequence of capsaicin-induced mucosal damage and that acid entering the tissue may in turn enhance aniline clearance (Whittle, 1977). However, such an explanation is very unlikely for a number of reasons. (i) Irritation causes damage to gastric surface cells (Silen, 1987; Takeuchi *et al.*, 1987) but neither light nor scanning electron microscopic examination of the mucosa revealed any capsaicin-induced lesion of the gastric epithelium. (ii) Disruption of the gastric mucosal barrier is associated with a fall in the gastric potential difference (Whittle, 1977; Takeuchi *et al.*, 1987) yet capsaicin failed to alter this parameter. (iii) The

back-diffusion of acid was not enhanced by capsaicin as would be expected if capsaicin were to break the mucosal barrier (Whittle, 1977). (iv) Intragastric capsaicin also fails to enhance vascular permeability in the stomach (Holzer & Lippe, 1988), which is another consequence of mucosal injury (Szabo *et al.*, 1986). (v) Capsaicin-induced disruption of the gastric mucosa would most probably be independent of the sensory innervation, yet ablation of capsaicin-sensitive afferent neurones abolished the gastric effects of capsaicin measured here. Thus, there is no evidence that purified capsaicin is a gastric irritant; on the contrary, intragastric capsaicin can counteract ethanol-induced lesion formation (Holzer & Lippe, 1988). As this protective action also requires an intact sensory innervation (Holzer & Lippe, 1988) it is probable that facilitation of gastric acid elimination and aniline clearance may be among the mechanisms by which afferent nerve-mediated protection of the gastric mucosa is accomplished.

The increase in aniline clearance produced by intragastric capsaicin was not inhibited by a combination of atropine, phentolamine, and propranolol. It would appear therefore that, like afferent nerve-mediated gastric mucosal protection against ethanol (Holzer & Lippe, 1988), afferent nerve-mediated facilitation of aniline clearance involves neither the autonomic nervous system nor the adrenal glands. This parallel suggests that a rise in gastric mucosal blood flow, as deduced from the aniline clearance, could be of importance for afferent nerve-mediated gastric mucosal protection and that both processes could be brought about by a local release of vasodilator mediators from afferent nerve endings within the gastric mucosa and submucosa. This view is further supported by the observation that blood vessels in the submucosa of the rat stomach receive a particularly dense innervation by capsaicin-sensitive afferent neurones (Sharkey *et al.*, 1984). An increase in mucosal blood flow is thought to be an essential factor in the ability of gastric mucosa to protect itself against acute injury (Cheung, 1984; Pihan *et al.*, 1986; Guth & Leung, 1987). Enhanced mucosal blood flow leads to enhanced disposal of acid entering the tissue (Cheung, 1984; Guth & Leung, 1987), and the apparent elimination of acid caused by capsaicin may conceivably be a consequence of facilitated mucosal blood flow. Vasodilatation and a rise in mucosal blood flow may also counteract the circulatory stasis in submucosal blood vessels, which for example is a characteristic of ethanol injury (Pihan *et al.*, 1986), and thus explain the protective effect of intragastric capsaicin against ethanol-induced haemorrhagic lesions (Holzer & Lippe, 1988).

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Inhibition of noradrenaline release by ω-conotoxin GVIA in the rat tail artery

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1 The perivascular nerves of isolated tail arteries from Wistar rats were stimulated with field pulses (1 Hz, 2 pulses, every 2 min). ω-Conotoxin 10 nmol l^{-1} depressed neurogenically mediated contractions, but did not influence the contractions to noradrenaline 0.1–0.3 μmol l^{-1}.

2 The inhibitory effect of ω-conotoxin was concentration-dependent (IC_{50} = 3.8 nmol l^{-1}). It did not reach a steady-state during 30 min incubation and could not be reversed upon subsequent washout for another 60 min.

3 A gradual increase in the Ca^{2+} concentration of the medium from 1.25 mmol l^{-1} to 10 mmol l^{-1} enhanced vasoconstriction and attenuated the action of ω-conotoxin 10 nmol l^{-1}. When a low stimulation intensity (120 mA) was used at high external Ca^{2+} (10 mmol l^{-1}), similar contractile responses were obtained as under normal conditions (200 mA current, 2.5 mmol l^{-1} Ca^{2+}). However, the inverse relationship between the effect of the toxin and external Ca^{2+} remained unchanged.

4 The time-course and degree of the inhibition by ω-conotoxin 3 nmol l^{-1} was identical in tail arteries of spontaneously hypertensive rats (SHR) and their normotensive controls (WKY).

5 When tail arteries of Wistar rats were preincubated with [^{3}H]-noradrenaline, field stimulation (0.4 Hz, 24 pulses, every 16 min) evoked tritium overflow and vasoconstriction. ω-Conotoxin 30 nmol l^{-1} inhibited both responses to a similar extent.

6 Our results suggest that ω-conotoxin selectively blocks Ca^{2+} channels in the terminals of perivascular nerves and thereby reduces the release, but not the contractile effect of the sympathetic transmitter.

Introduction

An action potential initiated in the cell body of noradrenergic neurones passes along the axon and actively or passively invades the varicosity (Haefely, 1972). The temporary depolarization of the varicosity membrane opens voltage-sensitive Ca^{2+} channels (VSCCs) and leads to an enhanced influx of the ion, which subsequently triggers transmitter release (Blaustein, 1979). Based on electrophysiological evidence three types of VSCCs have been identified in the somata of sensory neurones, namely T, L and N (Nowycky *et al.*, 1985; Fox *et al.*, 1987; McCleskey *et al.*, 1987; Hofmann *et al.*, 1987; Miller, 1987). All VSCCs could be blocked by some divalent cations, whereas organic Ca^{2+} agonists and antagonists with a dihyropyridine structure affected only the L-channel. A 27 amino acid peptide, ω-conotoxin GVIA (ω-conotoxin: Olivera *et al.*, 1984; 1985) inhibited L- and N-, but not T-currents. In sympathetic neurones the L- and N-type VSCCs were also sensitive to ω-conotoxin; the T-channel seemed to be absent (McCleskey *et al.*, 1987; Miller, 1987).

The L-VSCCs at the terminals of postganglionic sympathetic nerves may have only a minor significance (Godfraind *et al.*, 1986; Miller, 1987). This was suggested among others by results from experiments in which the effects of various organic ligands acting at the VSCC were investigated on [^{3}H]-noradrenaline release from sympathetically innervated smooth muscle and heart preparations. The nerve terminals were stimulated both electrically and by raised potassium. The dihydropyridine agonist Bay K 8644 slightly enhanced (rat vas deferens: Cena *et al.*, 1985) and the benzothiazepine antagonist diltiazem (rabbit pulmonary artery: Zelis *et al.*, 1985) inhibited the

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release of [^{3}H]-noradrenaline, but the required concentrations were in both cases rather high. Finally, the phenylalkylamine antagonists verapamil and D 600 (methoxyverapamil) reduced transmitter overflow from the heart (Göthert *et al.*, 1979), but not from the aorta of rabbits (Karaki *et al.*, 1984).

In contrast to L-VSCCs, ω-conotoxin-sensitive N-channels may be abundant, at least at some nerve terminals. Acetylcholine release at a skeletal neuromuscular junction of the frog was irreversibly blocked by the toxin (cutaneous pectoris nerve-muscle: Kerr & Yoshikami, 1984), while there was no effect at a similar junction of the mouse (phrenic nerve-diaphragm: Sano *et al.*, 1987). Furthermore, the outflow of diverse tritiated transmitters, including acetylcholine and noradrenaline from electrically stimulated hippocampal slices of the rabbit brain was also inhibited by ω-conotoxin (Dooley *et al.*, 1987; 1988). It should be mentioned, in addition, that in the above experiments, dihydropyridines have only a slight effect on acetylcholine release from motoneurone terminals (Sano *et al.*, 1987) and were completely inactive on transmitter outflow from the endings of central nordrenergic neurones (Dooley *et al.*, 1988).

The aim of the present study was to investigate the effect of ω-conotoxin on neuroeffector transmission in a sympathetically innervated vascular preparation, the rat tail artery. The release of noradrenaline in this organ has been previously shown to be insensitive to blockade by verapamil (Zsoter *et al.*, 1984) or diltiazem (Hicks *et al.*, 1985).

Methods

Measurement of vasoconstriction

Male Wistar rats (200–250 g), or in some experiments 12 week old (200–250 g) male spontaneously hypertensive rats (SHR) and their normotensive Wistar Kyoto controls (WKY) were decapitated. The systolic blood pressure, as measured by the tail-cuff method, was higher than 180 mmHg in SHR and below 135 mmHg in WKY. The proximal parts (1.5–2.5 cm) of the ventral tail arteries were removed, cannulated at both ends and set up in an organ bath containing 4 ml of medium. The preparation and experimental procedure were similar to those previously described (Illes *et al.*, 1987; Bucher *et al.*, 1987). The arteries were perfused with medium by means of a roller pump (Abimed, Minipuls 2); the perfusion rate was gradually increased from zero to 2.1 ml min^{-1} during the first 5 min after suspension of the tissue in the bath, and was kept constant thereafter. The inflow perfusion pressure was determined with a Statham P23Db transducer and recorded on a Watanabe WTR 281 pen recorder. Changes in perfusion pressure reflected changes in the resistance to flow, i.e. the degree of vasoconstriction. In most experiments both the incubation and perfusion medium consisted of (mmol l^{-1}): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 0.9, $NaHCO_3$ 25 and glucose 11. It was bubbled with 5% CO_2: 95% O_2 and maintained at 37°C. When the concentration of external Ca^{2+} was varied, a modified medium (NaCl 133, KCl 5.7, $NaHCO_3$ 10 and glucose 11 mmol l^{-1}, plus $CaCl_2$ 1.25, 2.5, 5 or 10 mmol l^{-1}) was used in order to prevent precipitation of the ion. With noradrenaline as the contractile agent, the medium also contained ascorbic acid 300 μmol l^{-1} and Na_2EDTA 30 μmol l^{-1}. The arteries were allowed to equilibrate for 1 h before an experiment was started. ω-Conotoxin (4–12 μl) and noradrenaline (40–120 μl) were added to the bathing medium.

The periarterial nerves were stimulated via platinum electrodes located at the top and the bottom of the organ bath. The current source was a Hugo Sachs stimulator I, which delivered pulses of 0.3 ms duration at 1 Hz every 2 min. The number of pulses was usually 2; it was, however, 5 at Ca^{2+} 1.25 mmol l^{-1}. The intensity of stimulation was, with the exception of a single series of experiments (Ca^{2+} 10 mmol l^{-1}, 120 mA) always supramaximal (200 mA). From the beginning of stimulation throughout the experiment the bathing medium was exchanged every 10 min.

After about 30 min of stimulation the vasoconstriction became stable. Then a further 10 min elapsed before ω-conotoxin was added for 30 min. As already mentioned, it was washed out and immediately reapplied at 10 min intervals. Thus, a major enzymatic degradation of the toxin is unlikely. Since a prolonged reduction of the contractile responses was observed even after a washout of ω-conotoxin, the effect of a single concentration of the substance was measured in each artery. The inhibitory action of the toxin gradually increased with time; in order to construct concentration-response curves, the effects were measured both 10 and 30 min after application. Depression of vasoconstriction was expressed as a percentage of the last response to stimulation before the addition of the respective peptide concentration. Concentrations which inhibited vasoconstriction by 50% (IC_{50} values) were graphically estimated. When the effect of external Ca^{2+} on the action of the toxin was tested, only a single concentration (10 nmol l^{-1}) of the peptide was applied, and its effect was evaluated after 4 or 10 min. Eventually, the depression produced by ω-conotoxin 3 nmol l^{-1} was compared in tail arteries of SHR and WKY.

In a few preparations, after an initial 30 min of electrical stimulation, every fifth train of impulses

was substituted by the addition of noradrenaline 0.1–0.3 μmol^{-1}. The amine was injected into the bath by means of a Braun Repetita pump, and was washed out 30 s later. Drug concentrations were chosen so as to match the nerve stimulation-evoked vasoconstriction. At least three stable responses to noradrenaline were obtained before ω-conotoxin 10 nmol l^{-1} was applied. The peptide was present for 30 min in the bath; it was washed out together with noradrenaline each time, but was subsequently readministered.

Most of the results are presented in such a manner that the last response to electrical stimulation or noradrenaline, before exposure of the preparation to ω-conotoxin, was termed 100%. Therefore, differences in the height of vasoconstriction before drug application could be eliminated and only percentage changes were considered.

Measurement of [^{3}H]-noradrenaline overflow and vasoconstriction

Rat tail arteries were cannulated at one end and preincubated at 37°C for 1 h in 1.5 ml of medium of the same composition as used in the previous experiments. This medium additionally contained ascorbic acid 300 μmol l^{-1}, Na_2EDTA 30 μmol l^{-1} and (−)-[^{3}H]-noradrenaline 2.2 μmol l^{-1} (specific activity 3.7 Ci mmol^{-1}). Then, the arteries were set up vertically between two platinum electrodes and perfused at 2.1 ml min^{-1} in such a way that the perfusate after having passed the lumen superfused the adventitial surface. The medium was prewarmed to 37°C and contained ascorbic acid 300 μmol l^{-1}, Na_2EDTA 30 μmol l^{-1} and cocaine 10 μmol l^{-1}, but not [^{3}H]-noradrenaline. Perfusion pressure was recorded as described.

Each artery was exposed to 6 stimulation periods (24 pulses at 0.4 Hz, 0.3 ms, 200 mA). The first stimulation period was after 96 min of perfusion and further ones followed at intervals of 16 min. Collection of the perfusate/superfusate started after 126 min of perfusion in 1 min, 6 min or 9 min samples (see Figure 6). The stimulation period beginning at 128 min is termed S_1 and subsequent periods S_2–S_4. ω-Conotoxin 30 nmol l^{-1} or its solvent was infused by a Braun injection pump into the perfusion stream from 10 min before S_3 for 15 min. The rate of infusion was 56 μl min^{-1}. At the end of the experiments the arteries were solubilized in 1 ml Soluene 100 (Packard Instruments, Frankfurt am Main, FRG). Tritium was determined by liquid scintillation spectrometry and quenching was measured by addition of known amounts of [^{3}H]-water to representative samples.

The outflow of tritium was calculated as fraction of the amount of tritium at the onset of the respective collection period (fractional rate of outflow). For evaluation of stimulation-evoked tritium overflow, the difference was calculated between the overall tritium outflow during stimulation plus the following 3 min and the estimated basal outflow. The latter was assumed to change linearly from the 1 min interval before the beginning of stimulation to the fourth collection period after the end of stimulation. The difference was expressed as percentage of the amount of tritium in the tissue at the onset of the respective stimulation period.

The effect of ω-conotoxin on evoked release was evaluated by calculating the ratio of the overflow evoked by S_3 (in the presence of the peptide) and S_2 (before peptide application). Possible influences on the basal release were checked by calculating the ratio (b_3/b_2) of the fractional rates of outflow from the last samples before the respective stimulation periods. Similarly, the S_4/S_2 and b_4/b_2 values were also determined in order to document whether a recovery after washout of the toxin occurs.

Materials

The drugs used were: ω-conotoxin GVIA (Peninsula, St. Helens, U.K.); (−)-noradrenaline hydrochloride (Hoechst, Frankfurt am Main, F.R.G.); cocaine hydrochloride (Merck, Darmstadt, F.R.G.). (−)-[Ring-2,5,6^{3}H]-noradrenaline, specific activity 37 Ci mmol^{-1} (New England Nuclear, Dreieich, F.R.G.) was diluted with unlabelled (−)-noradrenaline hydrochloride to obtain a specific activity of 3.7 Ci mmol^{-1}.

Stock solutions of ω-conotoxin were prepared with distilled water as 30 or 500 μl portions (10 μmol l^{-1}) and stored at −20°C until use. Further dilutions were made in medium. (−)-Noradrenaline hydrochloride was dissolved before use in medium containing ascorbic acid 300 μmol l^{-1} and Na_2EDTA 30 μmol l^{-1}; it was kept on ice during the experiment to prevent oxidation. The quantities of the solvent used had no effects of their own.

Results

Measurement of vasoconstriction

Effects of ω-conotoxin on electrically- and noradrenaline-induced vasoconstriction As in previous experiments, the average perfusion pressure of tail arteries taken from Wistar rats (Illes *et al.*, 1987) or from SHR and WKY (Bucher *et al.*, 1987) at the end of the 1 h equilibration period was about 20 mmHg. Electrical field stimulation with 2 pulses at 1 Hz produced vasoconstriction (Figure 1). Noradrenaline 0.1–0.3 μmol l^{-1} evoked responses of

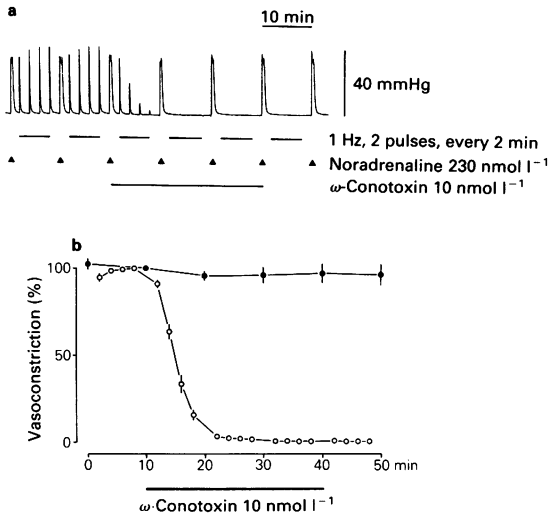


Figure 1 Inhibition by ω-conotoxin of contractile responses to electrical stimulation, but not to bath applied noradrenaline. Isolated tail arteries of rats were perfused at a constant rate and changes in perfusion pressure were taken as a measure of vasoconstriction. Noradrenaline was added in concentrations (0.1–0.3 μmol l^{-1}) that matched nerve stimulation-induced responses (1 Hz, 2 pulses, every 2 min, 0.3 ms, 200 mA). (a) Representative tracing. The contractile stimuli were applied as indicated by the upper horizontal bars (electrical pulses) and ▲ (noradrenaline). ω-Conotoxin 10 nmol l^{-1} was present for the period designated by the lower horizontal bar. (b) Depicts the mean results from 7 experiments similar to that shown in (a); vertical lines indicate s.e. A 10 min period preceding the contact with ω-conotoxin is displayed. The last vasoconstriction evoked by nerve stimulation (○: 53.0 ± 5.0 mmHg) or noradrenaline (●: 50.8 ± 6.3 mmHg) before the addition of ω-conotoxin was termed 100%. Thereby percentage changes of these responses could be evaluated.

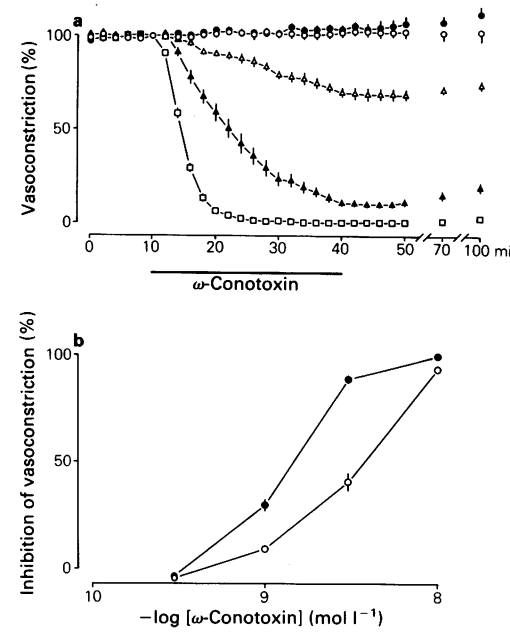


Figure 2 Inhibitory action of ω-conotoxin on electrically-evoked vasoconstriction. Stimulation was by 2 pulses at 1 Hz, every 2 min (0.3 ms, 200 mA). (a) Time-course and concentration-dependence of the effect of the toxin. Six stable responses preceding the contact with ω-conotoxin are shown. ω-Conotoxin or its solvent was present for the period indicated by the horizontal bar. The last vasoconstriction before the addition of the solvent (○: 48.3 ± 8.3 mmHg) or of ω-conotoxin 0.3 nmol l^{-1} (●: 43.1 ± 3.5 mmHg), 1 nmol l^{-1} (△: 65.2 ± 5.8 mmHg), 3 nmol l^{-1} (▲: 43.5 ± 6.5 mmHg) and 10 nmol l^{-1} (□: 53.9 ± 7.1 mmHg) was termed 100%. (b) Concentration-response curves of ω-conotoxin 10 min (○) and 30 min (●) after application. Ordinate scale: percentage inhibition of vasoconstriction; abscissa scale: logarithm of toxin concentration. Data are replotted from (a). In (a) and (b) each point represents the mean of 6 experiments; vertical lines indicate s.e.

similar amplitude. ω-Conotoxin 10 nmol l^{-1} depressed only the effect of nerve stimulation, but not that of the exogenously applied noradrenaline. The inhibition was maximal within 10–15 min and remained constant over an additional 20 min of incubation. When the toxin was washed out thereafter, no recovery occurred within 10 min.

The degree of the depression caused by ω-conotoxin 10 nmol l^{-1} was similar with time, when the responses to electrical pulses were not interrupted by the addition of noradrenaline (Figure 2a). Lower concentrations (1 and 3 nmol l^{-1}) were less active; their effects increased with time of incubation. ω-Conotoxin 0.3 nmol l^{-1} did not change vasoconstriction. When stimulation was performed in the absence of the peptide, the responses were stable for 100 min, i.e. over the duration of the whole experiment. The maximal inhibition reached at the end of the 30 min incubation period persisted, in spite of repeated washout for an additional hour. The concentration-response curve of ω-conotoxin is shown in Figure 2b; its position along the abscissa scale differed according to the contact time. The IC_{50} value was 3.8 nmol l^{-1} after 10 min and 1.5 nmol l^{-1} after 30 min (the average of 6 experiments).

Influence of external Ca^{2+} on the effect of ω-conotoxin A rise in the Ca^{2+} concentration of the medium from 2.5 to 10 mmol l^{-1} almost doubled the

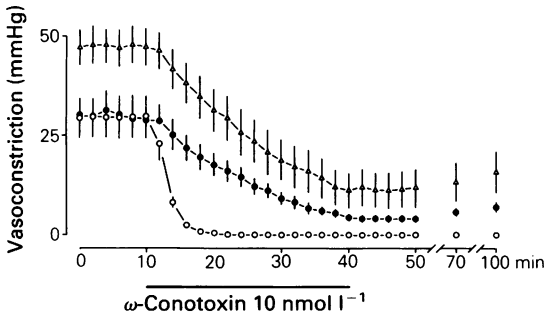


Figure 3 Effect of changes in the Ca^{2+} concentration on the action of ω-conotoxin. Stimulation was by 2 pulses of 0.3 ms duration, at 1 Hz, every 2 min. In contrast to previous and subsequent experiments, vasoconstriction is plotted in absolute values. ω-Conotoxin 10 nmol l^{-1} was present for the period indicated by the horizontal bar. When the stimulation current was kept at 200 mA, and the normal Ca^{2+} concentration of 2.5 mmol l^{-1} (○) was raised to 10 mmol l^{-1} (△), the responses were markedly enhanced. In order to obtain comparable vasoconstriction under both conditions, the stimulation current was decreased at Ca^{2+} 10 mmol l^{-1} to 120 mA (●). Means of 8 (○, ●) or 6 (△) experiments are shown; vertical lines indicate s.e.

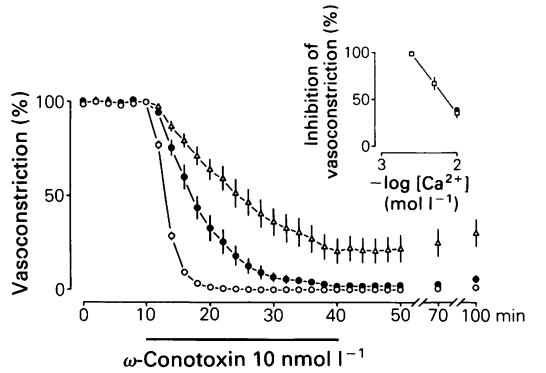


Figure 4 Effect of a gradual increase in the external Ca^{2+} concentration on the action of ω-conotoxin. Stimulation was by 2 pulses at 1 Hz, every 2 min (0.3 ms, 200 mA). ω-Conotoxin 10 nmol l^{-1} was present for the period indicated by the horizontal bar. The last vasoconstriction before the addition of ω-conotoxin at Ca^{2+} 2.5 mmol l^{-1} (○: 29.9 ± 5.3 mmHg), 5 mmol l^{-1} (●: 36.3 ± 8.5 mmHg) or 10 mmol l^{-1} (△: 47.5 ± 4.6 mmHg) was termed 100%. Means of 8 (○) or 6 (●, △) experiments are shown; vertical lines indicate s.e. Inset: percentage inhibition by ω-conotoxin 10 nmol l^{-1} after a 10 min contact time; the stimulation current was 200 mA (□). The effect of the toxin did not change at Ca^{2+} 10 mmol l^{-1}, when the current was decreased to 120 mA (■; replotted from Figure 3).

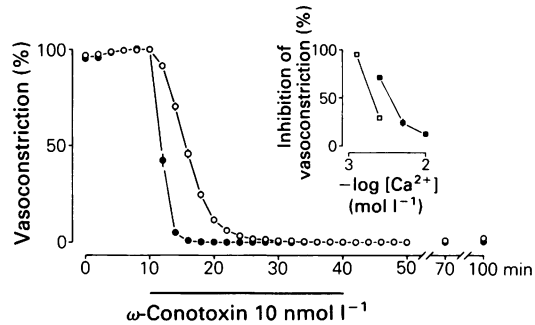


Figure 5 Effect of a decrease in the external Ca^{2+} concentration on the action of ω-conotoxin. Stimulation was by 5 pulses at 1 Hz, every 2 min (0.3 ms, 200 mA). Since the halving of the normal Ca^{2+} concentration of 2.5 mmol l^{-1} markedly reduced vasoconstriction, the previously used number of pulses was increased from 2 to 5. ω-Conotoxin 10 nmol l^{-1} was present for the period indicated by the horizontal bar. The last vasoconstriction before toxin application at Ca^{2+} 2.5 mmol l^{-1} (○: 97.0 ± 8.3 mmHg), or 1.25 mmol l^{-1} (●: 33.0 ± 4.4 mmHg) was termed 100%. Means of 6 experiments are shown at both Ca^{2+} concentrations; vertical lines indicate s.e. Inset: percentage inhibition by ω-conotoxin 10 nmol l^{-1} after a 4 min contact time; the number of pulses was 5 (□). The inverse relationship between the external Ca^{2+} concentration and the effect of the toxin was apparent also when the number of pulses was only 2 (■; replotted from Figure 4). However, in this case the inhibitory potency at Ca^{2+} 2.5 mmol l^{-1} decreased.

height of the vasoconstriction (Figure 3), and also markedly counteracted the effect of ω-conotoxin. When the stimulation current was decreased from 200 to 120 mA, in order to obtain comparable responses at elevated and normal Ca^{2+} concentrations, the attenuation of the effect was still prominent. Indeed, in a medium containing Ca^{2+} 10 mmol l^{-1}, the percentage inhibition of vasoconstriction by ω-conotoxin was identical, irrespective of the stimulation strength used (inset in Figure 4). Therefore, in the following experiments impulses of 200 mA amplitude were applied, and the contractions were normalized with respect to the last response before peptide application. The effect of ω-conotoxin depended inversely on the external Ca^{2+} concentration; a gradual rise in the Ca^{2+} concentration from 2.5 to 5 and then 10 mmol l^{-1} produced increasingly more antagonism (Figure 4). On the other hand a halving of the Ca^{2+} content of 2.5 mmol l^{-1} in a normal medium enhanced the inhibition by the toxin (Figure 5). Since at Ca^{2+} 1.25 mmol l^{-1} the previously used 2 pulses were not sufficient to evoke a reliably measurable vasoconstriction, we increased the number of pulses to 5. At

Ca^{2+} 2.5 mmol l^{-1} the responses grew with the length of the train, but their sensitivity to ω-conotoxin became less (inset in Figure 5). The inverse relationship between external Ca^{2+} and the effect of ω-conotoxin could still be demonstrated for a wide range of Ca^{2+} concentrations between 1.25 and 10 mmol l^{-1}.

Effect of ω-conotoxin on SHR and WKY tail arteries The time course and degree of the inhibition by ω-conotoxin 3 nmol l^{-1} of the vasoconstriction evoked by 2 pulses in SHR and WKY arteries were indentical, and also similar to those found in preparations obtained from Wistar rats (data not shown).

Measurement of tritium overflow and vasoconstriction The transmitter pools of rat tail arteries were loaded with [^{3}H]-noradrenaline and tritium overflow was induced by 24 field pulses at 0.4 Hz. The overflow was stable when stimulation periods (S_1 and S_2) were repeated with an interval of 16 min (Figure 6). In preliminary experiments the subsequent contact with ω-conotoxin 10 nmol l^{-1} for 10 min produced only moderate inhibition of the response to S_3; a higher concentration (30 nmol l^{-1}) of the toxin almost abolished it (Figure 6). Although the peptide was no longer present in the medium from 11 min before S_4, its effect still persisted. The basal tritium outflow seemed to be, during toxin application, somewhat lower compared with the preceding and subsequent values. However, these changes were not significant (Table 1).

In order to evaluate statistically the effect of ω-conotoxin on tritium outflow, the S_3/S_2 and b_3/b_2 ratios were calculated (Table 1). The additional S_4/S_2 and b_4/b_2 values give information about a possible recovery after washout. In the absence of the peptide, both ratios indicated stable responses to stimulation,

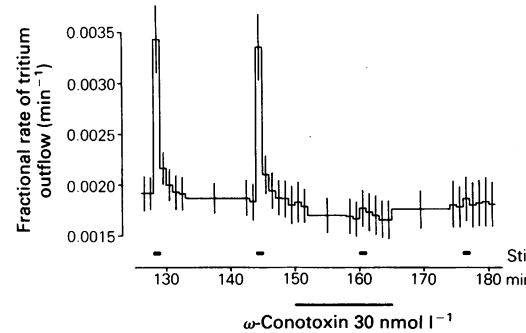


Figure 6 Effect of ω-conotoxin on tritium overflow from tail arteries preincubated with [^{3}H]-noradrenaline, and subsequently perfused/superfused with [^{3}H]-noradrenaline-free medium containing cocaine 10 μmol l^{-1}. Four periods of field stimulation (Stim: 24 pulses at 0.4 Hz, 0.3 ms, 200 mA) were delivered at intervals of 16 min. ω-Conotoxin 30 nmol l^{-1} was present for the period indicated by the horizontal bar. Means of 7 experiments are shown; vertical lines indicate s.e.

and a continuous fall in basal outflow with time. ω-Conotoxin 30 nmol l^{-1} markedly depressed the evoked overflow, without changing the basal values. The inhibition was not reversed in a toxin-free medium. Effects on vasoconstriction paralleled those on tritium overflow.

Discussion

The main finding of this paper is that ω-conotoxin inhibits transmitter release from the terminals of postganglionic sympathetic nerves in the rat tail artery. This was shown both by an indirect and a direct approach. Firstly, the toxin depressed the

Table 1 Effect of ω-conotoxin on basal tritium outflow, as well as on stimulation-induced ^{3}H overflow and vasoconstriction in the rat tail artery

Treatment	n	*^{3}H outflow*		*^{3}H overflow*		*Vasoconstriction*	
		b_3/b_2	b_4/b_2	S_3/S_2	S_4/S_2	S_3/S_2	S_4/S_2
None	7	0.97 ± 0.02	0.97 ± 0.05	1.01 ± 0.07	1.07 ± 0.11	1.01 ± 0.03	0.97 ± 0.05
ω-Conotoxin 30 nmol l^{-1}	7	0.91 ± 0.04	0.98 ± 0.04	0.15 ± 0.04*	0.11 ± 0.03*	0.11 ± 0.04*	0.09 ± 0.03*

Rat tail arteries were preincubated with [^{3}H]-noradrenaline and subsequently perfused/superfused with a [^{3}H]-noradrenaline-free medium containing cocaine 10 μmol l^{-1}. Four periods of field stimulation (S_1–S_4; 24 pulses at 0.4 Hz, 0.3 ms, 200 mA) were delivered at intervals of 16 min. ω-Conotoxin was added 10 min before S_3 for 15 min. Its effect was evaluated by calculating the ratio of overflow or vasoconstriction evoked by S_3 and S_2 (S_3/S_2). Effects on the basal release were checked by calculating the ratio of the fractional rate of outflow from the last samples before the respective stimulation periods (b_3/b_2). The S_4/S_2 values show that no recovery occurs after washout of the toxin. At S_2 the overflow amounted to 0.21 ± 0.02% of tissue tritium; the vasoconstriction was 120.3 ± 10.6 mmHg (n = 14 each). The b_2 value equalled 0.17 ± 0.02% min^{-1} (n = 14).

* $P < 0.001$; Student's t test. Statistical comparison was with the respective ratios in the absence of ω-conotoxin.

vasoconstriction evoked by nerve stimulation, but did not change the contractile effect of exogenous noradrenaline. In a number of blood vessels (dog mesenteric artery: Muramatsu, 1986; rabbit ileocolic artery: von Kügelgen & Starke, 1985; rabbit ear artery: Kennedy *et al.*, 1986; rabbit saphenous artery: Burnstock & Warland, 1987) including the tail artery of SHR (Vidal *et al.*, 1986) the neuroeffector transmitter is both noradrenaline and ATP. However, in tail arteries of Sprague-Dawley (Medgett & Langer, 1984) and Wistar rats (Brod, I., Rickman, H. & Illes, P., unpublished) electrically-induced contractions are solely due to the effect of endogenous noradrenaline. Thus, the abolition of the mechanical responses to nerve stimulation by ω-conotoxin 10 nmol l^{-1} indicates a comparable effect on the release of the catecholamine. Secondly, the presynaptic inhibitory effect of ω-conotoxin could be unequivocally proven by the measurement of tritium overflow from preparations preincubated with [^{3}H]-noradrenaline. Stimulation conditions in this series of experiments (24 pulses, 0.4 Hz) were different from those in previous ones (2 or 5 pulses, 1 Hz). At a frequency of 1 Hz, the increase in the number of pulses led to a decrease in the potency of ω-conotoxin. A further enhancement of the train length, despite the fact that the stimulation frequency was approximately halved, forced us to use a higher concentration of the toxin (30 nmol l^{-1} instead of 10 nmol l^{-1}) in order to obtain a complete inhibition. A similar inverse relationship between the duration of electrical stimulation and the activity of presynaptic modulators restricting transmitter release was repeatedly demonstrated (Vizi, 1979; Illes, 1986). A possible mode of action of these substances is a receptor-mediated blockade of Ca^{2+} entry via VSCCs; the reaction chain may utilize second messenger systems and/or guanine nucleotide binding proteins (Illes, 1986; Dunlap *et al.*, 1987).

Electrophysiological evidence indicates that ω-conotoxin reduces the entry of Ca^{2+} into the cell bodies of sympathetic neurones (Mochida & Kobayashi, 1986; McCleskey *et al.*, 1987; Miller, 1987). A similar effect on the nerve terminals may underlie the inhibition of transmitter release. In synaptosomes of rat brain, e.g., which are not accessible to electrophysiological methods, the toxin reduced the early phase of potassium-induced $^{45}Ca^{2+}$ uptake; this is generally attributed to an action on VSCCs (Reynolds *et al.*, 1986; Rivier *et al.*, 1987). The overflow of [^{3}H]-noradrenaline initiated by the depolarizing stimulus was also depressed (Reynolds *et al.*, 1986). Finally, selective binding of radioiodinated ω-conotoxin has been demonstrated in synaptic plasma membranes of various species (Abe *et al.*, 1986; Cruz & Olivera, 1986; Knaus *et al.*, 1987). The binding was not affected by organic Ca^{2+} agonists or antagonists, whereas divalent cations were potent inhibitors (Abe *et al.*, 1986; Knaus *et al.*, 1987), with a similar rank order of potency as they interfere with the Ca^{2+} channel involved in transmitter release from the brain (Nachshen, 1984). Thus, the presence of a neuronal site for ω-conotoxin, which is probably identical with the α-subunit of the channel protein (Cruz *et al.*, 1987) has been suggested. It is certainly different from the dihydropyridine site (Abe *et al.*, 1986; Cruz & Olivera, 1986; Cruz *et al.*, 1987) which does not seem to be situated at the axon terminals (Miller, 1987) and therefore may not influence transmitter release (Miller & Freedman, 1984; Godfraind *et al.*, 1986).

In good agreement with the above findings, we demonstrated that the inhibition of noradrenaline release by ω-conotoxin depends on the Ca^{2+} concentration of the medium. Apparently an increase of external Ca^{2+} enhances the driving force for the entry of the ion via the VSCC and thereby counteracts the effect of the toxin. A similar relationship between the concentration of Ca^{2+} in the perfusion fluid and the potency of ω-conotoxin at depressing [^{3}H]-noradrenaline overflow from rabbit hippocampal slices was obtained previously (Dooley *et al.*, 1987). It is interesting to note that the IC_{50} value of the peptide after 30 min incubation was, despite the different stimulation parameters used, almost identical in this study and our experiments. Finally, it seems to be a general phenomenon that the inhibitory effect of presynaptic modulators, which is supposed to be due to decreased availability of Ca^{2+} for the release process (see above), can be reversed by increasing the external Ca^{2+} concentration. Such modulators are, for example, in sympathetically innervated smooth muscle and heart preparations, muscarinic agonists (Dubey *et al.*, 1975), α_2-adrenoceptor agonists (Drew, 1978), adenosine (Wakade & Wakade, 1978) and opioids (Illes *et al.*, 1980).

In tail arteries of SHR and WKY, ω-conotoxin had the same potency on neurogenic vasoconstriction. It has been shown recently that some prejunctional inhibitory receptors (e.g. α_2-adrenoceptors: Galloway & Westfall, 1982; Westfall & Meldrum, 1985; A_1-adenosine-receptors: I. Brod, H. Rickmann, & P. Illes, unpublished) situated at the terminals of postganglionic sympathetic nerves innervating the vessels of hypertensive rats, become subsensitive to the effects of the respective endogenous agonists. However, no similar change occurred in the channel constituent which binds ω-conotoxin.

The last finding which requires an explanation is that the toxin did not affect the vasoconstriction evoked by exogenous noradrenaline. Receptor-operated Ca^{2+} channels are generally less sensitive to organic Ca^{2+} antagonists than VSCCs (Bülbring

& Tomita, 1987). Thus, in most cases these substances inhibit strongly the responses to high potassium, but only slightly those to catecholamines. The noradrenaline-induced contraction is mainly due to the release of intracellular Ca^{2+} from a pool which seems to be refilled directly through leak channels (Bou *et al.*, 1983). However, it should be noted that the susceptibility of vasoconstriction to a decrease in extracellular Ca^{2+} concentration or to channel blockade varies significantly according to the vascular muscle used and the subtype of α-adrenoceptor activated (Bou *et al.*, 1983; McGrath, 1983). The overall impression in the literature is that the effect of α_2- but not α_1-agonists depends on external Ca^{2+}. In tail arteries of Sprague-Dawley (Medgett & Langer, 1984) and spontaneously hypertensive rats (Medgett *et al.*, 1984) both subtypes of receptor are present. As expected, diltiazem reduced the contractile response to the mixed agonist noradrenaline in preparations from both strains (Medgett & Rajanayam, 1984; Hicks *et al.*, 1985). Since a similar adrenoceptor population is present in tail arteries of Wistar rats (I. Brod, H. Rickmann, & P. Illes, unpublished), organic Ca^{2+} blockers should counteract the catecholamine effect. Thus, in the present experiments, the failure of ω-conotoxin to affect vasoconstriction induced by noradrenaline, excludes an inhibition of VSCCs in this tissue. Previous experiments in the rat aorta demonstrated that the toxin has no effect on the potassium- and noradrenaline-induced contractions (Weinheimer, G. & Osswald, H., unpublished) or the calcium-dependent endothelium-mediated relaxation (Weinheimer *et al.*, 1988). All these findings are in accordance with the inability of the toxin to block L-VSCCs in skeletal, cardiac or smooth muscle (Hofmann *et al.*, 1987; McCleskey *et al.*, 1987).

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Role of endoperoxides in arachidonic acid-induced vasoconstriction in the isolated perfused kidney of the rat

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1 Administration of arachidonic acid caused dose-dependent vasoconstriction in the isolated rat kidney perfused *in situ* with Krebs-Henseleit solution.

2 Inhibition of cyclo-oxygenase with indomethacin or meclofenamate reduced the renal vasoconstrictor effect of arachidonic acid.

3 The renal vasoconstrictor effect of arachidonic acid was unaffected by CGS-13080 at concentrations that effectively reduced thromboxane A_2 (TxA_2) synthesis by platelets and the kidney.

4 The endoperoxide/TxA_2 receptor antagonist, SQ 29,548, abolished the renal vasoconstrictor effect of arachidonic acid and of U46619, an endoperoxide analogue. In contrast, SQ 29,548 did not affect the renal vasoconstrictor response to angiotensin II, prostaglandin E_2 or $F_{2\alpha}$.

5 These data suggest that the vasoconstrictor effect of arachidonic acid in the isolated kidney of the rat is mediated by its metabolites, including the prostaglandin endoperoxides.

Introduction

Arachidonic acid (AA), the precursor of prostaglandins, thromboxane and leukotrienes, elicits renal vasodilatation or renal vasoconstriction depending on the species. For example, the administration of AA produces vasodilatation in the dog (Chang *et al.*, 1975) and rabbit (Larson & Angaard, 1974) kidney and vasoconstriction in the rat kidney (Gerber & Nies, 1979). The renal vasodilator effect of AA in dogs and rabbits requires metabolism of the fatty acid by cyclo-oxygenase to vasodilator mediators, presumably prostaglandin E_2 (PGE_2) and PGI_2 (Larsson & Angaard, 1974; Chang *et al.*, 1975). The renal vasoconstrictor effect of AA in the rat also requires metabolism of the fatty acid by cyclo-oxygenase to a vasoconstrictor mediator(s) (Sakr & Dunham, 1982).

In the rat kidney autoperfused with blood, the renal vasoconstrictor effect of AA is incompletely inhibited by OKY-1581, an inhibitor of thromboxane synthetase, suggesting that thromboxane A_2 (TxA_2) of platelet or renal origin mediates, in part, the vascular effect of arachidonic acid in the rat kidney (Sakr & Dunham, 1982). The mediator(s) of AA-induced renal vasoconstriction which is not subject to attenuation by inhibitors of thromboxane synthetase is yet to be identified.

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The prostaglandin endoperoxides, PGG_2 and PGH_2, common precursors of prostaglandins and TxA_2, stimulate contraction of isolated vascular smooth muscle via interaction with TxA_2 receptors (Coleman *et al.*, 1981; Saussy *et al.*, 1985). Hence the possibility arises that the prostaglandin endoperoxides contribute to the renal vasoconstrictor effect of AA in the rat. To test this hypothesis we examined the vasoconstrictor effect of AA in the rat kidney perfused *in situ* with Krebs-Henseleit buffer, in the presence and the absence of cyclo-oxygenase inhibitors, thromboxane synthesis inhibitors or antagonists of TxA_2/PGH_2 receptors. If prostaglandin endoperoxides interacting with the TxA_2 receptor contribute to AA-induced vasoconstriction, treatment with blockers of TxA_2/PGH_2 receptors should be more effective than inhibitors of thromboxane synthetase in attenuating the vasoconstrictor effect of arachidonic acid.

Methods

Male Wistar rats (Charles River Labs, Wilmington, MA), 8–10 weeks of age (body weight 260–340 g) were used for these studies.

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Isolated perfused kidney in situ

Following pentobarbitone anaesthesia (60 mg kg^{-1}, i.p.) the right kidney was exposed by midline laparotomy and the mesenteric and right renal arteries cleared of surrounding tissue. Ties were loosely placed around these vessels and the vena cava just above and below the junction with the right renal vein. The right renal artery was then cannulated with a 19 gauge needle via the mesenteric artery to avoid interruption of blood flow. The vena cava was then occluded and cut for exit of the perfusate. The right ureter was also cut. The animal was then killed by an intracardiac injection of 10 mg pentobarbitone. The kidney was perfused by means of a Watson-Marlow pump (Model 5025) with Krebs-Henseleit buffer gassed with 95% O_2:5% CO_2 at 37°C, and the flow adjusted to approximately 13 ml min^{-1} to obtain a basal perfusion pressure of approximately 100–110 mmHg. The perfusion pressure was measured with a Harvard pressure transducer and recorded on a Soltec 1246 recorder.

In those preparations used to establish the effective concentration of CGS-13080 for inhibition of renal thromboxane synthesis, the vena cava was cannulated for the collection of renal venous effluent for the subsequent measurement of immunoreactive TxB_2.

Examination of renal vascular responses to arachidonic acid

AA was administered as random bolus doses (1–100 μg) given into the perfusate line just proximal to the kidney. Each kidney was used for 1 or 2 doses of AA only, depending on whether a high or low dose was given first. For example, if the first injection of AA was at a dosage of 10 μg or greater, no subsequent injections of the fatty acid were made. This procedure was adopted because of the appearance of a tachyphylactic-type effect upon repeated administration of AA.

Effect of cyclo-oxygenase and thromboxane synthase inhibition on the renal vascular response to arachidonic acid

The renovascular response to injections of AA was studied in rat kidneys perfused with Krebs-Henseleit buffer containing indomethacin (1 μM) or meclofenamate (3 μM) to inhibit cyclo-oxygenase, $n = 5$ (Flower, 1974), CGS-13080, $n = 7$ (5 and 25 μM) or OKY-046, $n = 3$ (5 μM) to inhibit thromboxane synthase (McNab *et al.*, 1984; Garcia-Szabo *et al.*, 1984), or with an appropriate vehicle, $n = 10$. When studying the effects of CGS-13080, in addition to adding the drug to the renal perfusate, the rats were treated 2 h prior to perfusion of the kidney with CGS-13080 at 2 mg kg^{-1}, i.p.

Effect of thromboxane A_2/endoperoxide receptor antagonism on the arachidonic acid renovascular response

Rat kidneys were perfused with Krebs buffer containing either SQ 29,548 at a final concentration of 1 μM ($n = 6$) or vehicle ($n = 7$) and dose-response curves to AA (1–30 μg) were constructed as described previously. In the isolated perfused kidneys the effectiveness of receptor antagonism by SQ 29,548 was tested by use of 100 ng U46619, an endoperoxide analogue. This dose of U46619 corresponded to an ED_{40} obtained from dose-response curves constructed from preliminary experiments. In addition, the response to 10 ng angiotensin II was compared in kidneys treated with SQ 29,548 or vehicle. In six other experiments the renal vasoconstrictor responses to PGE_2 or $PGF_{2\alpha}$ were compared before and after SQ29548 was added to the perfusate.

In 5 other preparations another TxA_2/PGH_2 receptor antagonist was used (Stegmeier *et al.*, 1984). Thus, BM13.177, at a concentration (20 μM) that inhibited pressor responses to U46619 (100 ng), was included in the perfusate and the responses to 10 μg AA and 10 ng angiotensin II recorded and compared to responses obtained in vehicle-treated kidneys.

Statistical analyses

Individual points on the curves were compared by Student's t test for unpaired data. Comparison of responses to U46619 and angiotensin II as well as basal perfusion pressure and flow in different groups was also made using an unpaired t test. Comparison of responses to PGE_2 and $PGF_{2\alpha}$ in the same preparations before and after SQ 29,548 were compared by a paired t test. Results are expressed as mean ± standard error of the mean (s.e.mean). A P value < 0.05 was considered statistically significant.

Materials

Sodium arachidonate was obtained from NuChek, Elysian, Mn., U.S.A. and dissolved in distilled water to give a concentration of 1 mg ml^{-1} which was stored under nitrogen at −70°C. A fresh solution was thawed and used for each kidney preparation. CGS-13080 (imidazo[1,5-α]pyridine-5-hexanoic acid), a gift from Ciba-Geigy, Summit, NJ, U.S.A. was dissolved in a minimal volume of Na_2CO_3 (4%) and diluted with saline. Solutions were freshly made each day. Indomethacin was a gift from Merck, Sharpe and Dohme and was dissolved in 4% Na_2CO_3 and diluted in distilled water; sodium

meclofenamate was obtained from Parke-Davis and Co., Holland, MI, U.S.A. and dissolved immediately before use in distilled water. SQ 29,548 ([1S-[1α, 2β(5Z),3β,4α]]-7-[3-[[2-[(phenylamino)carbonyl]-hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid) was a gift from E.R. Squibb and Sons, Inc., Princeton, NJ, U.S.A. and was dissolved in 95% ethanol to give a 10 mg ml^{-1} solution which was diluted with 9 volumes of 2 mM Na_2CO_3. The resulting 1 mg ml^{-1} stock solution was divided into aliquots, sealed under nitrogen and stored at −70°C until use. U46619 (11,9 epoxy methano-prostaglandin H_2), a gift from the UpJohn Co., was initially dissolved in ethanol, 1 mg ml^{-1}, diluted in distilled water to 50 μg ml^{-1}, and stored frozen in aliquots at −70°C. OKY-046 (sodium-(E)-3-[4-(1-imidazolylmethyl)phenyl]-2-propenoate) was obtained from Ono Pharmaceuticals Ltd., Japan, dissolved in distilled water to yield a stock solution of 2 mg ml^{-1} which was stored in aliquots at −70°C. BM 13,177 (4-[2-benzenesulphonamido)-ethyl]-phenoxyacetic acid) was obtained from Boehringer Mannheim, F.R.G.

Results

Effect of cyclo-oxygenase and thromboxane synthetase inhibitors on arachidonic acid responses in rat kidneys

The flow rates in the control group, CGS-13080 group and indomethacin or meclofenamate group were 13.1 ± 0.8 ml min^{-1}, 13.5 ± 1.5 ml min^{-1} and 13.8 ± 1.3 ml min^{-1}, respectively, and the basal perfusion pressures were 117 ± 5 mmHg, 110 ± 7 mmHg and 101 ± 3 mmHg ($P < 0.05$ vs control), respectively.

Figure 1 shows the effect of AA on renal perfusion pressure in rat kidneys perfused with Krebs-Henseleit buffer only or buffer containing 5 μM CGS-13080. In kidneys perfused without the enzyme inhibitors, injections of arachidonic acid resulted in dose-dependent increases in perfusion pressure which is indicative of vasoconstriction. This response is specific for arachidonic acid as linolenic acid has no effect on renal perfusion pressure when injected in doses of up to 30 μg ($n = 2$). The renal vasoconstrictor effect of arachidonic acid at all dose levels was greatly inhibited by indomethacin (1 μM) or meclofenamate (3 μM). Thus, after cyclo-oxygenase inhibition, 10 μg and 30 μg AA increased pressure by 7 ± 5 mmHg and 34 ± 15 mmHg, respectively, whereas in untreated kidneys 1 μg and 3 μg AA increased perfusion pressure by 38 ± 12 mmHg ($P < 0.01$) and 121 ± 22 mmHg ($P < 0.01$), respectively. In contrast, the thromboxane synthase inhibitor, CGS-13080 at 5 μM, had no effect on the dose-response curve to arachidonic acid. The effectiveness of CGS-13080 in inhibiting thromboxane synthase was ascertained in several experiments. First, we showed that CGS-13080 at 5–25 μM inhibits by more than 97% the synthesis of platelet TxB_2 during clotting of rat blood *in vitro* ($n = 2$). Thus, 1, 5 and 25 μM CGS-13080 reduced TxB_2 from 70.8 ng ml^{-1} to 3.5, 2.1 and 1.7 ng ml^{-1}, respectively. Second, we found that the pretreatment of rats with CGS-13080 at 2 mg kg^{-1}, i.p., reduces the levels of serum TxB_2 measured by radioimmunoassay from 87.6 ± 7.4 ng ml^{-1} ($n = 7$) to 15.6 ± 4.6 ng ml^{-1} ($n = 4$), an inhibition of more than 80% ($P < 0.01$). Third, in two control and two treated preparations the increase in renal venous efflux of immunoreactive TxB_2 following a renal arterial injection of 10 μg arachidonic acid fell from 817 and 449 pg min^{-1} in control kidneys to 72 and 237 pg min^{-1} in kidneys perfused with Krebs-Henseleit buffer containing 5 μM CGS-13080. Moreover, CGS-13080 at 25 μM did not affect the increase in perfusion pressure caused by 10 μg of AA, perfusion pressure being increased by 150 ± 38 mmHg and 152 ± 29 mmHg in kidneys perfused with and without the inhibitor of thromboxane synthesis, respectively. Similarly, OKY-046 at a concentration of 5 μM, was without effect on the perfusion pressure response obtained to 10 μg AA (176 ± 40 mmHg vs 183 ± 26 mmHg in control, $P > 0.05$).

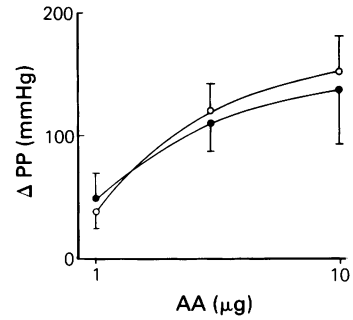


Figure 1 Effect of CGS-13080 (5 μM, ●) on the perfusion pressure responses to arachidonic acid (AA, 1–10 μg) in rat kidneys perfused with Krebs solution; (○) control. Data are presented as the mean with s.e.mean shown by vertical bars.

Effects of thromboxane A_2/prostaglandin H_2 receptor blockade on aracidonic acid responses in control rat kidneys

The flow rates in control and SQ 29,548-treated kidneys were 13.4 ± 0.7 ml min^{-1} and 12.4 ± 1.0 ml min^{-1}, respectively, which resulted in perfusion pressures of 102 ± 3 mmHg in both groups. Figure 2 shows the dose-response curves for

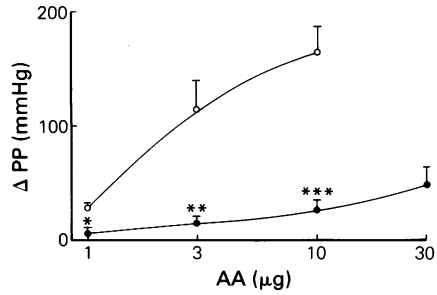


Figure 2 Effects of thromboxane A_2/prostaglandin H_2 receptor antagonism with SQ 29,548 (1 μM) on perfusion pressure responses to arachidonic acid (AA, 1–30 μg) in rat kidneys perfused with Krebs solution. Data are presented as the mean with s.e.mean shown by vertical bars; (○) Control; (●) SQ 29,548 1 μM. * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$ when compared to corresponding value in control.

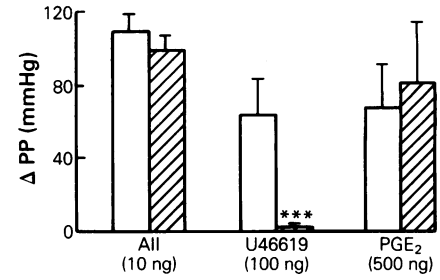


Figure 3 Changes in perfusion pressure to angiotensin II (AII, 10 ng), U46619 (100 ng) and prostaglandin E_2 (PGE_2, 500 ng) in isolated kidneys from rats. Kidneys were perfused with Krebs buffer containing vehicle (open columns) or 1 μM SQ 29,548 (hatched columns). Data are presented as means with s.e.mean shown by vertical bars. *** $P < 0.01$ compared to control.

arachidonic acid (1–30 μg) in kidneys perfused with Krebs buffer alone or buffer containing 1 μM SQ 29,548. This concentration of SQ 29,548 has been demonstrated to be effective in antagonizing responses to 9,11-azo PGH_2 in guinea-pig tracheal spirals and rat aortic strips (Ogletree *et al.*, 1985). SQ 29,548 significantly reduced the perfusion pressure responses to arachidonic acid at all doses tested, shifting the dose-response curve to the right. Thus in control rat kidneys, 10 μg AA increased pressure by 165 ± 21 mmHg compared to only 25 ± 9 mmHg in treated preparations. The responses obtained to U46619 (100 ng) in untreated and treated kidneys (Figure 3) show that the concentration of SQ 29,548 (1 μM) was sufficient to block TxA_2/PGH_2 receptors in the kidney. In contrast, SQ 29,548 was without effect on the perfusion pressure increases produced by 10 ng angiotensin II or 500 ng PGE_2 (Figure 3). Microgram quantities of $PGF_{2\alpha}$ were required to elicit increases in perfusion pressure which were unaffected by SQ 29,548.

Like SQ 29,548, 20 μM BM 13.177 significantly reduced the response to 10 μg AA, $P < 0.05$ (Figure 4). The concentration of BM 13.177 used, effectively blocked TxA_2/PGH_2 receptors shown by the response to U46619 (100 ng), $P < 0.02$ (Figure 4). However, BM 13.177 was without effect on the pressor response obtained to 10 ng AII, ($P > 0.05$).

Discussion

This study demonstrates that the vasoconstrictor effect of AA in the rat kidney involves interaction of one or more of its metabolites by cyclo-oxygenase with TxA_2/PGH_2 vascular receptors. The conclusion that products of AA metabolism by cyclo-oxygenase mediate the renal vasoconstrictor effect of the fatty acid is based on the finding that inhibitors of cyclo-oxygenase, indomethacin and meclofenamate, greatly inhibited the rise in perfusion pressure elicited by AA in the rat kidney perfused *in situ* with Krebs-Henseleit buffer. This finding confirms reports that inhibition of cyclo-oxygenase results in attenuation of AA-induced renal vasoconstriction in the rat (Sakr & Dunham, 1982). The conclusion that the eicosanoid(s) mediating the renal vasoconstrictor effect of AA does so via interaction with TxA_2/PGH_2 receptors is inferred from our finding that two dissimilar antagonists of TxA_2/PGH_2 receptors, SQ 29,548 and BM 13.177, greatly inhibited the rise in perfusion pressure caused by arachidonic acid in the rat kidney.

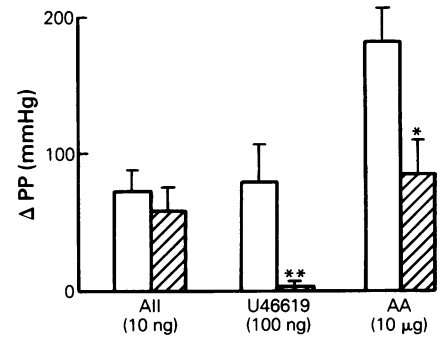


Figure 4 Changes in perfusion pressure to angiotensin II (AII, 10 ng), U46619 (100 ng) and arachidonic acid (AA, 10 μg) in rat isolated kidneys perfused with Krebs buffer containing vehicle (open columns) or 20 μM BM 13.177 (hatched columns). Data are presented as means with s.e.mean shown by vertical bars. * $P < 0.05$, ** $P < 0.02$ compared to control.

In the kidney, the metabolism of AA via the cyclo-oxygenase pathway results in the formation of various eicosanoids including PGI_2, PGE_2, $PGF_{2\alpha}$, TxA_2 and their common endoperoxide precursors, PGG_2 and PGH_2. PGE_2, $PGF_{2\alpha}$, TxA_2 and PGH_2 cause vasoconstriction in the rat isolated kidney perfused with a balanced salt solution and, consequently, are candidates for mediating the renal vasoconstrictor effect of AA (Malik & McGiff, 1975; Shibouta *et al.*, 1981). In the present study, the finding that blockade of TxA_2/PGH_2 receptors with SQ 29,548 or BM 13.177 inhibits the renal vasoconstrictor effect of AA and of U46619, a prostaglandin endoperoxide- and thromboxane-mimetic agent (Coleman *et al.*, 1981), suggests that TxA_2 and/or the prostaglandin endoperoxides mediate the effect of AA on the renal vasculature. The possibility that SQ 29,548 and BM 13.177 inhibit the renal vasoconstrictor effect of AA via a nonspecific mechanism can be excluded because neither receptor antagonist interfered with the renal vasoconstriction elicited by angiotensin II, PGE_2, and $PGF_{2\alpha}$. That the antagonists of TxA_2/PGH_2 receptors did not interfere with the renal vasoconstrictor effect of PGE_2 and $PGF_{2\alpha}$, while inhibiting that of AA, argues against any significant contribution of PGE_2 and $PGF_{2\alpha}$ to AA-induced renal vasoconstriction.

In the rat kidney autoperfused with blood, the lowering of renal blood flow caused by a renal arterial infusion of AA is partially inhibited by pretreatment with OKY-1581, an inhibitor of thromboxane synthetase, suggesting that TxA_2 contributes to the renal vasoconstrictor effect of AA (Sakr & Dunham, 1982). In the present study, however, the thromboxane synthetase inhibitor CGS-13080, at concentrations which significantly diminished the renal output of TxB_2 during AA administration, did not inhibit the renal vasoconstrictor effect of arachidonic acid. Another inhibitor of thromboxane synthesis, OKY-046, also was without effect on AA-induced renal vasoconstriction. One interpretation of these findings is that in the rat kidney perfused with Krebs-Henseleit buffer the vasoconstrictor effect of AA is mediated by a pressor eicosanoid(s) other than TxA_2, presumably a prostaglandin endoperoxide. Yet, based on our findings, one cannot exclude a role for TxA_2 as a mediator of AA-induced renal vasoconstriction. More specifically, the renal vasodilatation expected to result from the reduction in TxA_2 due to inhibition of thromboxane synthetase may be offset by the renal vasoconstriction caused by the increased levels of prostaglandin endoperoxides. If so, the renal vasoconstrictor effect of AA may be mediated by prostaglandin endoperoxides when thromboxane synthetase is inhibited, and by the prostaglandin endoperoxides and/or TxA_2 when it is not. Be that as it may, our finding that AA-induced renal vasoconstriction in the rat is inhibited by antagonists of TxA_2/PGH_2 receptors but not by inhibitors of thromboxane synthetase suggests a role for the prostaglandin endoperoxides in the mediation of the vasoconstrictor response to AA in the rat kidney.

In summary, this study demonstrates that the vasoconstrictor effect of AA in the rat kidney perfused *in situ* with Krebs-Henseleit buffer is inhibited by cyclo-oxygenase inhibitors and by antagonists of TxA_2/PGH_2 receptors, but not by inhibitors of thromboxane synthetase. We conclude that the renal vasoconstrictor response to AA is mediated by the prostaglandin endoperoxides when thromboxane synthetase is inhibited, and by the prostaglandin endoperoxides and/or TxA_2 when it is not.

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Co-axial bioassay of a smooth muscle relaxant factor released from guinea-pig tracheal epithelium

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1 The ability of guinea-pig trachea to release an epithelium-derived relaxant factor (EpDRF) was assessed in a co-axial bioassay system.

2 Histamine (100 μM) and methacholine (25 μM) caused endothelium-dependent relaxation of rat isolated aorta, presumably via the release of endothelium-derived relaxant factor (EDRF). In contrast, endothelium-denuded rat aorta did not relax in response to these agents.

3 EDRF release was detected in response to methacholine in a co-axial bioassay system, consisting of intact rabbit aorta tube (EDRF donor) and endothelium-denuded rat aorta strip (assay preparation). These results indicated the transfer of EDRF from a donor to an assay preparation, thereby validating the co-axial bioassay method.

4 Substitution of endothelium-intact rabbit aorta tube by epithelium-intact guinea-pig tracheal tube tissue in co-axial assemblies, still allowed the assay preparation to relax in response to histamine or methacholine. Removal of the intact tracheal tube from the system, or removal of the epithelium from the donor tracheal tube in co-axial preparations, abolished such relaxant responses. These observations are consistent with histamine- or methacholine-induced release of an epithelium-derived relaxant factor (EpDRF) from the trachea.

5 In the co-axial assembly comprising intact guinea-pig trachea and endothelium-denuded rat aorta, histamine and methacholine produced concentration-dependent, EpDRF-induced aortic relaxation. Mean concentrations of histamine and methacholine producing 50% of the maximum relaxation (EC_{50}) were 39.8 μM and 2.7 μM respectively. Histamine-induced relaxation was inhibited in the presence of mepyramine (2 μM) and responses to methacholine were inhibited by atropine (0.1 μM).

6 Methylene blue (50 μM) had no effect on such relaxant responses, indicating that EpDRF does not activate guanylate cyclase. Furthermore, the cyclo-oxygenase inhibitor indomethacin (5 μM), the cyclo-oxygenase/lipoxygenase inhibitor BW 755C (150 μM) and the leukotriene receptor antagonist FPL 55712 (10 μM) each failed significantly to alter EpDRF-mediated relaxation of vascular smooth muscle suggesting that EpDRF is not a prostanoid. Platelet activating factor (Paf) failed to cause relaxation of endothelium-denuded rat aorta, indicating that this mediator was also not EpDRF.

7 EpDRF was also released from human bronchial segments.

8 This study provides direct evidence for the release of an EpDRF from non-diseased airway tissue and further suggests that healthy airway reactivity to spasmogens is modulated by the release of an endogenous protective, spasmolytic substance. The bronchial hyperreactivity of asthma may be partly caused by attenuated production of such an inhibitory signal.

Introduction

The cause of asthma and of the characteristic bronchial hyperreactivity to airway spasmogens is unknown (Boushey *et al.*, 1980; Barnes, 1986). Inhalation of spasmogens in normal subjects causes bronchoconstriction which quickly reaches a plateau. In asthmatics, not only does bronchoconstriction occur at lower doses, but it continues in a dose-related manner as if some protective inhibitory mechanism is lacking (Woolcock *et al.*, 1984). Our group (Goldie *et al.*, 1986a) and others (Flavahan *et al.* 1985; Barnes *et al.*, 1985; Murlas, 1986; Hay *et al.*, 1986), have recently suggested that the airway epithelium in healthy lung produces a relaxant factor, perhaps similar to vascular endothelium-derived relaxant

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factor (EDRF) (Furchgott & Zawadzki, 1980) which modulates the responsiveness of the underlying smooth muscle to spasmogens. Such an airway epithelium-derived relaxant factor (EpDRF) generated in response to airway smooth muscle spasmogens as well as to osmotic stimuli (Munakata *et al.*, 1988) may limit bronchoconstriction in healthy lung. Conversely, in asthma when the airway epithelium is damaged (Laitinen *et al.*, 1985), the production of protective EpDRF would be attenuated or abolished.

Smooth muscle reactivity to spasmogens including histamine and methacholine, was significantly increased in epithelium-denuded isolated airway preparations from several animal species as well as from man (Raeburn *et al.*, 1986). However, studies describing smooth muscle reactivity changes following epithelium removal provide only indirect evidence for the existence of EpDRF. In contrast, Ilhan & Sahin (1986) and Guc *et al.* (1988) have demonstrated the release and transfer of a smooth muscle relaxant substance from airway epithelium. In the present study, we have directly measured the relaxant activity of a substance derived from guinea-pig trachea in a co-axial bioassay system utilizing rat thoracic aorta. These data further establish the existence of EpDRF, and support the concept that asthma-induced airway hyperreactivity may be at least partly due to the loss of such an inhibitory autacoid.

Methods

Tissue preparation for organ bath experiments

Male Wistar rats (220–250 g) were killed by a blow to the head and the thoracic aorta removed. After removal of adhering fat and connective tissue, a segment of the vessel was cut open along the longitudinal axis. For some experiments, care was taken to preserve the integrity of the endothelium during the production of cross-cut vessel strip preparations (7–8 mm in length). For other experiments, the endothelium was removed by gentle rubbing with a cotton wool probe. All preparations were suspended under 500 mg tension in Krebs solution maintained at 37°C and gassed with 5% CO_2 in O_2 and equilibrated for 1 h in the presence of indomethacin (5 μM), which was present thereafter in all experiments.

Aortae were then precontracted with the α-adrenoceptor agonist phenylephrine (Phe). Initially, all preparations were challenged with 0.2 μM Phe. After washout and equilibration for 45 min, preparations were re-challenged with 0.05 μM Phe. This second challenge was repeated to confirm the production of a stable, submaximal increase in tone. Preparations were then exposed to histamine (100 μM) or methacholine (25 μM). These tests were designed to confirm the ability of histamine and methacholine to induce the release of EDRF from endothelium-intact rat aorta as assessed by relaxation responses and to verify the successful deliberate removal of the endothelium by the absence of such effects in appropriate preparations. In some experiments, endothelium-intact and endothelium-denuded vessel strips were challenged with the β-adrenoceptor agonist (−)-isoprenaline (13 nM–1 μM) or the inflammatory mediator platelet activating factor (Paf, 1–100 nM).

Co-axial bioassay assemblies

Guinea-pig trachea Male guinea-pigs (SR/C Tricolour) weighing 450–500 g were killed by cervical dislocation and the tracheae removed, dissected free of surrounding tissue and cut into tubular segments approximately 7 mm in length. The epithelium of some tube segments was deliberately removed by gentle rubbing of the luminal surface with a cotton wool-coated probe as previously described for tracheal ring preparations (Goldie *et al.*, 1986a). Complete removal of the airway epithelium from all such preparations was verified by subsequent histological examination.

Following initial pharmacological verification of the presence or absence of endothelium, aorta preparations were set-up in co-axial assemblies under 500 mg resting tension, within guinea-pig tracheal tube segments. In such assemblies, the tracheal tube freely surrounds the vessel preparation without any direct contact between the tissues. After a 45 min equilibration period, the vessel within the assembly was precontracted with Phe (0.05 μM) and rechallenged with either histamine (5–200 μM) or methacholine (2.5–200 μM) in the presence or absence of the histamine receptor antagonists mepyramine (H_1-selective; 2 μM) or cimetidine (H_2-selective; 25 μM), the muscarinic cholinoceptor antagonist atropine (0.1 μM), the cyclo-oxygenase/lipoxygenase inhibitor BW 755C (150 μM), the leukotriene receptor antagonist FPL 55712 (10 μM), or the guanylate cyclase inhibitor methylene blue (50 μM). Indomethacin (5 μM) was also present throughout these experiments.

Human bronchus Bronchial tubes (2–3 mm, i.d.) were dissected as previously described (Goldie *et al.*, 1986b), from samples of non-diseased human lung obtained 2–4 h post-mortem from the victims of motor vehicle accidents. Tube segments (approximately 7 mm) were mounted co-axially with endothelium-denuded rat aorta and tested pharmacologically as described for guinea-pig trachea.

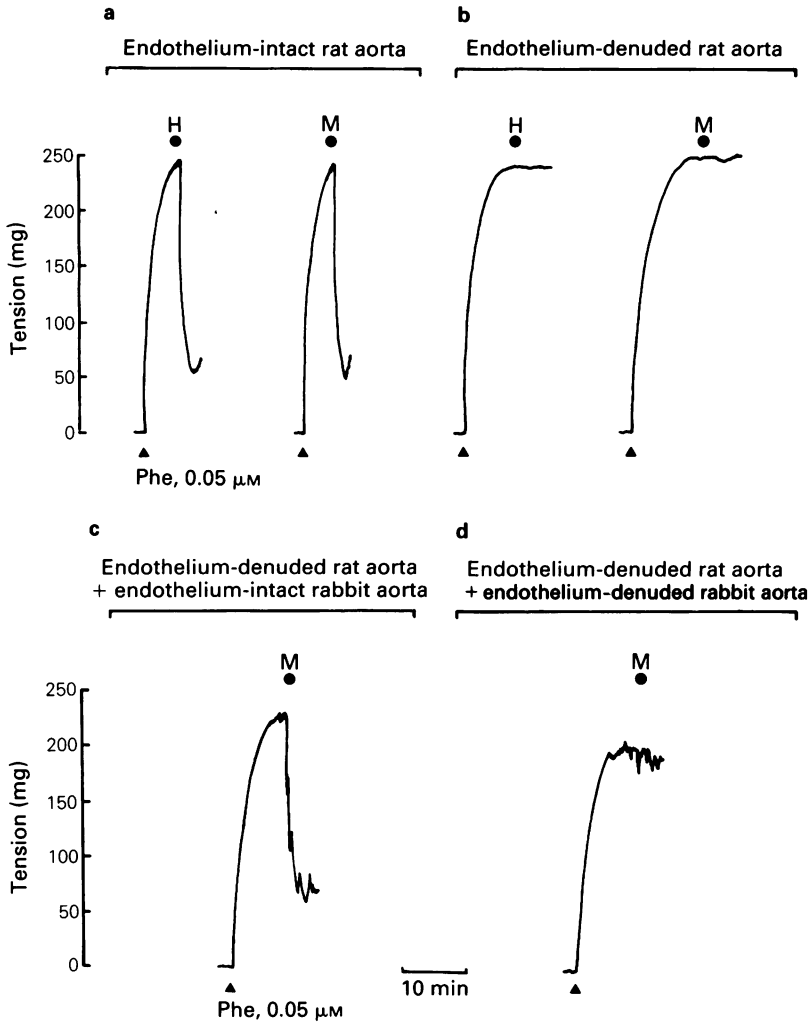


Figure 1 Responses to histamine (H: 100 μM) or methacholine (M; 25 μM) in (a) an endothelium-intact rat aorta preparation, (b) an endothelium-denuded rat aorta preparation, (c) an endothelium-denuded rat aorta preparation mounted co-axially within the lumen of an endothelium-intact rabbit aortic tube and (d) an endothelium-denuded rat aorta preparation mounted co-axially within the lumen of an endothelium-denuded rabbit aortic tube. All rat aortic preparations were precontracted with phenylephrine (Phe, 0.05 μM; ▲). Indomethacin (5 μM) was present throughout all the experiments.

Rabbit aorta The smooth muscle relaxant activity of EDRF released from rabbit aorta was also measured on endothelium-denuded rat aorta in co-axial bioassay. Endothelium-denuded aorta preparations were suspended under 500 mg tension within the lumen of tube segments (approximately 7 mm in length) of intact and endothelium-denuded rabbit aorta. Aortae were precontracted with Phe (0.05 μM) and the assemblies then challenged with methacholine (25 μM). These tests were conducted with a view to validating the co-axial bioassay method with respect to the transfer of an endogenously produced and secreted relaxant substance.

Analysis of results

Unless otherwise indicated, numerical data are presented as mean ± standard error of the mean (± s.e.mean). The potencies of agonists causing concentration-dependent responses of rat aorta

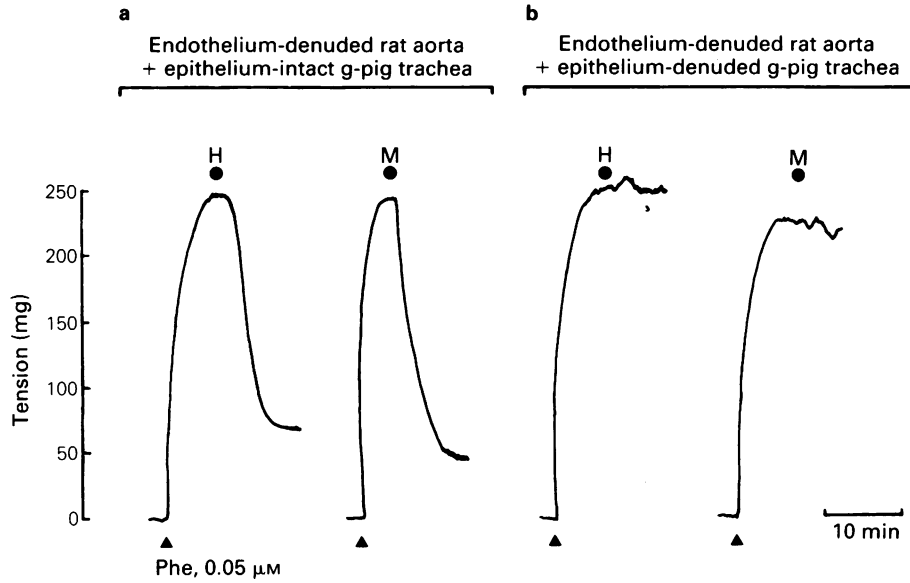


Figure 2 Responses to histamine (H, 100 μM) and methacholine (M, 25 μM) in endothelium-denuded rat aorta (a) mounted co-axially within the epithelium-intact guinea-pig trachea or (b) mounted co-axially within epithelium-denuded guinea-pig trachea. All preparations were pre-contracted with phenylephrine (Phe, 0.05 μM; ▲). Indomethacin (5 μM) was present throughout all the experiments.

preparations are expressed as pD_2 values or as molar EC_{50} values, i.e. concentrations producing 50% of the maximum response, derived from mean estimates of pD_2 where $pD_2 = -\log_{10}(EC_{50})$. The effects of competitive antagonists on the potency of agonists were assessed as mean shifts (with 95% confidence limits) derived from paired data i.e. from pD_2 values for the agonists obtained in the presence and absence of the appropriate antagonist.

Drugs and solutions

Drugs used were: cimetidine, histamine diphosphate, indomethacin, isoprenaline hydrochloride, methacholine chloride, mepyramine maleate, phenylephrine hydrochloride, L-α-phosphatidylcholine β-acetyl-γ-O-alkyl (Paf) (each from Sigma); atropine sulphate (D.H.A. Laboratories); 3-amino-1[*m*-(trifluoromethyl)-phenyl]-2 pyrazoline (BW 755C, Wellcome); 7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid (FPL 55712, Fisons); methylene blue (Gurr). Indomethacin was prepared in 5 mg ml^{-1} sodium carbonate solution. Isoprenaline was prepared in 0.9% w/v NaCl solution (saline) containing ascorbic acid 20 μg ml^{-1}. Stock solutions of Paf were prepared in 0.25% bovine serum albumin (BSA; Sigma) in polypropylene containers. Dilutions of Paf stock solution were also prepared using 0.25% BSA. The Krebs solution used throughout the study had the following composition (mM): NaCl 117.6, KCl 5.4, $NaHCO_3$ 25, KH_2PO_4 1.03, $MgSO_4$ 0.57, D-glucose 11.1 and $CaCl_2$ 2.5.

Results

Endothelium-dependent rat thoracic aorta preparations were precontracted with phenylephrine (Phe; 0.05 μM). This concentration of Phe produced stable increases in tone averaging 233 $\pm$ 8 mg of force (n = 42 preparations from 7 animals). This was equivalent to 98 $\pm$ 1% (n = 6) of the maximum response to this agonist. In endothelium-intact aortic strips, histamine (100 μM) and methacholine (25 μM) reduced Phe-induced tone by 78 $\pm$ 5% (n = 15 preparations from 6 animals) and 77 $\pm$ 2% (n = 14 preparations from 4 animals), respectively, presumably as a result of the release of EDRF (Figure 1a). In contrast, endothelium-denuded rat aorta failed to respond to these agents, thereby verifying the absence of endothelial cells and of EDRF (Figure 1b). Nevertheless, the β-adrenoceptor agonist (−)-isoprenaline (13 nM–1 μM) caused complete concentration-dependent relaxation of the

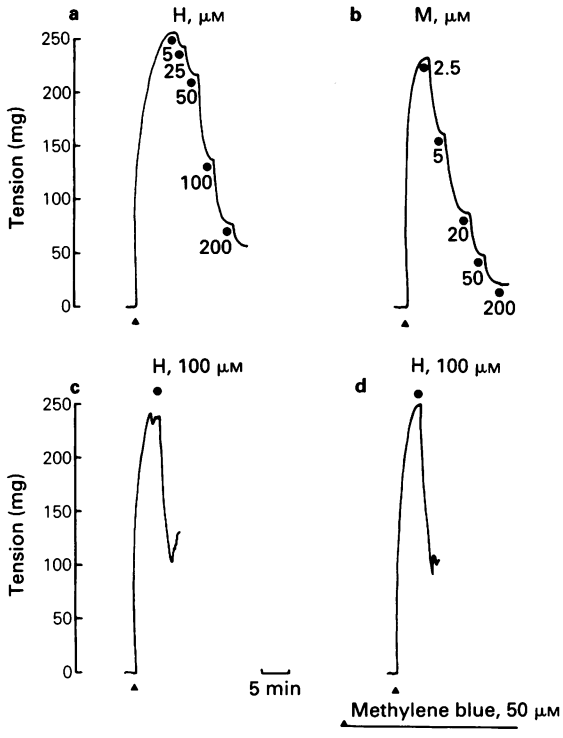


Figure 3 Concentration-dependent relaxation of endothelium-denuded rat aorta caused by (a) histamine (H; 5–200 μM) or (b) methacholine (M; 2.5–50 μM) induced release of epithelium-derived relaxing factor (EpDRF) from co-axially mounted guinea-pig trachea. Histamine-induced relaxation of rat aorta caused by EpDRF released from guinea-pig trachea in the (c) absence or (d) presence of the guanylate cyclase inhibitor methylene blue (50 μM). All preparations were pre-contracted with phenylephrine (▲; 0.05 μM). Indomethacin (5 μM) was present throughout all the experiments.

endothelium-denuded tissue. The mean pD_2 value for isoprenaline-induced relaxation was 7.77 ± 0.05 ($n = 8$). Paf (1 nM–100 nM) was virtually inactive in endothelium-denuded rat aorta.

When endothelium-denuded rat aortic preparations were set-up co-axially within the lumen of endothelium-intact tubular segments of rabbit aorta, methacholine again caused relaxation equivalent to 59 ± 5% ($n = 16$ preparations from 5 animals) of Phe-induced tone (Figure 1c). Thus, these co-axial assemblies could function as bioassay systems, with the intact vessel tubular segment acting as a donor of EDRF and the endothelium-denuded preparation responding with relaxation. Removal of the endothelium from tubular rabbit aorta donor preparations, or exposure to the guanylate cyclase antagonist methylene blue (50 μM) abolished the relaxant effect of methacholine. This demonstrated the endothelium dependency of this response in this co-axial system (Figure 1d).

As seen in Figure 2a, when a segment of epithelium-intact guinea-pig trachea was substituted for the endothelium-intact rabbit aortic tube, endothelium-denuded rat aorta again relaxed in response to both histamine and methacholine in the presence of the cyclo-oxygenase inhibitor indomethacin (5 μM). On average, these preparations lost 78 ± 5% ($n = 36$ preparations from 9 animals) and 75 ± 3% ($n = 31$ preparations from 10 animals) of their phenylephrine-induced tone in response to histamine (100 μM) and methacholine (25 μM) respectively. When the surrounding tracheal ring segments were removed, the relaxant effect of these agents was abolished. Similarly, if the luminal surface of intact tracheal rings was subsequently rubbed with a cotton wool swab to remove the epithelium and remounted in co-axial assemblies, both histamine and methacholine again failed to cause relaxation of rat aortae (Figure 2b).

Ring samples of guinea-pig and human airway tubular preparations in which the epithelium had not been deliberately denuded, were examined histologically prior to and following their use in co-axial bioassay assemblies. Despite the handling of these airway tubes during assembly and dismantling of co-axial preparations, the epithelium remained virtually intact around the luminal circumference. Histological examination revealed that the endothelium of rabbit aorta preparations was also apparently intact prior to testing in the organ bath. Furthermore, the endothelium was similar in appearance before and after such testing.

It can be seen that both histamine (Figure 3a) and methacholine (Figure 3b) caused concentration-dependent release of EpDRF from guinea-pig trachea as indicated by the concentration-dependent relaxations produced in response to these agonists. The mean pD_2 for histamine was 4.40 ± 0.08 ($n = 13$ preparations) and for methacholine was 5.57 ± 0.08 ($n = 14$ preparations). The histamine H_1-receptor antagonist mepyramine (2 μM) caused a mean reduction in the relaxant potency of histamine of 13 fold (95% confidence limits: 7–23; $n = 6$ preparations), while the histamine H_2-receptor antagonist cimetidine (25 μM) was without effect. Atropine (0.1 μM) reduced the relaxant potency of methacholine by 52 fold (95% confidence limits: 27–102; $n = 7$ preparations).

At 300 μM the cyclo-oxygenase/lipoxygenase inhibitor BW 755C has been shown to cause 80% inhibition of endothelium-dependent, acetylcholine-induced relaxation of rabbit aorta pre-contracted with noradrenaline. However, vessel tone was depressed by 40% (Forstermann *et al.*, 1988). In the

present study, BW 755C (150 μM) significantly reduced phenylephrine-induced tone, but histamine and methacholine-induced relaxation was not inhibited. Similarly, the leukotriene receptor antagonist FPL 55712 (10 μM), which has been shown to be active between 2 and 20 μM while not altering drug-induced vascular tone (Jancar *et al.*, 1987), also failed to affect such relaxations. Thus EpDRF was not a prostanoid substance. Relaxant responses to histamine and methacholine were also unaltered in the presence of the guanylate cyclase inhibitor methylene blue (50 μM) (Figure 3c,d), which is known to selectively inhibit EDRF-induced responses (Martin *et al.*, 1985).

Figure 4 shows the rat aorta again relaxed in response to both histamine and methacholine in the presence of indomethacin when surrounded by epithelium-intact, human bronchial ring preparations in co-axial assemblies. Removal of the bronchial epithelium caused abolition of the vascular relaxant response. Thus, an EpDRF was also released from human airway tissue *in vitro*.

Discussion

This study has provided direct evidence for the production and transfer of EpDRF from intact guinea-pig trachea and human bronchi in response to airway spasmogens, in a co-axial bioassay system. The validity of this approach was demonstrated by the successful transfer of EDRF produced in response to methacholine, from intact rabbit aortic tube donor to cause relaxation of endothelium-denuded rat aorta. In contrast, methacholine did not relax isolated endothelium-denuded rat aorta preparations in the absence of rabbit aortic tube tissue. The failure of histamine (100 μM) or methacholine (25 μM) to cause relaxation following removal of the endothelium suggests that these agonists also did not cause non-specific spasmolytic effects in these preparations. Previous studies have also demonstrated specific endothelium-dependent relaxation of rat aorta in response to histamine at concentrations up to 1 mM and to acetylcholine up to 100 μM (Van de Voorde & Leusen, 1983; Rapoport & Murad, 1983).

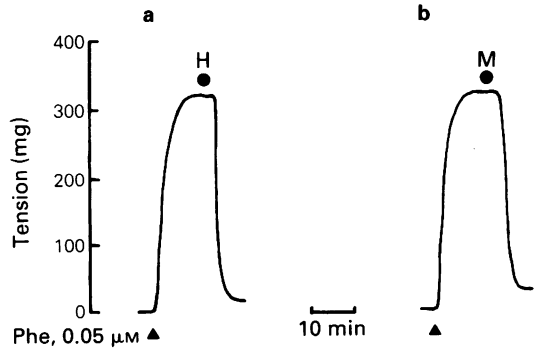


Figure 4 Histamine (H; 100 μM) and methacholine (M; 25 μM) induced relaxation of phenylephrine-contracted (Phe, 0.05 μM; ▲), endothelium-denuded rat aorta mounted co-axially within the lumen of segments of epithelium-intact human bronchial tube preparations. Indomethacin (5 μM) was present throughout all the experiments.

Isoprenaline caused complete relaxation of isolated endothelium-denuded rat aorta preparations, indicating that removal of the endothelium had not caused significant damage to the underlying smooth muscle. Most importantly, histamine and methacholine caused concentration-dependent relaxation of endothelium-denuded rat aorta when these preparations were placed in co-axial assemblies with intact guinea-pig tracheal or intact human bronchial tubes. Removal of the epithelium from airway tubes caused complete abolition of the relaxant response. Clearly, relaxation observed in co-axial assemblies resulted from the release of a substance derived from the tracheal epithelium i.e. epithelium-derived relaxant factor (EpDRF).

EpDRF-induced relaxation of assay preparations in response to histamine was inhibited by mepyramine (2 μM; H_1-selective) but not by cimetidine (25 μM; H_2-selective). Similar concentrations of these agents have previously been shown to cause histamine receptor subtype-selective antagonism in guinea-pig trachea (Duncan *et al.*, 1980). Similarly, the muscarinic cholinoceptor antagonist atropine (0.1 μM) inhibited the relaxant effect of methacholine. Thus, the generation of EpDRF was probably initiated following the activation of epithelial histamine H_1-receptors or muscarinic cholinoceptors, as is apparently the case for EDRF (Furchgott & Zawadzki, 1980).

While the identity of vascular EDRF remains the subject of debate (Palmer *et al.*, 1987; Shikano *et al.*, 1987), some of its properties are established. EDRF behaves like a nitrodilator, in that relaxation of vascular smooth muscle results from increased levels of intracellular cyclic-GMP. Thus, responses to EDRF were selectively inhibited by the guanylate cyclase inhibitor methylene blue (50 μM) (Martin *et al.*, 1985). In contrast, methylene blue (50 μM) failed to alter relaxation responses of rat aorta produced by histamine-induced release of EpDRF from guinea-pig trachea. Thus, EpDRF was not a guanylate cyclase stimulant, and therefore was neither EDRF nor nitric oxide. In addition, EpDRF does not appear to be a cyclo-oxygenase or lipoxygenase product of arachidonic acid metabolism, since indomethacin, BW 755C and FPL 55712 all failed to alter significantly the relaxation induced by this

autacoid. The failure of Paf to induce relaxation of rat aorta preparations suggests that EpDRF cannot be Paf.

This study has demonstrated that guinea-pig tracheal epithelium produced a non-prostanoid inhibitory factor capable of attenuating smooth muscle responsiveness to spasmogens. The endothelium-denuded rat thoracic aorta strip provides a sensitive assay preparation for this spasmolytic substance. The loss of such an inhibitory mediator in airway preparations deliberately denuded of epithelium most probably explains their increased sensitivity to spasmogens including histamine, methacholine, acetylcholine, 5-hydroxytryptamine and prostaglandin $F_{2\alpha}$ (Flavahan *et al.*, 1985; Barnes *et al.*, 1985; Goldie *et al.*, 1986a; Aizawa *et al.*, 1988; Raeburn *et al.*, 1988). This relaxant autacoid may help to protect the healthy respiratory tree against inhaled spasmogens as well as mediators released within the lung. Reduced EpDRF activity due to epithelial damage such as is commonly observed in asthma (Hogg & Eggleston, 1984; Laitinen *et al.*, 1985), may partially explain the bronchial hyperreactivity associated with this disease.

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Carotid haemodynamics in pigs during infusions of 8-OH-DPAT: reduction in arteriovenous shunting is mediated by 5-HT_1-like receptors

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1 The effects of intracarotid infusions of 8-hydroxy-2-[di-n-propyl-amino]-tetralin (8-OH-DPAT) on heart rate, blood pressure and carotid blood flow and its distribution were studied in anaesthetized pigs by use of radioactive microspheres of 15 μm diameter.

2 Control experiments with physiological saline showed that systemic and carotid haemodynamics remain essentially unchanged during the experimental period. In contrast to results obtained in rat, cat and dog experiments, 8-OH-DPAT did not decrease arterial blood pressure.

3 8-OH-DPAT, which has a high affinity and is selective for the 5-HT_{1A} recognition site, caused a dose-related decrease in arteriovenous anastomotic (non-nutrient) blood flow, resulting in a decrease in carotid blood flow. At the highest dose used, a small increase in arteriolar (nutrient) blood flow was observed.

4 The decrease in arteriovenous anastomotic and carotid blood flow induced by 8-OH-DPAT was not significantly modified by pretreatment with the 5-HT_2 receptor antagonist ketanserin (0.5 mg kg^{-1}), but was markedly reduced by pretreatment with methiothepin (1 mg kg^{-1}), which blocks both the 5-HT_1-like and 5-HT_2 receptors.

5 It is concluded that the effects of 8-OH-DPAT on arteriovenous anastomotic blood flow are mediated by 5-HT_1-like receptors. These receptors, however, cannot yet be classified as belonging to 5-HT_{1A} receptor subtype. Since a number of antimigraine drugs reduce arteriovenous shunting, it is tempting to suggest that 8-OH-DPAT may have similar clinical efficacy.

Introduction

Intracarotid administration of 5-hydroxytryptamine (5-HT) in the anaesthetized pig decreases arteriovenous anastomotic (non-nutrient) blood flow but increases arteriolar (nutrient) blood flow. The increase in arteriolar blood flow is especially marked in the ears and skin which change to bright red colour (Saxena & Verdouw, 1982). These effects of 5-HT are antagonized by methiothepin, which blocks both 5-HT_1-like and 5-HT_2 receptors, but not by the 5-HT_2 receptor antagonist ketanserin or the 5-HT_3 receptor antagonist MDL 72222 (Verdouw *et al.*, 1984). Furthermore, the effects of 5-HT are mimicked by 5-carboxamidotryptamine (Saxena & Verdouw, 1985). Therefore, according to the recent criteria for the classification of functional 5-HT receptors (Bradley *et al.*, 1986), 5-HT_1-like receptors mediate the constriction of arteriovenous anastomoses as well as the dilatation of arterioles (Saxena *et al.*, 1986).

The 5-HT_1-like receptors in the carotid circulation appear to be heterogeneous. Methysergide (Saxena & Verdouw, 1984) and BEA 1654 (Verdouw *et al.*, 1985), which have affinity for 5-HT_1-like binding sites, reduce arteriovenous shunting but, unlike 5-HT (Saxena & Verdouw, 1982) or 5-carboxamidotryptamine (Saxena & Verdouw, 1985), only slightly increase arteriolar blood flow. More recently, another 5-HT_1-like receptor agonist, GR 43175, has been described. This compound lacks 5-HT_1-like receptor-mediated vascular and intestinal smooth muscle relaxant actions, but contracts dog saphenous vein and inhibits noradrenaline release

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from sympathetic nerve terminals (Humphrey et al., 1988). GR 43175 also reduces arteriovenous anastomotic blood flow without affecting arteriolar blood flow in the carotid circulation of the cat (Feniuk et al., 1987).

The subdivision of functional 5-HT receptors into 5-HT_1-like, 5-HT_2 and 5-HT_3 receptors has been partially based on results from radioligand binding studies. Peroutka & Snyder (1979) demonstrated that two distinct 5-HT recognition sites exist in the central nervous system. The sites labelled by [^{3}H]-5-HT were designated as 5-HT_1-'receptors' and those labelled by [^{3}H]-spiperone were called 5-HT_2-'receptors'; the former have been further subdivided into 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} and 5-HT_{1D} recognition sites (Pedigo et al., 1981; Pazos & Palacios, 1985; Heuring & Peroutka, 1987). In contrast to the good agreement between 5-HT_2 recognition sites and functional 5-HT_2 receptors, it is still unclear whether the subtypes of the 5-HT_1 recognition site correspond to 5-HT_1-like receptors, which undoubtedly, are heterogeneous (Bradley et al., 1986; Humphrey & Richardson, 1988). The barrier here is the lack of sufficiently selective compounds to discriminate unequivocally between the different 5-HT_1 recognition site subtypes. One possible exception appears to be the 5-HT_{1A} recognition site where 8-OH-DPAT displays a high affinity and about 1000 fold selectivity compared to the other subtypes of 5-HT_1 recognition sites (Middlemiss & Fozard, 1983; Hoyer et al., 1985; Heuring & Peroutka, 1987). Therefore, in an attempt to characterize further the 5-HT_1-like receptors in the porcine carotid circulation we studied the effects of 8-OH-DPAT on carotid blood flow and its distribution in the anaesthetized pig.

Methods

General

After an overnight fast young Yorkshire pigs (body weight: 19–29 kg) were sedated with 120 mg (i.m.) azaperone and 120–150 mg (i.v.) metomidate, intubated and connected to a respirator for intermittent positive pressure ventilation with a mixture (1:2) of oxygen and nitrous oxide. A continuous infusion of pentobarbitone sodium (20–26 mg kg^{-1} h^{-1}, i.v.) completed the anaesthesia. Respiratory rate and tidal volume were adjusted to keep arterial blood gases and pH within normal limits (pH, 7.35–7.45; Po_2, 90–150 mmHg; Pco_2, 35–45 mmHg). Aortic blood pressure was recorded with a Statham pressure transducer (P23 ID) connected to a cannula inserted into the right femoral artery. The common carotid arteries were dissected free and a bilateral cervical vagosympathectomy was performed to reduce reflex influences on the carotid circulation. Blood flow in one of the common carotid arteries was measured with a precalibrated flow probe (2.5 or 3 mm, i.d.) connected to a sine-wave electromagnetic blood flow meter (Transflow 601-system, Skalar, Delft, The Netherlands). After removing its hub, a 0.5 mm (external diameter) needle, connected to suitable polyethylene tubing, was inserted into this common carotid artery for the administration of microspheres and drugs. The animals were insulated with a blanket to maintain body temperature at 37–38°C.

Distribution of common carotid blood flow

The distribution of common carotid blood flow was determined with the radioactive microsphere method using 15 ± 1 (s.d.) μm diameter spheres labelled with either ^{141}Ce, ^{113}Sn, ^{103}Ru, ^{95}Nb or ^{46}Sc (NEN Company, Dreieich, Federal Republic of Germany). For each measurement a suspension of microspheres, labelled with one of the nuclides, was mechanically agitated and injected against the direction of the carotid blood flow to facilitate uniform mixing with the blood. At the end of the experiment the animal was killed and the heart, kidneys, lungs and the various tissues of the head were dissected out, weighed and placed in vials. The radioactivity in these vials was counted for 5–10 min in a γ-scintillation counter (Packard, Minaxi Autogamma 5000) equipped with a multichannel analyzer using suitable windows for discriminating the different isotopes. The amount of carotid blood flow distributed to the individual tissues ($Q_{tis[car]}$) was calculated as described earlier (Saxena et al., 1980; Saxena & Verdouw, 1982) by: $Q_{tis[car]}$ (ml min^{-1}) = (I_{tis}/I_{tot}) × $Q_{[car]}$, where I_{tis} and I_{tot} are, respectively, the radioactivity (c.p.m.) in a particular tissue and that detected in all tissues, and $Q_{[car]}$ is carotid blood flow (ml min^{-1}). Because there was complete trapping of microspheres passing through the cranial circulation in the capillaries of the lungs (no significant amounts of radioactivity were detected in the heart or kidneys), the values determined for the lungs provide an index of the arteriovenous anastomotic fraction of the common carotid arterial blood flow (see Johnston & Saxena, 1978; Saxena & Verdouw, 1982).

Experimental protocol

Four series of experiments ($n = 6$ each) were performed. In the first series the effects of 4 consecutive intracarotid infusions of physiological saline on carotid blood flow and its distribution were determined. In the following series, the effects of intracarotid infusions of 8-OH-DPAT on carotid blood

flow distribution were assessed in untreated animals (Series 2) and in animals that were pretreated with either 0.5 mg kg^{-1} ketanserin (Series 3) or 1 mg kg^{-1} methiothepin (Series 4) administered at a rate of 1 ml min^{-1} during a period of 10 min, followed by a 10 min period of intracarotid infusion of physiological saline.

In all experiments the baseline values were determined after the preparation had been stable for at least 45 min after completion of the surgical procedure. The measurements consisted of the recording of the electrocardiogram, arterial blood pressure and common carotid blood flow, while a batch of microspheres (100,000–150,000) was injected for the determination of tissue (arteriolar) and arteriovenous anastomotic blood flows. Subsequently, in Series 2–4 the measurements were repeated after consecutive 10 min intracarotid infusions of 8-OH-DPAT, 0.3, 1.0, 3.0 and 10.0 μg kg^{-1} min^{-1} at the rate of 1 ml min^{-1}, always given in this order. In the first series, in which only physiological saline was infused, an identical protocol was followed.

Statistical analysis

The significance of the differences between variables was evaluated by Duncan's new multiple range test, once an analysis of variance (randomized block design) had revealed that the samples represented different populations (Steel & Torrie, 1980). Statistical significance was accepted at $P < 0.05$ (two-tailed). All data are expressed as means ± s.e.mean.

Drugs

Apart from the anaesthetics, the drugs used in this study were: 8-OH-DPAT (8-hydroxy-2-[di-n-propylamino]-tetralin (courtesy of Dr. J. Traber, Troponwerke, Cologne, Federal Republic of Germany), ketanserin tartrate (courtesy of Dr J.M. Van Nueten, Janssen Pharmaceutica, Beerse, Belgium) and methiothepin maleate (courtesy of Dr P.J. Pijper, Hoffman La Roche B.V., Mijdrecht, The Netherlands). The doses of 8-OH-DPAT are in terms of the base, whereas those of ketanserin and methiothepin refer to the salt.

Results

Physiological saline experiments

The effects of four consecutive infusions of physiological saline on heart rate, mean arterial blood pressure, carotid blood flow and its distribution are summarized in Table 1. Heart rate and blood pressure showed a tendency to decrease, but a statistically significant change was only observed in heart rate after the last saline infusion. Total carotid blood flow and arteriovenous anastomotic blood flow tended to increase, but the changes were not statistically significant. Nutrient (arteriolar) blood flow remained unaffected.

8-OH-DPAT experiments

Systemic haemodynamics The values for heart rate, mean arterial blood pressure and carotid blood flow before and after the infusion of either ketanserin or methiothepin are shown in Table 2. Both mean arterial blood pressure and heart rate decreased after intracarotid infusion of ketanserin (0.5 mg kg^{-1}), whereas after methiothepin infusion (1 mg kg^{-1}) only mean arterial blood pressure was lowered. Carotid blood flow was not changed significantly by infusion of either antagonist. In Table 3 are summarized the values of mean arterial blood pressure and heart rate recorded during the intracarotid infusions of 8-OH-DPAT. 8-OH-DPAT did not affect mean arterial blood pressure, but decreased heart rate in both

Table 1 Mean arterial blood pressure, heart rate and carotid blood flow and its distribution during four consecutive infusions of physiological saline

		Saline infusions			
	Baseline	*First*	*Second*	*Third*	*Fourth*
MAP	97 ± 12	92 ± 11	91 ± 11	88 ± 11	87 ± 11
HR	109 ± 13	109 ± 12	105 ± 12	102 ± 11	99 ± 10*
CBF	108 ± 17	108 ± 20	115 ± 20	123 ± 22	124 ± 23
AVA	87 ± 18	89 ± 20	95 ± 21	100 ± 23	102 ± 24
Nutrient	20 ± 3	19 ± 2	20 ± 3	22 ± 3	22 ± 3

Saline was infused for 10 min periods at a rate of 1 ml min^{-1} ($n = 6$). Abbreviations and units: MAP, mean arterial blood pressure (mmHg); HR, heart rate (beats min^{-1}); CBF, total carotid blood flow (ml min^{-1}); AVA, arteriovenous anastomotic blood flow (ml min^{-1}); Nutrient, arteriolar blood flow (ml min^{-1}).

* Significantly different ($P < 0.05$) from the baseline value.

Table 2 Mean arterial blood pressure, heart rate and carotid blood flow before and after ketanserin and methiothepin

	Antagonist pretreatment	*Baseline*	*Immediately after antagonist infusion*	*10 min after antagonist infusion*
MAP	Ketanserin	94 ± 7	85 ± 7*	84 ± 7*
	Methiothepin	98 ± 10	86 ± 10*	80 ± 7*
HR	Ketanserin	114 ± 8	107 ± 6	100 ± 5*
	Methiothepin	100 ± 8	97 ± 8	96 ± 8
CBF	Ketanserin	125 ± 14	111 ± 14	112 ± 13
	Methiothepin	102 ± 8	105 ± 11	97 ± 9

Ketanserin (0.5 mg kg^{-1}; $n = 6$) or methiothepin (1 mg/kg^{-1}; $n = 6$) was infused into the carotid artery at a rate of 1 ml min^{-1} during a period of 10 min. Thereafter an infusion of physiological saline (1 ml min^{-1}) was given. Abbreviations and units: MAP, mean arterial blood pressure (mmHg); HR, heart rate (beats min^{-1}); CBF, carotid blood flow (ml min^{-1}).

* Significantly different ($P < 0.05$) from the baseline value.

untreated and ketanserin- or methiothepin-pretreated animals during intracarotid infusions of the three highest doses (1, 3 and 10 μg kg^{-1}).

Carotid blood flow and its distribution The effects of intracarotid infusions of 8-OH-DPAT on carotid blood flow and its fractionation over non-nutrient, arteriovenous anastomotic blood flow and nutrient, arteriolar blood flow are shown in Figure 1. As was the case in the experiments with physiological saline, a large fraction (70%) of the total carotid blood flow was diverted through the arteriovenous anastomoses in untreated as well as in ketanserin- or methiothepin-pretreated animals before the infusions of 8-OH-DPAT were started. In the untreated and ketanserin-pretreated animal, 8-OH-DPAT infusions induced a dose-dependent decrease in total carotid blood flow, which was directly related to a decrease in arteriovenous anastomotic blood flow. Only at the highest dose tested did arteriolar blood flow show an increase in the untreated animals, whereas in the ketanserin-pretreated animals arteriolar blood flow tended to decrease. In the methiothepin-pretreated animals the effects of 8-OH-DPAT infusions on total carotid and arteriovenous anastomotic blood flow were markedly reduced. Furthermore, a small but dose-related decrease in arteriolar blood flow was observed.

Analysis of regional carotid blood flow distribution showed that 8-OH-DPAT infusion at the highest dose caused an increase in blood flow to the skin, ears, tongue, bones and brain in the untreated animals (Figure 2). No changes in colour of the skin and ears, as seen with 5-HT and 5-carboxamidotryptamine (Saxena & Verdouw, 1982; 1985), were observed during the experiments. In the group pretreated with ketanserin, blood flow to the skin and ears increased at the highest dose of 8-OH-

Table 3 Effect of intracarotid infusions of 8-hydroxy-2[di-n-propyl-amino]tetralin (8-OH-DPAT) on mean arterial blood pressure and heart rate

	Antagonist pretreatment	8-OH-DPAT (μg kg^{-1} min^{-1}) 0	0.3	1.0	3.0	10.0
MAP	None	79 ± 7	78 ± 7	76 ± 6	76 ± 7	75 ± 6
	Ketanserin	84 ± 7	84 ± 8	84 ± 8	84 ± 8	85 ± 8
	Methiothepin	80 ± 7	80 ± 7	78 ± 7	74 ± 5	74 ± 6
HR	None	109 ± 7	104 ± 6	99 ± 5*	93 ± 4*	88 ± 3*
	Ketanserin	100 ± 5	96 ± 6	92 ± 5*	90 ± 4*	85 ± 2*
	Methiothepin	96 ± 8	94 ± 8	90 ± 8*	86 ± 6*	82 ± 5*

8-OH-DPAT was infused in 10 min periods at a rate of 1 ml min^{-1} in untreated ($n = 6$), ketanserin (0.5 mg kg^{-1}; $n = 6$) or methiothepin (1 mg kg^{-1}; $n = 6$) pretreated animals. Abbreviations and units: MAP, mean arterial blood pressure (mmHg); HR, heart rate (beats min^{-1}).

* Significantly different ($P < 0.05$) from the value before the administration of 8-OH-DPAT.

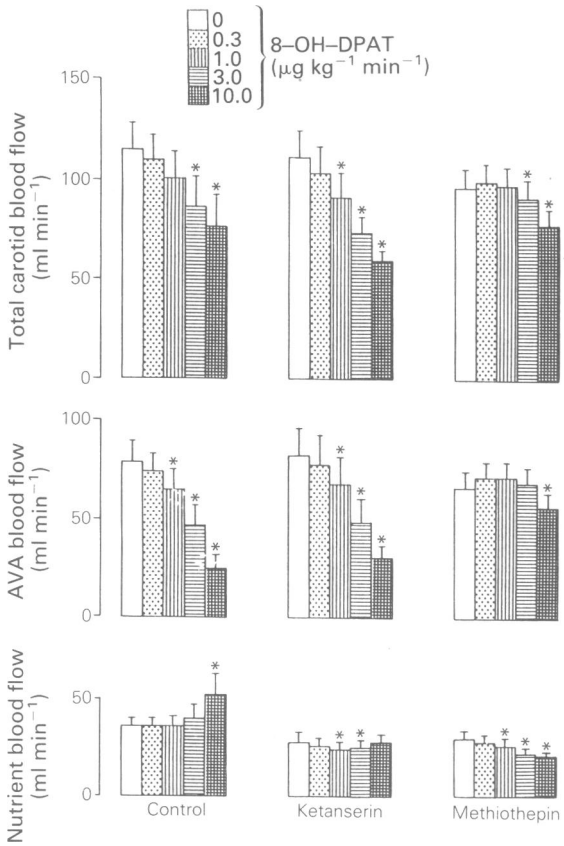


Figure 1 Total carotid blood flow, arteriovenous anastomotic (AVA) blood flow and arteriolar (nutrient) blood flow in untreated (control) and ketanserin ($0.5\,mg\,kg^{-1}$) or methiothepin ($1\,mg\,kg^{-1}$) pretreated pigs ($n = 6$ each) before and after intracarotid infusions of increasing doses of 8-hydroxy-2-[di-n-propylamino]tetralin (8-OH-DPAT). * $P < 0.05$ vs baseline.

DPAT, but blood flow to the tongue, bones and brain remained unchanged, while that to the skeletal muscles and eyes was reduced. Pretreatment with methiothepin prevented the 8-OH-DPAT-induced increase in blood flow to the skin, ears, tongue and brain, whereas blood flow to the muscles, bones and eyes decreased.

Discussion

Validity of the experimental model

Our experiments, in which four consecutive intracarotid infusions of physiological saline were given to the anaesthetized pig, clearly show that no appreciable changes occur in either systemic or carotid haemodynamics over a period of about 40–60 min. Similarly, we have previously shown that within minutes of stopping the infusions of 5-HT, which is quickly metabolised, the distribution of carotid blood flow is restored to that observed during the baseline period (Saxena & Verdouw, 1982; 1984; Verdouw *et al.*, 1984). Therefore, we can conclude that no time-dependent changes or changes due to the injection of microspheres themselves are seen in carotid haemodynamics during the experimental period in the animal model used here.

Effects of 8-OH-DPAT

In contrast to the rat (Gradin *et al.*, 1985; Martin & Lis, 1985; Fozard *et al.*, 1987), cat (Ramage & Fozard, 1987; McCall *et al.*, 1987; Doods *et al.*, 1988) and dog (Di Francesco *et al.*, 1988), 8-OH-DPAT did not lower blood pressure in the anaesthetized pig. Whether this is caused by differences in species, anaesthesia or the level of baseline arterial blood pressure, remains unclear. The decrease in heart rate observed in our experiments cannot be unequivocally attributed to an action of 8-OH-DPAT, since this was also observed in experiments in which only physiological saline was infused. The use of nitrous oxide as an anaesthetic might be an explanation for this phenomenon. Nitrous oxide is reported to reduce heart rate and cardiac output with systemic arterial blood pressure being maintained by increased total peripheral resistance (Eisele & Smith, 1972).

Intracarotid infusion of 8-OH-DPAT, which is a selective agonist at $5\text{-}HT_{1A}$ receptors (Middlemiss & Fozard, 1983; Hoyer *et al.*, 1985), caused a dose-related decrease in carotid blood flow in the anaesthetized pig. This effect on carotid blood flow, unrelated to changes in mean arterial blood pressure, was associated with a selective decrease in the arteriovenous anastomotic fraction. Pretreatment with methiothepin, but not with ketanserin, considerably reduced the effects of 8-OH-DPAT, indicating that 8-OH-DPAT was acting on $5\text{-}HT_1$-like receptors.

The increase in tissue blood flow to the skin and ears observed during the infusion of the highest dose of 8-OH-DPAT is probably also mediated by $5\text{-}HT_1$-like receptors since this effect was antagonized by methiothepin but not be ketanserin. However, it should be noted that like methysergide and BEA 1654 (Saxena & Verdouw, 1984; Verdouw *et al.*, 1985) and GR 43175 (Feniuk *et al.*, 1987), but in contrast to 5-HT and 5-carboxamidotryptamine (Saxena & Verdouw, 1982, 1985), the effect of 8-OH-DPAT on total nutrient (arteriolar) blood flow was not marked. Compatible with this difference, intracarotid infusions of 5-HT and 5-carboxamidotryptamine changed the colour of the

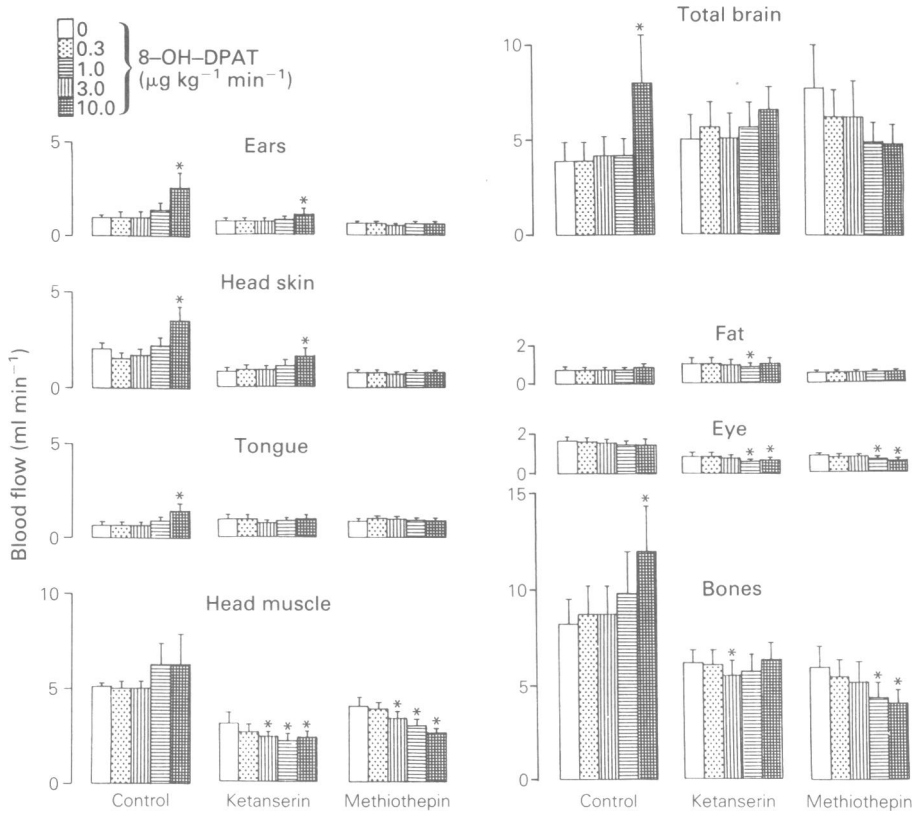


Figure 2 The distribution of carotid blood flow to various cranial tissues in untreated (control), ketanserin (0.5 mg kg^{-1}) or methiothepin (1 mg kg^{-1}) pretreated pigs ($n = 6$ each) before and after intracarotid infusions of increasing doses of 8-hydroxy-2-[di-n-propyl-amino]tetralin (8-OH-DPAT). * $P < 0.05$ vs baseline.

skin and ears to bright red (Saxena & Verdouw, 1982; 1985), but infusions of 8-OH-DPAT did not. The above findings are suggestive of heterogeneity of the 5-HT$_1$-like receptors mediating the constriction of arteriovenous anastomoses and the dilatation of arterioles in the carotid vascular bed.

The selectivity of 8-OH-DPAT for the 5-HT$_{1A}$ recognition sites (Middlemiss & Fozard, 1983; Hoyer *et al.*, 1985) obviously tempts one to suggest that the 5-HT$_1$-like receptors mediating the reduction of arteriovenous blood flow belong to the 5-HT$_{1A}$ subtype. However, it is still unclear whether this is so, since no selective antagonists for the putative 5-HT$_{1A}$ receptor are as yet available. Moreover, in initial experiments, ipsapirone, which also has a high affinity for the 5-HT$_{1A}$ recognition site (Peroutka, 1986; Traber & Glaser, 1987) and reduces blood pressure in anaesthetized cats by an agonist action on putative 5-HT$_{1A}$ receptors in the brain (Ramage & Fozard, 1987), was found to be inactive in our experimental model (Bom *et al.*, 1988). It remains possible that the inactivity of ipsapirone is due to a low efficacy of the drug at the putative 5-HT$_{1A}$ receptors or to some differences in 5-HT$_1$ recognition sites in the brain and in the carotid circulation.

Lastly we would like to draw some attention to the possible clinical relevance of the main finding of this study, i.e. the constriction of arteriovenous anastomoses by 8-OH-DPAT. It should be recalled that dilatation of arteriovenous anastomoses has been suggested to form part of the pathophysiology of migraine headache (Heyck, 1969; Saxena, 1978; 1987). Indeed, a number of proven and effective antimigraine drugs, including ergotamine, methysergide and isometheptene (Johnston & Saxena, 1978; Spierings & Saxena, 1980a,b; Saxena & Verdouw, 1984; Saxena, 1987), constrict arteriovenous anastomoses. Also, GR 43175, the newly developed agonist at 5-HT$_1$-like receptors (Humphrey *et al.*, 1988), reduces arteriovenous anastomotic blood flow (Feniuk *et al.*, 1987) and has shown promise in the treatment of acute attacks of migraine (Doenicke *et al.*, 1988). It is therefore possible that 8-OH-DPAT

may also have an antimigraine action. However, we do not know how the behavioural effects of 8-OH-DPAT described in animals (Tricklebank, 1985) would manifest in man.

In conclusion the present study demonstrates that 8-OH-DPAT, which has a high affinity and is selective for 5-HT_{1A} recognition sites, decreases common carotid arterial blood flow in the pig by selectively reducing arteriovenous anastomotic blood flow. Since methiothepin, but not ketanserin, antagonized the 8-OH-DPAT-induced constriction of arteriovenous anastomoses, this response is mediated by 5-HT_1-like receptors. It is, however, not yet certain whether these 5-HT_1-like receptors correlate with the 5-HT_{1A} recognition sites.

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The thermogenic actions of α_2-adrenoceptor agonists in reserpinized mice are mediated via a central postsynaptic α_2-adrenoceptor mechanism

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1 The dose-related effects of the selective α_2-adrenoceptor agonists clonidine, UK-14,304 and B-HT 933 on the body temperature of untreated and reserpine-treated mice were investigated.

2 In untreated mice all three agonists induced a dose-related hypothermia. The highest doses of UK-14,304 and B-HT 933, 3 and 100 mg kg^{-1} respectively, elicited a marked (10°C) hypothermia, whereas the maximal hypothermic effect of clonidine (5.5°C) was less pronounced and reached a plateau at a dose of 0.5 mg kg^{-1} i.p.

3 Reserpine (2.5 mg kg^{-1}, s.c.) induced a marked hypothermia in the mouse; 18 h after injection body temperature had decreased to only slightly (0.5–1.5°C) above ambient (19°C).

4 All three α_2-agonists produced a partial dose-related reversal of reserpine-induced hypothermia; maximal thermogenic responses (9–10°C increases in body temperature) were elicited by doses of 0.2, 0.5 and 16 mg kg^{-1} i.p. of clonidine, UK-14,304 and B-HT 933 respectively, and the log dose-response curves for all 3 agonists were bell-shaped.

5 Following intracerebroventricular administration to reserpine-treated mice, the thermogenic response to clonidine was more rapid in onset, and the agonist was 20 fold more potent than when injected i.p.

6 The selective α_2-adrenoceptor antagonists, idazoxan (0.05–0.5 mg kg^{-1}), Wy 26392 (0.3–5.0 mg kg^{-1}) and yohimbine (0.1–1.6 mg kg^{-1}) given orally attenuated the thermogenic responses to all 3 agonists in reserpinized mice in a dose-related manner. Pretreatment with a single dose of idazoxan (0.3 mg kg^{-1}, orally) elicited a 6 fold parallel shift to the right in the dose-response curve to clonidine.

7 The selective α_1-adrenoceptor antagonists, prazosin (10 mg kg^{-1}) and indoramin (3–10 mg kg^{-1}), and the β-adrenoceptor antagonist, propranolol (10 mg kg^{-1}), only partially attenuated the thermogenic responses to the α_2-agonists in reserpinized mice. These effects were variable and not clearly dose-related.

8 Pretreatment of reserpinized mice with the catecholamine synthesis inhibitor, α-methyl-*p*-tyrosine, markedly attenuated (60–95%) the thermogenic response to the noradrenaline uptake inhibitor, desipramine (0.13–12.5 mg kg^{-1}, i.p.), but only slightly reduced (10–35%) that to clonidine (0.032–0.5 mg kg^{-1}, i.p.).

9 These results suggest that α_2-adrenoceptor agonists reverse reserpine-induced hypothermia via a central mechanism involving activation of postsynaptic α_2-adrenoceptors.

Introduction

Recent evidence suggests that α_2-adrenoceptors, similar or identical to the α_2-autoreceptors described by Langer (1974), are not confined to presynaptic sites but are also located on postsynaptic membranes (see review by Timmermans & Van Zwieten, 1981). The majority of the results which led to this conclusion were derived from studies on vascular smooth muscle preparations (Timmermans & Van Zwieten, 1981). It is now apparent, however, that α_2-adrenoceptors are also found at postsynaptic sites

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within the central nervous system. For example, clonidine is thought to produce its hypotensive action and to stimulate growth hormone secretion via activation of central postsynaptic α_2-adrenoceptor mechanisms (Hoefke, 1980; Eriksson *et al.*, 1982). Indeed, radioligand binding studies with [^{3}H]-clonidine after the selective destruction of central noradrenergic neurones by 6-hydroxydopamine imply that the majority of α_2-adrenoceptors within the central nervous system are located postsynaptically rather than presynaptically on noradrenergic nerve terminals (U'Prichard *et al.*, 1979; Dausse *et al.*, 1982).

One of the many pharmacological effects induced by reserpine in rodents is a profound hypothermia (Askew, 1963), which is thought to result from the depletion of central and peripheral neuronal stores of catecholamines (noradrenaline (NA) and dopamine) and 5-hydroxytryptamine (5-HT) (Shore, 1962). In this paper we present evidence suggesting that the thermogenic action of a series of selective α_2-adrenoceptor agonists in reserpine-treated mice is mediated via activation of a central postsynaptic α_2-adrenoceptor mechanism. A preliminary account of this work was presented to the British Pharmacological Society (Bill & Stephens, 1984).

Methods

Animals

Female, albino, T/O strain mice (18–27 g) supplied by Tuck were used. Before experimentation the animals were housed in groups of 15 at an ambient temperature of 21–23°C on a 12 h light/dark cycle (light period 08 h 00 min to 20 h 00 min) with free access to standard laboratory diet (CRM pellets) and tap water.

Reserpine treatment and measurement of body temperature

Experiments were performed on groups of 8 mice per treatment unless otherwise specified. Animals were injected s.c. with 2.5 mg kg^{-1} of reserpine and maintained overnight for 18 h, and for the duration of the subsequent experimental period in plastic cages (30 × 15 × 12 cm) with sawdust in the bottom at an ambient temperature of 19.0 ± 0.5°C.

Body temperatures were measured with a rectal thermistor probe inserted to a standard depth of 2 cm into the colon and a digital thermometer (Comark Electronics Limited, Sussex), while the mice were lightly restrained in the hand by the scruff and the tail.

Intracerebroventricular (i.c.v.) injection techniques

Clonidine or vehicle (sterile isotonic saline) were injected into the lateral cerebral ventricles of reserpine-treated mice (weighing 18–22 g) in a dose volume of 5 μl per mouse according to the method of Brittain (1966). The site of injection was within 1 mm of a point on the midline 2 mm rostral to a line joining the anterior bases of the ears. Injections were made with a sterile 25 μl Hamilton syringe with a 26/10 mm gauge needle inserted perpendicularly through the skull into the brain, and penetrated the brain to a depth of 2.0–2.5 mm below the top surface of the skull. The location of the injection site was verified by injection of 5 μl of a 1 in 10 dilution of Indian ink in isotonic saline. Brains were then removed, sectioned and examined histologically for the presence of particles of ink in the cerebral ventricles. These examinations revealed a success rate for the i.c.v. injections of approximately 90%.

Antagonist studies

Antagonists were administered orally to reserpine-treated mice 17 h after reserpine over the dose ranges shown in Table 1. Thirty minutes later clonidine (0.2 mg kg^{-1}), UK-14,304 (0.4 mg kg^{-1}), B-HT 933 (16 mg kg^{-1}) or vehicle were injected i.p. Body temperatures were recorded immediately before each drug injection and at 1 or 2 h intervals for 6 h, post-injection of the agonists. In all experiments vehicle/vehicle, vehicle/agonist and antagonist/vehicle control groups were included. For each treatment group the mean maximum increase in body temperature over the 6 h period post injection of the agonist was calculated. From these data the percentage inhibition by the antagonists of the response to each agonist was determined and ED_{50} values for the antagonists (doses to inhibit the thermogenic response to each agonist by 50%) together with 95% confidence limits obtained by linear regression analysis.

Catecholamine synthesis inhibition

The effects of pretreatment with the catecholamine synthesis inhibitor, α-methyl-*p*-tyrosine (α-MPT), on the responses of reserpine-treated mice to clonidine and the noradrenaline uptake inhibitor, desipramine, were examined. Groups of 8 mice were dosed i.p. with either vehicle or 300 mg kg^{-1} of α-MPT one hour before the s.c. injection of reserpine (2.5 mg kg^{-1}). A further dose of vehicle or α-MPT (100 mg kg^{-1}) was administered i.p. 16 h after reserpine. Clonidine (0.013–1.25 mg kg^{-1}) and desipramine (0.13–12.5 mg kg^{-1}) were then injected i.p. 2 h after the last dose of α-MPT. Body temperatures

were measured before clonidine and desipramine administration and at 1 h intervals for the following 6 h.

Measurement of brain monoamines and metabolites

The same dosing schedule as that described above was used to determine the effects of reserpine alone and combined treatment with reserpine and α-MPT on brain levels of noradrenaline, dopamine, 5-HT, 3,4-dihydroxyphenylacetic (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA). Whole mouse brain levels of these materials were assayed by high performance liquid chromatography (h.p.l.c.) with electrochemical detection 18 h after injection of reserpine, by the method of Wagner *et al.* (1982).

Experimental design and statistics

In all experiments animals were dosed according to a predetermined sequence such that they were assessed in a balanced order with respect to time and treatment received. Also, with the exception of reserpine, the experimenter was unaware of the treatment(s) that each animal had received.

Body temperatures and temperature changes are shown as mean ± s.e.mean and were analysed by Student's unpaired *t* test. Temperature changes are calculated as the mean maximum increases occurring over the 6 or 8 h period post injection of the agonists or desipramine.

Drugs

The following drugs were used in this study: α-methyl-*p*-tyrosine methylester hydrochloride (Sigma), B-HT 933 ((azepexole), 2-amino-6-ethyl-4,5,7,8-tetrahydro-6H-oxazolo {5,4-d} azepine dihydrochloride, (Boehringer-Ingelheim)), clonidine hydrochloride (Boehringer-Ingelheim), desipramine hydrochloride (Geigy Pharmaceuticals), idazoxan hydrochloride (Reckitt and Colman), indoramin hydrochloride (Wyeth), prazosin hydrochloride (Pfizer), (±)-propranolol hydrochloride (Sigma), reserpine (Sigma), UK-14,304 (5-bromo-6-[2-imidazolin-2-yl-amino]-quinoxaline tartrate, (Pfizer)), Wy 26392 (N-methyl-N-(1,3,4,6,7,11bα-hexahydro-2H-benzo-{a}-quinolizin-2β-yl)-propan-1-sulphonamide hydrochloride, (Wyeth)), yohimbine hydrochloride (Sigma).

For oral administration the antagonists were dissolved or suspended in 0.4% hydroxypropylmethylcellulose (HPMC) in distilled water. Other drugs (except reserpine) were dissolved in isotonic saline for i.p. injection. Reserpine was prepared in a stock solution containing 4% benzyl alcohol, 0.5% citric acid and 20% Tween 80 in distilled water to give a final concentration of reserpine of 5 mg ml^{-1}. This stock solution was diluted 20 fold to give the required dose of reserpine for s.c. injection. All drugs were administered in a dose volume of 10 ml kg^{-1}, apart from i.c.v. injections when the dose volume was 5 μl per mouse. Drug doses in the text refer to the salt.

Results

Effects of α_2-adrenoceptor agonists on the body temperature of non-reserpinized mice

Groups of previously untreated mice were dosed i.p. with either vehicle, clonidine, UK-14,304 or B-HT 933 and body temperatures were recorded before drug injection and at 30 min intervals for the next 2 h.

The mean body temperature of non-reserpinized mice before drug treatment was 37.5 ± 0.2°C (n = 108). Clonidine (0.045–0.5 mg kg^{-1}), UK-14,304 (0.3–3.0 mg kg^{-1}) and B-HT 933 (10–100 mg kg^{-1}) all induced a dose-dependent hypothermia (Figure 1). The hypothermic effects of all three α_2-agonists were rapid in onset, with maximal decreases in body temperature observed at 30–60 min post-injection. After 60 min the body temperature of the drug-treated animals returned gradually towards that of the vehicle-controls. The highest doses of UK-14,304

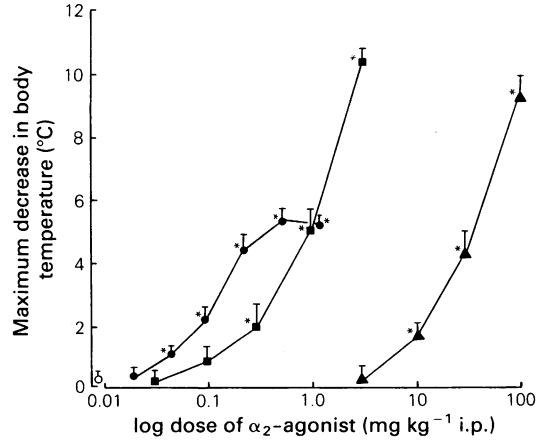


Figure 1 Dose-response relationships for the hypothermia induced by clonidine, UK-14,304 and B-HT 933 in non-reserpinized mice. Groups of 8 mice were dosed i.p. with clonidine (●), UK-14,304 (■), B-HT 933 (▲) or saline (○). Body temperatures were recorded at 30 min intervals for the next 2 h, and the mean maximum decrease in body temperature occurring over this time period calculated for each treatment group. * $P < 0.05$ (Student's unpaired *t* test cf. vehicle (saline) control group). Vertical bars denote ± s.e.mean.

and B-HT 933, 3 and 100 mg kg^{-1} respectively, elicited marked (10–10.5°C) decreases in temperature (Figure 1). In contrast, the maximal hypothermic effect of clonidine (a 5.5°C decrease in body temperature) was less pronounced and reached a plateau at a dose of 0.5 mg kg^{-1} i.p. Clonidine and UK-14,304 were 30–100 fold more potent than B-HT 933 as hypothermic agents.

Effects of reserpine treatment

Reserpine (2.5 mg kg^{-1}, s.c.) evoked a marked and long lasting hypothermia in the mouse. By 18 h after injection the animals were completely immobile, full blepharoptosis was present and body temperatures had decreased to only slightly (0.5–1.5°C) above ambient (19°C), a level of hypothermia that persisted for at least the following 12 h. The s.c. administration of the vehicle for reserpine (10 ml kg^{-1}) had no significant effect on mouse body temperature. At 18 h post-injection the mean body temperature of a group of 8 mice treated with the reserpine-vehicle was 35.5 ± 0.2°C compared to a mean body temperature of 35.8 ± 0.1°C for a group of 8 untreated mice maintained at 19°C for 18 h.

Effects of α_2-adrenoceptor agonists on the body temperature of reserpinized mice

Groups of 8 reserpinized mice were injected i.p. with either vehicle or various doses of clonidine, UK-14,304 or B-HT 933 18 h after reserpine. Body temperatures were recorded prior to drug injection and at 1 or 2 h intervals for the next 8 h.

The i.p. injection of saline alone had no significant effect on body temperature (Figure 2). Clonidine, UK-14,304 and B-HT 933 all evoked a moderate (maximum 10°C) and dose-related reversal of reserpine-induced hypothermia (Figure 3), with lowest doses inducing a significant increase in body temperature of 0.012, 0.02 and 2.5 mg kg^{-1} i.p. respectively. The time-dependent effects of various doses of clonidine (0.032, 0.2 and 1.15 mg kg^{-1}) on the body temperature of reserpinized mice are shown in Figure 2. Similar time-response curves were obtained with appropriate doses of UK-14,304 and B-HT 933. The thermogenic actions of the lower doses of all three agonists were slow in onset, such that the most rapid increases in body temperature were recorded 3–5 h after treatment with peak effects observed at 5–7 h. Maximal thermogenic responses (9–10°C increases in body temperature) were elicited by doses of 0.2, 0.5 and 16 mg kg^{-1} i.p. of clonidine, UK-14,304 and B-HT 933 respectively, and did not differ appreciably in magnitude. Higher doses of the agonists elicited a more even change in temperature with time, but the peak effects were progressively

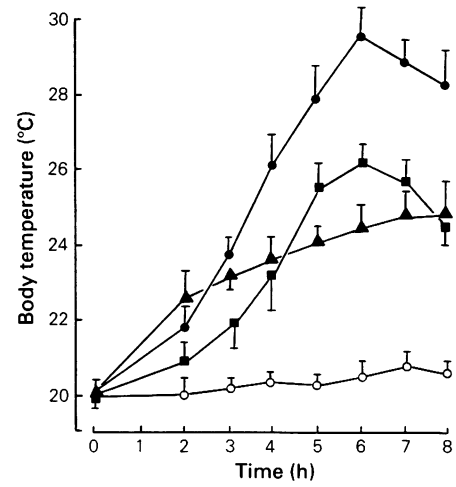


Figure 2 Time-dependent effects of various doses of clonidine on the body temperature of reserpinized mice. Groups of 8 mice were injected i.p. with vehicle (○), or 0.032 (■), 0.2 (●) and 1.15 mg kg^{-1} (▲) of clonidine 18 h after administration of reserpine (2.5 mg kg^{-1}, s.c.). Body temperatures were recorded prior to clonidine and at 1 or 2 h intervals for the next 8 h. The mean body temperature of each treatment group was calculated at each time point. Peak thermogenic responses to 0.032 and 0.2 mg kg^{-1} of clonidine were clearly defined and occurred at 6 h post-injection. Vertical bars denote ± s.e.mean.

smaller, i.e. the dose-response curves for the thermogenic actions of all three agonists were bell-shaped (Figure 3). In molar terms, clonidine and UK-14,304 were approximately equipotent as thermogenic agents, and both drugs were considerably (30–100 fold) more potent than B-HT 933.

Effects of intra-cerebroventricular injection of clonidine on the body temperature of reserpinized mice

Groups of reserpinized mice were injected i.c.v. with sterile isotonic saline or clonidine (0.8–200 μg kg^{-1}). Body temperatures were recorded prior to clonidine and at 1 h intervals for the next 6 h.

The i.c.v. injection of saline alone produced a slight (2–3°C), but statistically significant ($P < 0.05$) increase in the body temperature of reserpinized mice. The dose-related effects of clonidine on body temperature following i.c.v. and i.p. (0.012–1.25 mg kg^{-1}) administration are compared in Figure 4. Central injection of clonidine (0.8–12 μg kg^{-1}) evoked a dose-dependent and marked reversal (maximum effect, 11°C increase) of reserpine-induced hypothermia. This thermogenic effect was more rapid in onset than when the agonist

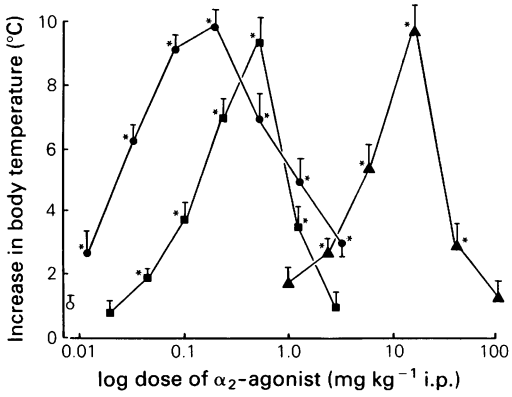


Figure 3 Dose-response relationships for the reversal of reserpine-induced hypothermia by clonidine, UK-14,304 and B-HT 933. Groups of 8 reserpinized mice were injected i.p. with clonidine (●), UK-14,304 (■), B-HT 933 (▲) or saline vehicle (○) 18 h after administration of reserpine (2.5 mg kg^{-1}, s.c.). Body temperatures were recorded prior to the injection of agonist and at 1 or 2 h intervals for the next 8 h. The mean maximum increase in body temperature occurring over this 8 h period was calculated for each dose of the agonist. The dose-response curves for all three agonists were bell-shaped. * $P < 0.05$ (Student's unpaired t test cf. vehicle (saline) control group). Vertical bars denote ± s.e.mean.

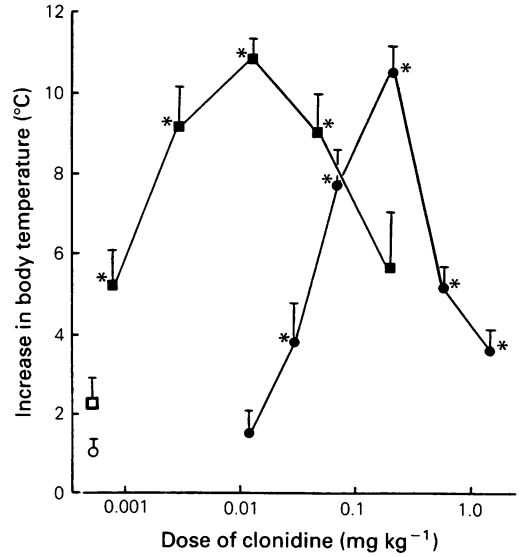


Figure 4 Dose-related effects of clonidine on the body temperature of reserpinized mice following intracerebroventricular (i.c.v.) and i.p. administration. Groups of 8 reserpinized mice were injected i.c.v. with 5 µl per mouse of sterile saline (□) or 0.8–200 µg kg^{-1} of clonidine (■), or i.p. with vehicle (○) or clonidine (0.012–1.25 mg kg^{-1}) (●) 18 h after reserpine (2.5 mg kg^{-1}, s.c.). Body temperatures were recorded at 1 h intervals for the next 6 h. The mean maximum increase in body temperature occurring over this 6 h period was calculated for each treatment group. Clonidine was 20 fold more potent as a thermogenic agent following i.c.v. injection. * $P < 0.05$ (Student's unpaired t test cf. i.c.v. saline or i.p. saline). Vertical bars denote ± s.e.mean.

was given peripherally, with peak increases in body temperature observed at 3–4 h after i.c.v. injection. As when the drug was injected i.p., the dose-response curve for reversal of reserpine-induced hypothermia by i.c.v. clonidine was bell-shaped, with higher doses of clonidine (50–200 µg kg^{-1}) inducing progressively smaller increases in temperature. Comparison of doses to elicit maximal thermogenic responses revealed that clonidine was 20 fold more potent when injected i.c.v. than i.p.

Effects of α- and β-adrenoceptor antagonists on the thermogenic actions of α_2-adrenoceptor agonists in reserpinized mice

The doses of clonidine (0.2 mg kg^{-1}), UK-14,304 (0.4 mg kg^{-1}) and B-HT 933 (16 mg kg^{-1}) used in the antagonist interaction studies were selected on the basis of previous experiments as being doses that would induce near maximal thermogenic responses. The results are summarised in Table 1.

None of the antagonists alone significantly affected the body temperature of reserpinized mice. Pretreatment with the selective α_2-adrenoceptor antagonists, idazoxan (0.05–0.5 mg kg^{-1}), yohimbine (0.1–1.6 mg kg^{-1}) and Wy 26392 (Lattimer *et al.*, 1982) (0.3–5.0 mg kg^{-1}) attenuated the thermogenic responses to all three α_2-agonists in a dose-dependent manner; the ED_{50} values for antagonism of responses to UK-14,304 were 0.15, 0.3 and 1.0 mg kg^{-1} p.o. respectively. Similar doses of these antagonists also inhibited the thermogenic actions of B-HT 933 and clonidine by 50% (Table 1). The rank order of potency of the antagonists, idazoxan > yohimbine > Wy 26392 was the same against all three agonists. In a further experiment, pretreatment of reserpinized mice with idazoxan also attenuated the increase in body temperature elicited by the central injection of clonidine (12 µg kg^{-1}), with an ED_{50} value of 0.1 mg kg^{-1} orally.

The selective α_1-adrenoceptor antagonists, prazosin and indoramin, at doses ⩾3 mg kg^{-1} orally also partially (approx. 50%) inhibited the thermogenic responses to clonidine, UK-14,304 and B-HT 933. These effects, however, were variable, were not clearly dose-related and only reached statistical significance ($P < 0.05$) against UK-14,304 and B-HT 933 at the highest dose of antagonist tested

Table 1 Summary of the effects of selective adrenoceptor antagonists on the thermogenic responses evoked by clonidine, UK-14,304 and B-HT 933 in reserpinized mice

Antagonist	Dose range ($mg\,kg^{-1}$)	*Clonidine* ($0.2\,mg\,kg^{-1}$) Effect	ED_{50}	*UK-14,304* ($0.4\,mg\,kg^{-1}$) Effect	ED_{50}	*B-HT 933* ($16\,mg\,kg^{-1}$) Effect	ED_{50}
Yohimbine (α_2)	0.1–1.6	+++	0.6 (0.4–0.8)	+++	0.3 (0.2–0.4)	+++	0.3 (0.2–0.4)
Idazoxan (α_2)	0.05–0.5	+++	0.25 (0.2–0.3)	+++	0.15 (0.05–0.2)	+++	0.15 (0.1–0.2)
Wy 26392 (α_2)	0.3–5.0	+++	1.2 (0.9–1.6)	+++	1.0 (0.5–2.0)	+++	0.75 (0.5–1.0)
Indoramin (α_1)	0.3–10	++	≃5†	+	>10	+	>10
Prazosin (α_1)	0.3–10	++	≃5†	+	>10	+	>10
Propranolol ($\beta_{1\&2}$)	0.3–10	+	>10	+	>10	+	>10

The antagonists were administered to groups of 8 reserpinized mice orally 30 min prior to the i.p. injection of the agonists. The mean maximum increase in body temperature over the 6 h period after injection of the agonist was calculated for each treatment group and % inhibition by the antagonists determined. (+++) = complete dose-related inhibition, (++) = partial inhibition (approx. 50% inhibition at 2 dose levels), (+) = slight inhibition (significant effect at top dose tested only). For each treatment group the mean maximum increase in body temperature over the 6 h period post injection of the agonist was calculated. From these data the percentage inhibition by the antagonists of the response to each agonist was determined and ED_{50} values (doses to inhibit the thermogenic response to each agonist by 50%) and 95% confidence limits obtained by linear regression analysis. † denotes the ED_{50} value was estimated from data where the effect of the antagonist was not clearly dose-related.

($10\,mg\,kg^{-1}$). Similarly, the β-adrenoceptor antagonist, propranolol, significantly ($P < 0.05$) attenuated (30–50%) the thermogenic responses to the α_2-agonists only at the relatively high dose of $10\,mg\,kg^{-1}$ (Table 1).

Effects of single doses of idazoxan, indoramin and propranolol on the dose-related effects of clonidine in reserpinized mice

In a further series of experiments, groups of reserpinized mice received single doses of either idazoxan ($0.3\,mg\,kg^{-1}$), indoramin ($10\,mg\,kg^{-1}$), propranolol ($7.5\,mg\,kg^{-1}$) or vehicle, orally, 17 h after reserpine. Thirty minutes later saline or a series of doses of clonidine ($0.032–3.1\,mg\,kg^{-1}$) were injected i.p. Body temperatures were recorded before each drug treatment and at 2 h intervals for 6 h post-clonidine. The results of these experiments are summarized in Figures 5a, b and c.

Pretreatment with idazoxan completely abolished the thermogenic responses to low doses ($\leqslant 0.2\,mg\,kg^{-1}$) of clonidine, and produced a 6 fold, parallel shift to the right in the bell-shaped dose-response curve to the drug (Figure 5a). Indoramin pretreatment significantly ($P < 0.05$) reduced the thermogenic responses to all but the lowest dose of clonidine ($0.032\,mg\,kg^{-1}$) by approximately 45–55% (Figure 5b). Pretreatment with propranolol produced a slight attenuation of the responses to all but the highest dose of clonidine ($3.1\,mg\,kg^{-1}$), an effect which reached a significant ($P < 0.05$) level only against a dose of $0.2\,mg\,kg^{-1}$ of the agonist (Figure 5c).

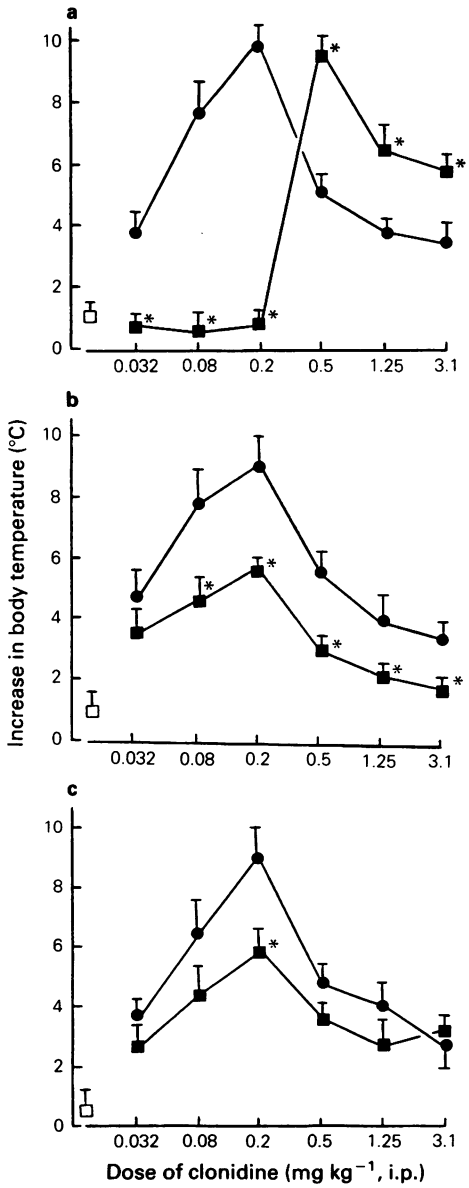
Effects of catecholamine synthesis inhibition on the responses to clonidine and desipramine in reserpinized mice

Desipramine ($0.13–12.5\,mg\,kg^{-1}$ i.p.) evoked a marked (3–13°C) and dose-related increase in the body temperature of mice that had received reserpine alone. Pretreatment with the catecholamine synthesis inhibitor, α-MPT ($300 + 100\,mg\,kg^{-1}$, i.p.), markedly attenuated (60–95%) the thermogenic responses to all doses of desipramine (Figure 6a). In contrast, the thermogenic action of clonidine ($0.032–1.25\,mg\,kg^{-1}$, i.p.) was only slightly decreased (10–35%) by treatment with α-MPT (Figure 6b), an effect which reached statistical significance ($P < 0.05$) only against responses to 0.2 and $0.5\,mg\,kg^{-1}$ of the agonist.

Effects of reserpine and α-methyl-p-tyrosine on brain monoamine and metabolite levels

By 18 h after the injection of reserpine ($2.5\,mg\,kg^{-1}$, s.c.) brain levels of NA and dopamine had decreased to only 5% and 5-HT levels to only 17% of those measured in the brains of vehicle-treated control

mice (Table 2). In contrast, a 130% increase in DOPAC and a 62% increase in 5-HIAA levels were observed 18 h after reserpine. The combined treatment with α-MPT and reserpine further depleted brain levels of NA and dopamine to only 3% of control values, but had no further effect on brain 5-HT levels. Pretreatment with α-MPT did, however, completely attenuate the production of DOPAC (Table 2), indicating that catecholamine synthesis had been almost completely inhibited.



Discussion

The present study demonstrates that clonidine and two highly selective α_2-adrenoceptor agonists, B-HT 933 (Kobinger & Pichler, 1977) and UK-14,304 (Cambridge, 1981), evoke a marked and dose-related reversal of reserpine-induced hypothermia in the mouse. The data presented also suggest that this thermogenic action is mediated through activation of α_2-adrenoceptor mechanisms. Thus, clonidine was a slightly more potent thermogenic agent than UK-14,304, which is in agreement with the relative potencies of the two agonists for inducing mydriasis in the anaesthetized rat (Berridge *et al.*, 1983), an effect thought to be mediated via central α_2-adrenoceptors. The finding that clonidine was approximately 100 fold more potent than B-HT 933 also compares well with estimates of their relative potencies as agonists at α_2-adrenoceptors in cardiovascular preparations obtained by Kobinger & Pichler (1977), who found B-HT 933 to be between 80–150 fold less potent than clonidine. Furthermore, the observation that low doses of the α_2-adrenoceptor antagonists, idazoxan, Wy 26392 (Lattimer *et al.*, 1982) and yohimbine attenuated the thermogenic responses to all three α_2-agonists in a dose-dependent manner also suggests the involvement of α_2-adrenoceptor mechanisms. The rank order of potency of the antagonists, idazoxan > yohimbine > Wy 26392 for antagonism of the responses to the α_2-agonists is in agreement with their relative potencies as antagonists at α_2-adrenoceptors in the pithed rat preparation (Doxey *et al.*, 1983; Paciorek *et al.*, 1984). That the α_2-adrenoceptor antagonists did not attenuate the thermogenic action of the agonists by limiting their passage into the central nervous system, is suggested by the fact that low doses of idazoxan abolished the increase in body temperature produced by central

Figure 5 Effects of single doses of (a) idazoxan (0.3 mg kg^{-1}), (b) indoramin (10 mg kg^{-1}) and (c) propranolol (7.5 mg kg^{-1}) on the thermogenic dose-response relationship for clonidine in reserpinized mice. The antagonists or vehicle were administered orally to groups of 8 mice 17 h after reserpine (2.5 mg kg^{-1}, s.c.); 30 min later saline or clonidine (0.032–3.1 mg kg^{-1}) were injected i.p. Body temperatures were recorded prior to each drug treatment at 2 h intervals and for 6 h post clonidine. Data points represent the mean maximum increase in body temperature over the 6 h period post-clonidine. (●) Represent the dose-related effects of clonidine in reserpine/vehicle pretreated animals and (■) the effect of the agonist in reserpine/antagonist pretreated animals; (□) indicate the effect of the antagonist alone. Idazoxan evoked a parallel shift to the right in the dose-response curve to clonidine. * $P < 0.05$ (Student's unpaired t test cf. vehicle/clonidine treated group). Vertical bars denote ±s.e.mean.

injection of clonidine. In addition, pretreatment of reserpinized mice with a single dose of idazoxan shifted the dose-response curve for reversal of reserpine-induced hypothermia by clonidine to the right in a parallel manner; an indication of competitive pharmacological antagonism at the α_2-adrenoceptor level.

The finding that the selective α_1-adrenoceptor antagonists, prazosin and indoramin, at doses $\geqslant 3\,mg\,kg^{-1}$ orally partially antagonized the thermogenic responses to the α_2-agonists might, at first sight, suggest the involvement of α_1-adrenoceptor mechanisms. This seems unlikely for a number of reasons. First, although neurochemical studies show that clonidine is selective for α_2-adrenoceptors only at doses $<0.1\,mg\,kg^{-1}$ and that higher doses stimulate α_1-adrenoceptors (Anden *et al.*, 1976), the other two α_2-agonists are highly selective agents. Both UK-14,304 and B-HT 933 exhibit a greater than 300 fold selectivity for the α_2- over the α_1-adrenoceptor in isolated tissue preparations (Cambridge, 1981; Rhodes, 1986). Second, the doses at which the α_1-antagonists elicited a partial antagonism of the responses to the α_2-agonists can be considered rather high. Deniard *et al.* (1983) found that prazosin attenuated the reversal of reserpine-induced ptosis by the α_1-agonists, methoxamine and phenylephrine in the mouse over the dose range 0.01–0.1 $mg\,kg^{-1}$, i.p. Prazosin at a dose of $3\,mg\,kg^{-1}$ also completely abolished the hyperactivity induced by the central injection of phenylephrine in the mouse (Heal, 1984).

In the present study, the inhibitory effects of prazosin and indoramin were slight and not clearly dose-related, in contrast to the clearly dose-related and complete antagonism produced by the selective α_2-adrenoceptor antagonists. Also, pretreatment with a single dose of indoramin inhibited the responses to a range of doses of clonidine to a similar degree. Furthermore, at high dose levels indoramin and prazosin have been shown to induce a moderate hypothermia in their own right (unpublished observations). These findings suggest that the partial inhibition by the α_1-antagonists of the thermogenic response to the α_2-agonists in reserpinized mice may

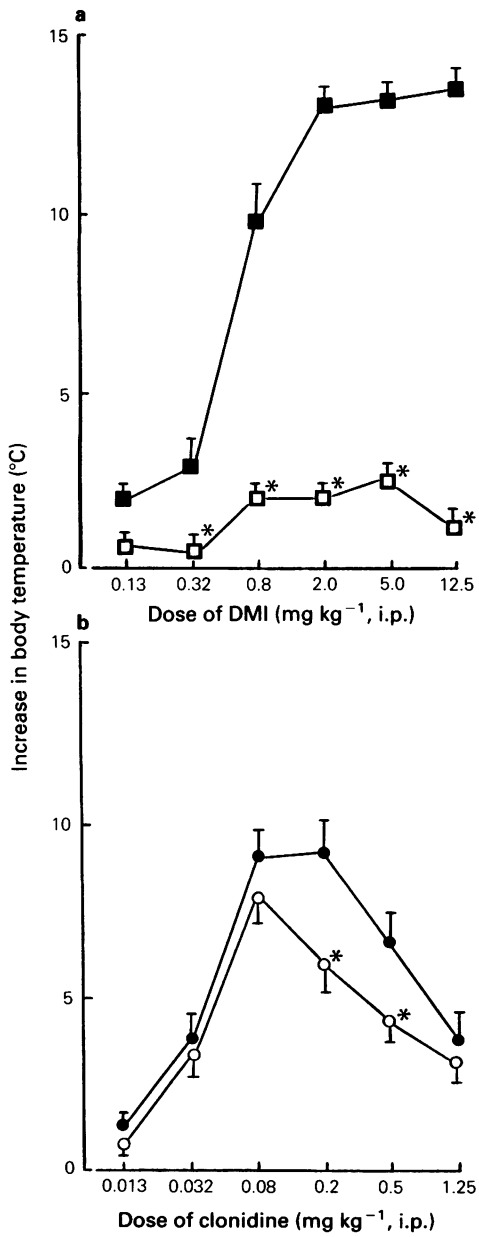


Figure 6 The effects of pretreatment with the catecholamine synthesis inhibitor, α-methyl-*p*-tyrosine (α-MPT) on the thermogenic dose-response relationships to (a) desipramine (DMI) and (b) clonidine in reserpinized mice. Mice ($n = 8$) were dosed i.p. with vehicle or α-MPT ($300\,mg\,kg^{-1}$) 1 h before reserpine ($2.5\,mg\,kg^{-1}$, s.c.). Further doses of vehicle or α-MPT ($100\,mg\,kg^{-1}$) were administered i.p. 16 h after reserpine. DMI (0.13–$12.5\,mg\,kg^{-1}$) and clonidine (0.013–$1.25\,mg\,kg^{-1}$) were injected i.p. 2 h later and body temperatures measured at 1 h intervals for the next 6 h. Solid symbols represent the dose-related effects of DMI and clonidine in animals that received reserpine and vehicle, and open symbols their effects in animals that were treated with reserpine and α-MPT. Pretreatment with α-MPT markedly attenuated the thermogenic responses to all doses of DMI, but only partially attenuated those to 0.2 and $0.5\,mg\,kg^{-1}$ of clonidine. * $P < 0.05$ (Student's unpaired *t* test cf. reserpine alone/DMI or reserpine alone/clonidine treated groups). Vertical bars denote ±s.e.mean.

Table 2 Effects of reserpine and reserpine + α-methyl-*p*-tyrosine treatment on mouse brain monoamine and metabolite levels

Treatments (mg kg^{-1})	*Mouse brain monoamine and metabolite levels* (ng g^{-1} wet weight)				
	NA	*Dopamine*	*DOPAC*	*5-HT*	*5-HIAA*
Vehicle + vehicle	423 ± 16	901 ± 23	107 ± 7	407 ± 20	249 ± 8
Reserpine (2.5 s.c.) + vehicle (i.p.)	22 ± 2*** (−95%)	46 ± 4*** (−95%)	232 ± 11*** (+130%)	69 ± 6*** (−83%)	386 ± 24*** (+62%)
Reserpine (2.5 s.c.) + α-MPT (300 + 100 i.p.)	15 ± 2*** (−97%)	27 ± 2*** (−97%)	ND	92 ± 11*** (−80%)	509 ± 31*** (+114%)

Mice received α-methyl-*p*-tyrosine (α-MPT, 300 mg kg^{-1}, i.p.) or vehicle (saline) 1 h before reserpine (2.5 mg kg^{-1}, s.c.) or vehicle. Further doses of α-MPT (100 mg kg^{-1}, i.p.) or saline were administered 16 h after reserpine. Animals were killed 2 h later (18 h after reserpine) and whole brain levels of monoamines and metabolites assayed as described in the methods section. Reported values are group means ± s.e.mean (n = 5–6). Figures in parentheses represent % change in levels cf. vehicle/vehicle-treated control group.
ND = no DOPAC detected. *** $P < 0.001$ (Student's unpaired t test cf. vehicle-treated control group).

be due to an indirect physiological interaction rather than a direct antagonism at the receptor level. Thus, it is possible that prazosin and indoramin partially opposed the thermogenic effect of the α_2-agonists by blocking α_1-adrenoceptors located on vascular smooth muscle, thereby preventing vasoconstriction and reducing heat conservation.

In the present experiments a dose of 10 mg kg^{-1} of the β-adrenoceptor antagonist, propranolol, also slightly attenuated the thermogenic response to the α_2-agonists. This dose of propranolol may be considered somewhat high for β-adrenoceptor blockade, since Ross (1980) showed that the reversal of reserpine-induced hypothermia in the mouse by clenbuterol, a potent and selective β_2-agonist, was completely abolished by pretreatment with a dose of 2.5 mg kg^{-1}, i.p. of the antagonist. It is therefore possible that propranolol also partially opposed the thermogenic response to the α_2-agonists through a physiological mechanism, i.e. via blockade of β-adrenoceptors located in brown adipose tissue, thereby reducing thermogenesis.

The observation that α_2-adrenoceptor agonists produce a marked increase in the body temperature of reserpinized mice may at first sight be somewhat surprising. In normal laboratory rodents, α_2-agonists induce signs of CNS depression, including overt sedation (Drew *et al.*, 1979), hypoactivity (Delini-Stula *et al.*, 1979) and a moderate to marked hypothermia (Gorka & Zacny, 1981; Colpaert, 1986). These effects are thought to be mediated via stimulation of central and peripheral presynaptic α_2-autoreceptors and a consequent decrease in NA release from noradrenergic nerve terminals (Langer, 1974). Interestingly, clonidine has also previously been reported to inhibit the onset of reserpine-induced hypothermia and ptosis in the mouse when injected prior to reserpine (Gouret *et al.*, 1977). The mechanism responsible for these anti-reserpine effects of clonidine would appear to be stimulation of α_2-autoreceptors. The rate of depletion of intraneuronal stores of catecholamine by reserpine is known to be largely dependent on the degree of neuronal activity (Wakade, 1980). Activation of α_2-autoreceptors by clonidine reduces the rate of firing of noradrenergic neurones (Cedarbaum & Aghajanian, 1977), inhibits NA release and decreases NA turnover, and would therefore be expected to reduce the rate of depletion of NA and inhibit the onset of the behavioural and autonomic signs induced by reserpine. Recent neurochemical studies have confirmed that pretreatment of rats with low doses of clonidine (< 0.2 mg kg^{-1}, i.p.) decreases the rate of depletion of cortical NA by tetrabenazine, a rapidly acting reserpine-like compound (Pettibone *et al.*, 1984).

It seems unlikely, for a number of reasons, that such a presynaptic mechanism is involved in the thermogenic responses to α_2-agonists in animals, in which a marked hypothermia has already been established by the previous administration of reserpine. Firstly, brain NA was markedly depleted (95%) by reserpine prior to the injection of the α_2-agonists. Secondly, stimulation of presynaptic α_2-adrenoceptors might be expected to enhance rather than reverse the hypothermia. Recently, however, the existence of postsynaptic as well as presynaptic α_2-adrenoceptors has been demonstrated in both the periphery and central nervous system (see Timmermans & Van Zwieten, 1981). In fact, radioligand binding studies using [^{3}H]-clonidine suggest that the majority of central α_2-adrenoceptors are located postsynaptically (Dausse *et al.*, 1982).

The observation that pretreatment with the cate-

cholamine synthesis inhibitor, α-MPT, prevented the reversal of reserpine-induced hypothermia by the NA uptake inhibitor, desipramine, is in agreement with the previous work of Sulser & Bickel (1962). This result supports the view that the thermogenic action of desipramine in reserpinized mice is dependent upon the presynaptic-release of newly synthesized NA and the consequent prolongation of the action of the neurotransmitter in the synaptic cleft. In contrast, the thermogenic response to clonidine in reserpinized mice does not appear to require this phenomenon, since treatment with α-MPT had very little effect on the increases in body temperature evoked by the α_2-agonist. This finding suggests that the α_2-adrenoceptor mechanisms involved in mediating the thermogenic action of α_2-agonists are located postsynaptically. Furthermore, centrally injected clonidine was considerably more potent than peripherally administered clonidine at increasing the body temperature of reserpinized mice. This strongly implies that the α_2-adrenoceptors in question are situated in the central nervous system. A recent report has also suggested that the hyperthermia elicited by clonidine in rats maintained at a high ambient temperature is mediated via central postsynaptic α_2-adrenoceptors (Mogilnicka *et al.*, 1985).

The results of the present study provide no clue to the mechanisms beyond the level of the central α_2-adrenoceptor ultimately responsible for producing an increase in the body temperature of reserpinized mice after the administration of α_2-agonists. It may be envisaged that activation of heat production and conservation pathways together with a 'switching off' of heat loss effectors is required. However, the precise nature of the mechanisms involved remains to be determined.

The observation that the dose-response relationships for reversal of reserpine-induced hypothermia by clonidine, UK-14,304 and B-HT 933 were bell-shaped is worthy of comment. Although the mechanism responsible for the down slopes of the dose-response curves is not obvious, the finding that pretreatment with a low dose of the α_2-antagonist, idazoxan, shifted the entirety of the bell-shaped dose-response curve to clonidine to the right, suggests that α_2-adrenoceptor mechanisms may also be involved in this aspect of the response to α_2-agonists. Furthermore, it should be noted that the dose-ranges over which clonidine, UK-14,304 and B-HT 933 induced hypothermia in non-reserpinized mice were almost coincident with the dose-ranges of the down slopes of their dose-response curves in reserpinized mice. Since, the hypothermic response to α_2-agonists in normal laboratory rodents is thought to be mediated via α_2-adrenoceptor stimulation (Gorka & Zacny, 1981), the above findings further imply that the mechanism of the down slopes of the thermogenic dose-response curves to the α_2-agonists in reserpinized mice may also involve α_2-adrenoceptors. Precisely how activation of two different α_2-adrenoceptor mechanisms might have opposing effects on the body temperature of the reserpinized mice appears obscure, and remains the subject of further study.

In summary, we have shown that three selective α_2-adrenoceptor agonists produce a marked reversal of reserpine-induced hypothermia in the mouse. The results of this study strongly suggest that the thermogenic action of these agonists is mediated via activation of a central mechanism involving postsynaptic α_2-adrenoceptors.

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The effects of acute and chronic desipramine on the thermogenic and hypoactivity responses to α_2-agonists in reserpinized and normal mice

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1 The effects of acute and chronic (14 day) administration of the noradrenaline uptake inhibitor, desipramine (DMI), on the thermogenic responses to clonidine in reserpine-treated mice, and on the hypothermic and hypoactivity responses to the α_2-agonist, UK-14,304, in untreated mice were examined.

2 Taking the capacity of DMI to delay the onset of reserpine-induced hypothermia as an indicator of noradrenaline (NA) uptake inhibition, the lowest dose of DMI to inhibit uptake significantly for 12 h in the mouse was shown to be between 10 and 20 mg kg^{-1} orally.

3 Chronic (every 12 h for 14 days), but not acute treatment with DMI (15 mg kg^{-1}, orally), attenuated the thermogenic responses to low doses (0.02–0.225 mg kg^{-1}, i.p.) of clonidine (injected 20 h after the last dose of DMI) in reserpinized mice.

4 Acute DMI administration slightly attenuated the hypothermia and hypoactivity induced by UK-14,304 (0.25–1.0 mg kg^{-1}, i.p.) when injected 2 h, but not when injected 18–21 h before the agonist. In contrast, 18–21 h after withdrawal from chronic DMI both of these responses to UK-14, 304 were markedly attenuated.

5 As the thermogenic response to clonidine in reserpinized mice appears to involve central postsynaptic α_2-adrenoceptors, these results suggest that prolonged inhibition of NA uptake decreases the sensitivity of postsynaptic α_2-adrenoceptors. The results of the studies using UK-14,304 indicate that central α_2-adrenoceptors involved in mediating other behavioural and pharmacological responses to α_2-agonists are also down-regulated by chronic inhibition of NA uptake.

Introduction

Although many studies indicate that a down-regulation of central β-adrenoceptor sensitivity accompanies chronic antidepressant administration, the consequences of such treatment on α_2-adrenoceptor function are more equivocal (for reviews see Charney *et al.*, 1981; Sugrue, 1983). The development of α_2-adrenoceptor subsensitivity has been indicated by the finding that chronic, but not acute treatment, with the noradrenaline (NA) uptake inhibitor desipramine (DMI) attenuates the abilities of clonidine to inhibit NA turnover in rat brain (McMillen *et al.*, 1980; Sugrue, 1981) and to induce sedation in rodents (Spyraki & Fibiger, 1980; Heal *et al.*, 1983). Although a decrease in α_2-autoreceptor sensitivity is thought to be responsible for the former observation, it is not clear whether clonidine-induced sedation is mediated via central pre- (Zebrowska-Lupina *et al.*, 1977) or postsynaptic (Spyraki & Fibiger, 1982) α_2-adrenoceptors.

In contrast to the above findings, radioligand binding studies provide no consistent evidence of a down-regulation of α_2-adrenoceptor number during chronic treatment with NA uptake inhibitors. Thus, increases (Johnson *et al.*, 1980), decreases (Campbell & McKernan, 1982) and no change (Sugrue, 1982) in the binding of [^{3}H]-clonidine to rat brain preparations have been reported after repeated tricyclic antidepressant administration. A complicating factor in these binding studies is the precise neuronal location of the recognition sites for the radioligand. Selective destruction of central noradrenergic neurones with 6-hydroxydopamine is associated with an

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increase (Dausse *et al.*, 1982) or only a slight decrease (U'Prichard *et al.*, 1979) in the binding of [^{3}H]-clonidine in rat cortex. This implies that α_2-autoreceptors on noradrenergic nerve terminals represent only a small proportion of the total population of α_2-adrenoceptors in the rat cortex. Arguably, therefore, the apparent inconsistencies mentioned above may be indicative of a differential effect of long term NA uptake inhibition on the sensitivity of pre- and postsynaptic α_2-adrenoceptors.

The thermogenic actions of α_2-adrenoceptor agonists in reserpinized mice appear to be mediated primarily via activation of central postsynaptic α_2-adrenoceptors (Bill & Stephens, 1984; Bill *et al.*, 1989). In the following study this response to α_2-agonists has been used to try to gain further insight into the effects of chronic administration of a NA uptake inhibitor on the sensitivity of central postsynaptic α_2-adrenoceptors. The effects of this long term treatment on the hypothermia and hypoactivity induced by the α_2-agonist, UK-14,304 (Cambridge, 1981), in non-reserpinized mice were also examined for comparison.

Methods

Animals

Female albino T/O strain mice (20–26 g) supplied by Tuck were used. Before all experiments and during the period of chronic dosing they were housed in groups of 15 at an ambient temperature of 21–23°C on a 12 h light/dark cycle (light period 08 h 00 min to 20 h 00 min) with free access to standard laboratory diet (CRM pellets) and tap water.

Measurement of body temperature

Body temperatures were measured with a thermistor probe inserted 2 cm into the colon and a digital thermometer (Comark Electronics Limited, Sussex) while the mice were lightly restrained in the hand by the scruff and the tail. Experiments were performed on groups of 8 mice per treatment which were housed in plastic boxes (30 × 15 × 12 cm) with sawdust in the bottom and at an ambient temperature of 19 ± 0.5°C.

Measurement of locomotor activity

Locomotor activity was recorded in individual mice in an automated activity monitor apparatus, designed and produced by the Equipment Development Laboratory at Wyeth Research (UK) Limited. The apparatus consisted of a cylindrical field, with dark outer and inner walls (50 and 17 cm in diameter respectively and 34 cm tall) forming an annular track. Six pairs of infra red transmitter and receiver beam units were arranged at 30° intervals around the outside of the outer wall in line with windows in both outer and inner walls, such that the transmitters and receivers formed diametrically opposite pairs. For each transmitter/receiver pair therefore, there were two positions around the annular field where their beam could be broken. Beam breaks were counted automatically by a counter/printer unit, and number of beam breaks for each transmitter/receiver pair were printed out at 2 min intervals. The output from the six pairs was summed to give total number of beam breaks (locomotor activity) per 2 min period. A delay was built into the counters which prevented a further count for 0.5 s after a beam was re-made. This effectively limited the counting rate to 2 counts per second for each transmitter/receiver pair, or 12 counts per second for the overall system.

Effects of desipramine (DMI) on the onset of reserpine-induced hypothermia

In order to determine the dose- and time-dependent effects of DMI treatment on the rate of onset of reserpine-induced hypothermia, vehicle or various doses of DMI (5, 10 and 20 mg kg^{-1}) were administered orally to groups of 10 mice at intervals of 2, 6 or 12 h before the s.c. injection of reserpine (2.5 mg kg^{-1}). Body temperatures were recorded immediately before each treatment and at 1, 2, 4, 6 and 8 h after the injection of reserpine. The onset of reserpine-induced hypothermia was also recorded in a similar manner in mice that had received either vehicle or 10 or 20 mg kg^{-1} of DMI orally every 12 h for 6 days before challenge with reserpine.

Effect of DMI on the thermogenic response to clonidine in reserpinized mice

Two groups of mice, each containing 64 animals were dosed orally every 12 h for 14 days with either vehicle or DMI (15 mg kg^{-1}). A further 2 groups, each containing 64 mice, received a single acute dose of DMI or vehicle. Twelve hours after the last chronic or acute treatment each animal received reserpine (2.5 mg kg^{-1}, s.c.). Eight hours later, subgroups ($n = 8$) from each treatment group were injected i.p. with saline or one of seven doses of clonidine (0.02, 0.045, 0.1, 0.225, 0.51, 1.15 and 2.6 mg kg^{-1}). Body temperatures were recorded immediately before clonidine administration and at 1 h intervals for the following 6 to 8 h.

Effects of DMI on UK-14,304-induced hypolocomotion and hypothermia

Two groups of mice, each containing 32 animals, were dosed orally twice daily for 14 days with either DMI (15 mg kg^{-1}) or vehicle. Eighteen to 21 h after the last dose, sub-groups ($n = 8$) from each treatment group were injected i.p. with either saline or various doses of UK-14,304 (0.25, 0.5 and 1.0 mg kg^{-1}). Thirty minutes later each animal was placed individually in the automated open field and locomotor activity was recorded during the first 2 min of exposure to this novel environment. Body temperatures were measured immediately prior to and at 35 min post injection of UK-14,304. The dose-related effects of UK-14,304 on locomotor activity and body temperature were also determined in a similar manner in mice at 2 h and at 18–21 h after acute treatment with either DMI (15 mg kg^{-1}, orally) or vehicle.

Experimental design and statistics

In all experiments animals were dosed according to a predetermined sequence such that they were assessed in a balanced order with respect to time and treatment received. Also, with the exception of reserpine, the experimenter was unaware of the treatment(s) that each animal had received throughout.

Body temperatures, temperature changes and locomotor activity scores are given as mean ± s.e.mean. The results from the experiments to determine the effects of DMI on the onset of reserpine-induced hypothermia (Figure 1) and the dose-related effects of UK-14,304 on the locomotor activity and body temperature of previously untreated mice (Table 1) were analysed by analysis of variance followed by Duncan's method for multiple

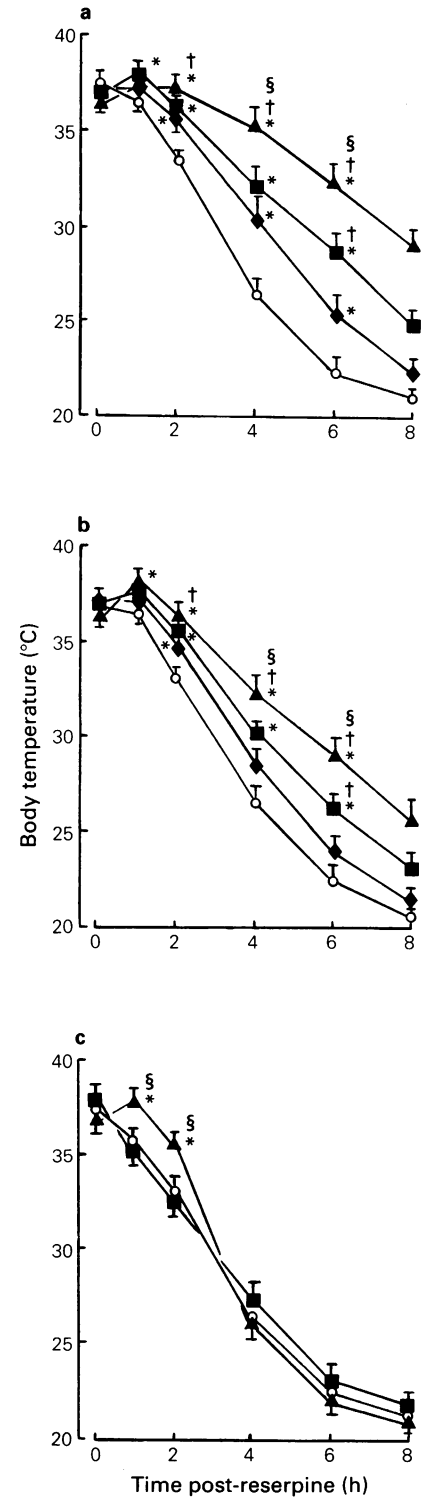


Figure 1 Effect of acute treatment with desipramine (DMI) on the onset of reserpine-induced hypothermia. Mice ($n = 8$ per treatment) were dosed orally with either vehicle (○), or DMI at doses of 5 (◆), 10 (■) or 20 (▲) mg kg^{-1}. Reserpine (2.5 mg kg^{-1}) was injected s.c. at (a) 2 h, (b) 6 h and (c) 12 h after DMI. Body temperatures were measured prior to reserpine and at 1 or 2 h intervals for 8 h. Data points show mean body temperature. Error bars denote ± s.e.mean. All doses of DMI significantly inhibited the onset of hypothermia when given 2 h and 6 h prior to reserpine. Only a dose of 20 mg kg^{-1} was effective when given 12 h before reserpine. * $P < 0.05$ (5, 10 and 20 mg kg^{-1} of DMI vs vehicle control); † $P < 0.05$ (10 and 20 vs 5 mg kg^{-1}); § $P < 0.05$ (20 vs 10 mg kg^{-1}), ANOVAR followed by Duncan's method for multiple comparisons. The acute effects of 10 and 20 mg kg^{-1} of DMI on the onset of reserpine-induced hypothermia did not differ significantly from those seen after repeated (twice daily for 6 days) treatment with 10 and 20 mg kg^{-1} of the drug.

comparisons (Duncan, 1955). All other data were analysed by nested analysis of variance followed by Student's t test (unpaired).

Drugs

The following drugs were used: clonidine hydrochloride (Boehringer Ingelheim), desipramine hydrochloride (Geigy Pharmaceuticals), reserpine (Sigma), UK-14,304 (5-bromo-6-[2-imidazolin-2-yl-amino]-quinoxaline tartrate, (Pfizer)).

For oral administration desipramine was dissolved in distilled water. Clonidine and UK-14,304 were dissolved in isotonic saline. Reserpine was solubilized in 4% benzyl alcohol, 0.5% citric acid and 20% Tween 80 in distilled water to give a final concentration of reserpine of 5 mg ml^{-1}. This stock solution was diluted with distilled water immediately before use to give a concentration appropriate for the dose volume used for all treatments, i.e. 10 ml kg^{-1}. Drug doses in the text refer to salt.

Results

Effects of DMI on the onset of reserpine-induced hypothermia

Reserpine (2.5 mg kg^{-1}, s.c.) evoked a marked and long lasting hypothermia in vehicle-pretreated mice, the intensity of which increased with time (Figure 1). By 8 h after injection of reserpine body temperatures had decreased from a normal value of 37.0 $\pm$ 0.5°C to only 1–2°C above ambient (19°C), an effect that persisted for at least the following 24 h.

Doses of 5, 10 and 20 mg kg^{-1} orally of DMI administered 2 h before reserpine markedly reduced the rate of onset of hypothermia in a dose-dependent manner (Figure 1a). These doses of DMI also significantly inhibited the onset of hypothermia when given 6 h before reserpine (Figure 1b), although the effects were less marked than those observed after the 2 h pretreatment. However, when the interval between treatments was extended to 12 h, the minimum dose to attenuate the initial hypothermic response to reserpine significantly ($P < 0.05$) was 20 mg kg^{-1} (Figure 1c). In mice treated twice daily with DMI (10 and 20 mg kg^{-1}) for 6 days, the rate of onset of the hypothermia induced by reserpine injected 2–12 h after the last dose of DMI did not differ significantly from that in animals given these doses acutely. None of the doses of DMI used in these experiments significantly affected the body temperature of non-reserpinized mice.

Effects of acute and chronic DMI treatment on the thermogenic response to clonidine in reserpinized mice

When reserpine was injected 12 h after the last chronic dose of DMI (15 mg kg^{-1}, orally, twice daily for 14 days) the rate of onset of hypothermia in these mice did not differ significantly from that observed in animals, which received reserpine 12 h after the last chronic treatment with vehicle or from that in animals given reserpine 12 h after a single dose of DMI or vehicle (data not illustrated). Thus, there was no evidence of an effect of residual drug 12 h after the last dose of chronic DMI.

A dose-dependent, partial reversal (maximum increase 10°C) of reserpine-induced hypothermia was elicited by clonidine over the dose range 0.02–0.225 mg kg^{-1} i.p. in animals pretreated acutely or chronically with vehicle. Peak thermogenic responses to these doses of clonidine were recorded 4–5 h after treatment (Figures 2a and b). Larger doses of the agonist (0.51–2.6 mg kg^{-1}) evoked progressively smaller responses, with peak effects occurring 6–7 h after injection (Figure 2c), i.e. the dose-response curve to clonidine was bell-shaped (Figure 3).

Acute treatment with DMI (15 mg kg^{-1}), 12 h before reserpine, failed to modify significantly the dose-response relationship for clonidine-induced reversal of reserpine-hypothermia (Figure 3a). In contrast, long term DMI treatment substantially attenuated the thermogenic responses evoked by low doses of clonidine (0.02–0.1 mg kg^{-1}) (Figure 2a and b), and produced a slight shift to the right of the maximally effective dose of clonidine. Responses to higher doses (0.51–2.6 mg kg^{-1}) of the agonist, however, were not significantly affected (Figure 2c). Thus, the up-slope but not the down-slope of the dose-response curve to clonidine was shifted to the right following repeated administration of DMI (Figure 3b).

Effects of acute and chronic DMI treatment on UK-14,304-induced hypolocomotion and hypothermia

In untreated or vehicle-pretreated animals, UK-14,304 (0.25–1.0 mg kg^{-1}, i.p.) induced a dose-related inhibition of locomotor activity and a dose-dependent hypothermia (Table 1). A dose of 1.0 mg kg^{-1} of the agonist consistently elicited a 70–80% reduction of locomotor activity and a 5–5.5°C decrease in body temperature. The mean body temperatures of all groups of animals before injection of saline or UK-14,304 were in the range 36.5–37.2°C.

Acute treatment with DMI (15 mg kg^{-1}) 2 h before injection of UK-14,304 produced a slight attenuation of the sedative and hypothermic responses to all doses of the agonist (Figures 4a and 5a). This effect,

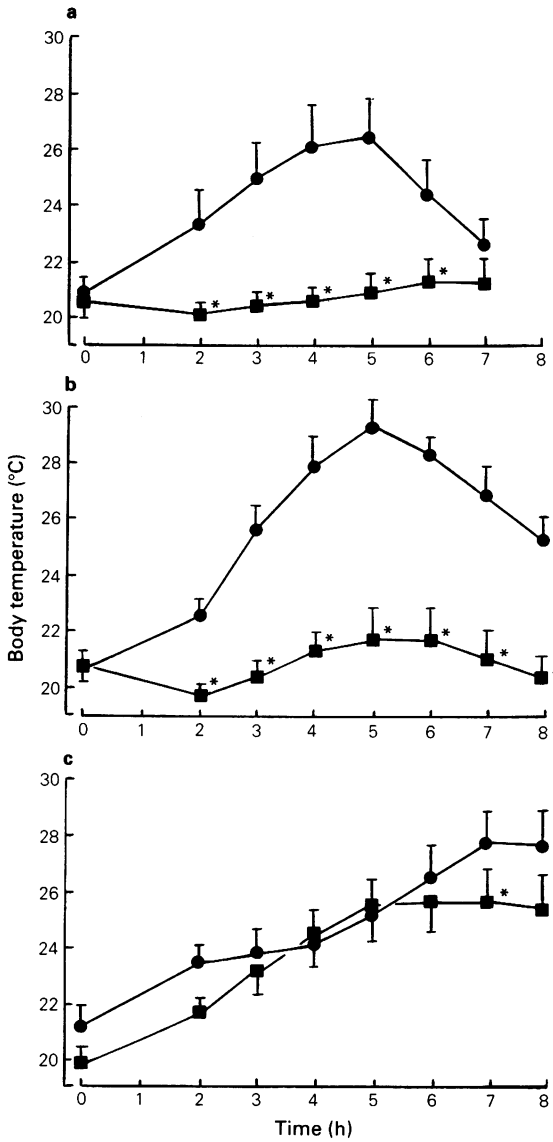


Figure 2 Effect of chronic desipramine treatment (twice daily for 14 days) on the time-related thermogenic responses to (a) 0.045, (b) 0.1 and (c) 1.15 mg kg^{-1} i.p. of clonidine in reserpinized mice. The animals were dosed with reserpine (2.5 mg kg^{-1}, s.c.) 12 h after the last dose of desipramine (DMI) (■) or vehicle (●). Clonidine was injected i.p. 8 h later. Body temperatures were recorded before clonidine and at 1 or 2 h intervals for the following 6 to 8 h. Each point is the mean body temperature of a group of 8 mice injected with clonidine. Error bars denote ±s.e.mean. Chronic DMI markedly attenuated thermogenic responses to 0.045 and 0.1 mg kg^{-1} of clonidine. * $P < 0.1$ (Nested analysis of variance followed by Student's t test cf. chronic vehicle/clonidine treatment).

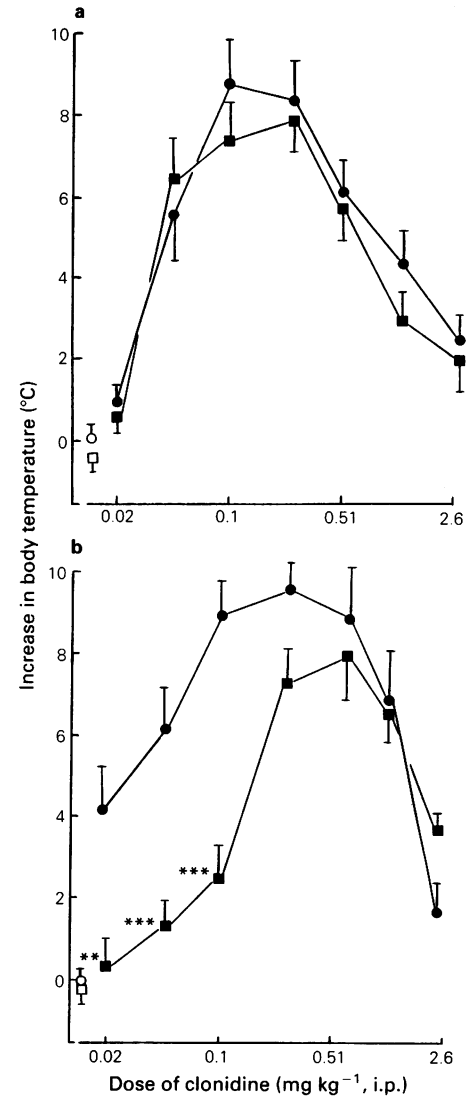
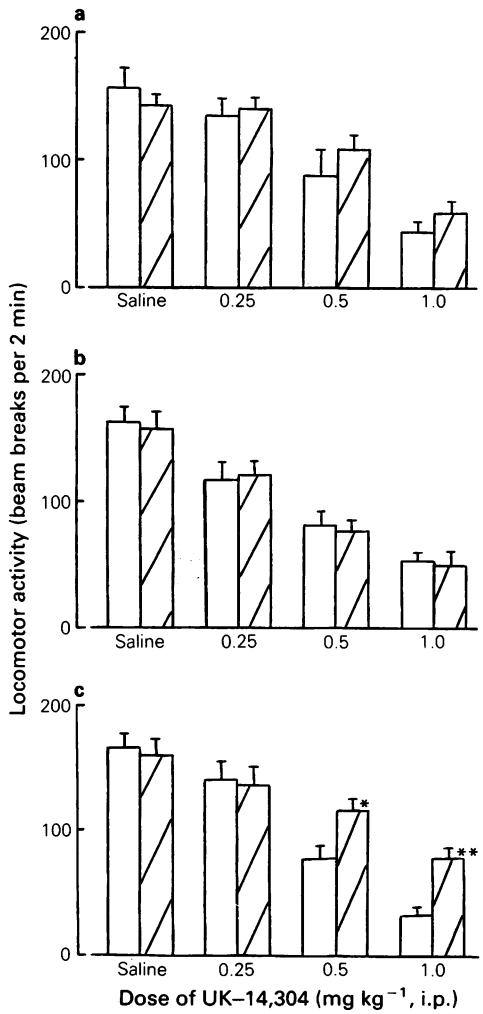


Figure 3 Effects of (a) acute and (b) chronic (twice daily for 14 days) treatment with desipramine (DMI, 15 mg kg^{-1}, orally) on the dose-response curve for clonidine-induced reversal of reserpine-hypothermia. Mice received reserpine (2.5 mg kg^{-1}, s.c.) 12 h after the last dose of DMI (■) or vehicle (●). Saline or clonidine (0.02–2.6 mg kg^{-1} i.p.) were injected 8 h later. Body temperatures were recorded before clonidine and at 1 or 2 h intervals for 6 to 8 h post-clonidine. Each point represents the mean maximum increase in temperature in this period in a group of 8 mice. Error bars denote ± s.e.mean. Open symbols show change in temperature after injection of saline. Chronic DMI significantly attenuated the thermogenic response to low doses of clonidine (0.02–0.1 mg kg^{-1}). ** $P < 0.01$; *** $P < 0.001$ (Nested analysis of variance followed by Student's t test cf. chronic vehicle treated groups).

Table 1 Dose-related effects of UK-14,304 on locomotor activity and body temperature in the mouse

Dose of UK-14,304 (mg kg^{-1}, i.p.)	*Locomotor activity* (counts per 2 min) *30 min post UK-14,304*	*Body temperature* (°C) *Pre*	*Post*
0 (Saline)	163 ± 7	37.1 ± 0.2	37.2 ± 0.2
0.25	143 ± 16	37.1 ± 0.3	35.6 ± 0.4*
0.5	77 ± 12*†	36.9 ± 0.2	33.7 ± 0.4*†
1.0	31 ± 4*†§	37.1 ± 0.2	31.5 ± 0.4*†§

Groups of mice ($n = 8$) were injected i.p. with saline or UK-14,304. Thirty minutes later the locomotor activity of each animal was recorded over a 2 min period as described in the methods section. Body temperatures were measured prior to and 35 min post injection of UK-14,304. Results expressed as mean ± s.e.mean. * $P < 0.01$ (0.25, 0.5 and 1.0 mg kg^{-1} of UK-14,304 vs saline-control), † $P < 0.01$ (0.5 and 1.0 vs 0.25 mg kg^{-1}), § $P < 0.01$ (1.0 vs 0.5 mg kg^{-1}), ANOVAR followed by Duncan's method for multiple comparisons.



however, only reached a statistically significant level ($P < 0.05$) against the hypothermic response to the lowest dose of UK-14,304 used (0.25 mg kg^{-1}). When the interval between treatments was increased to 18–21 h there was no significant difference between the dose-related effects of UK-14,304 on locomotor activity and body temperature in the DMI and vehicle pretreated groups (Figures 4b and 5b).

Repeated administration of DMI twice daily for 14 days, however, significantly attenuated the hypoactivity induced by doses of 0.5 and 1.0 mg kg^{-1} of UK-14,304 injected 18–21 h after the last dose of the uptake inhibitor (Figure 4c), and markedly reduced the hypothermic responses to all three doses of the agonist (Figure 5c).

Neither the acute nor the chronic DMI pretreatments alone significantly affected locomotor activity or body temperature of mice relative to vehicle-treated controls.

Discussion

The induction of a marked hypothermia in rodents by reserpine is thought to result from the depletion

Figure 4 Effect of acute (a and b) and chronic treatment (c) with desipramine (DMI, 15 mg kg^{-1}, orally) on hypoactivity responses to UK-14,304. Mice ($n = 8$ per treatment) were injected with saline or UK-14,304 at either (a) 2 h after acute, (b) 18–21 h after acute or (c) 18–21 h after the last chronic (twice daily for 14 days) treatment with DMI. Open columns show mean locomotor activity following acute or chronic vehicle pretreatment. Hatched columns show activity after DMI treatment. Error bars denote ± s.e.mean. Chronic DMI significantly attenuated responses to 0.5 and 1.0 mg kg^{-1} i.p. of UK-14,304. * $P < 0.05$, ** $P < 0.01$ (Nested analysis of variance followed by Student's t test cf. chronic vehicle-treated groups).

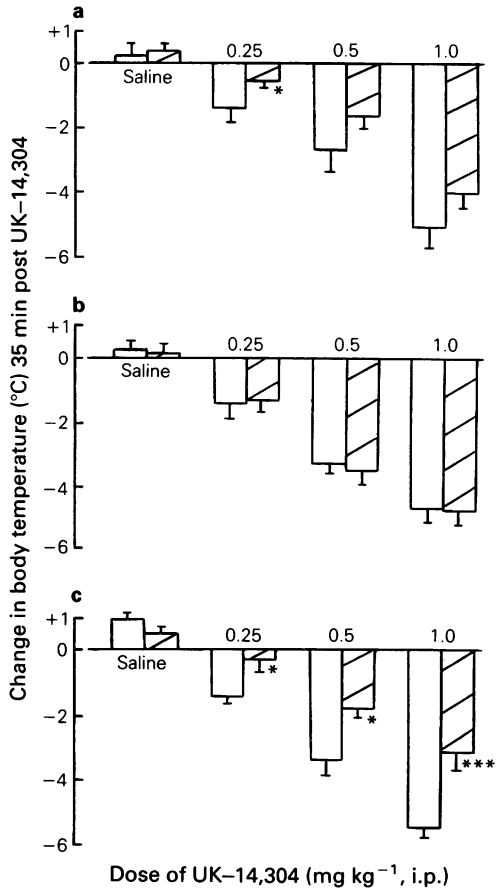


Figure 5 Effects of acute (a and b) and chronic (c) treatment with desipramine (DMI, 15 mg kg^{-1}, orally) on hypothermic responses to UK-14,304. Mice ($n = 8$ per treatment) were injected with saline or UK-14,304 at either (a) 2 h after acute, (b) 18–21 h after acute or (c) 18–21 h after the last chronic (twice daily for 14 days) treatment with DMI. Open columns show mean decreases in body temperature induced by UK-14,304 following acute or chronic vehicle pretreatment. Hatched columns show decreases induced by the agonist after DMI treatment. Error bars denote ± s.e.mean. Chronic DMI significantly attenuated hypothermic responses to all doses of UK-14,304. *$P < 0.05$; ***$P < 0.001$ (Nested analysis of variance followed by Student's t test cf. chronic vehicle-treated groups).

of intraneuronal vesicular stores of noradrenaline (NA) and dopamine in the CNS and periphery and their subsequent destruction by monoamine oxidase (Carlsson, 1975). The ability of classical tricyclic antidepressants to reverse or inhibit the onset of this hypothermia is believed to be achieved by the blockade of the neuronal re-uptake of NA, thereby prolonging the action of the neurotransmitter in the synaptic cleft (Sulser *et al.*, 1969). Taking the capacity of DMI to delay the onset of reserpine-induced hypothermia as an indicator of NA uptake inhibition, the initial studies suggested that the lowest dose of DMI to inhibit uptake significantly for 12 h in the mouse was approximately 15 mg kg^{-1} orally. It was assumed, therefore, that during repeated dosing, almost continuous inhibition of the NA re-uptake process would be afforded by treating mice every 12 h with this dose. This assumption seemed justified by the finding that the initial hypothermic response to reserpine was attenuated to a similar degree by single or repeated treatment with DMI.

Previously presented results suggest that the thermogenic actions of α_2-agonists in reserpinized mice are mediated primarily via activation of a central postsynaptic α_2-adrenoceptor mechanism (Bill & Stephens, 1984; Bill *et al.*, 1989). In the present study, chronic but not acute treatment with DMI markedly attenuated the thermogenic response to low doses of clonidine in reserpinized animals. This effect would not seem to be due to an increase in free concentrations of DMI in the brain during repeated administration and a consequent direct antagonist action of the drug, since (a) DMI is only a very weak α_2-antagonist (Maggi *et al.*, 1980) and (b) the finding that after repeated twice daily administration for 14 days, DMI (15 mg kg^{-1}, orally) failed to inhibit significantly the onset of hypothermia induced by reserpine given 12 h after the final dose of the uptake inhibitor suggests that there was no accumulation of DMI during chronic administration. It seems probable, therefore, that long term persistent inhibition of the NA re-uptake process results in an adaptive decrease in the sensitivity of central postsynaptic α_2-adrenoceptors. This conclusion agrees with that of Mogilnicka *et al.* (1985), who found that chronic treatment with DMI attenuated the hyperthermia induced by clonidine in rats maintained at a high ambient temperature. A complicating factor with this latter model, however, is that the hyperthermic response to clonidine in rats may also involve stimulation of central α_1-adrenoceptors as well as postsynaptic α_2-adrenoceptors.

In the present study, acute 2 h pretreatment with DMI slightly inhibited the hypoactivity and hypothermia elicited by UK-14,304 in the mouse. A more marked attenuation of these responses was observed, however, at 18–21 h after withdrawal from chronic DMI treatment, whereas no effect was seen 18–21 h after a single dose of DMI. Heal *et al.* (1983) found similar actions of acute and repeated DMI administration on the hypoactivity induced in both mice and rats by clonidine. The use of UK-14,304 as an agonist in these models may have certain advantages over that of clonidine. First, UK-14,304 shows con-

siderably greater selectivity for the α_2 over the α_1-adrenoceptor than clonidine; in isolated tissue preparations, UK-14,304 has a greater than 300 fold selectivity for the α_2-adrenoceptor as opposed to the 10–40 fold selectivity exhibited by clonidine (Cambridge, 1981). Second, UK-14,304 elicits a more marked and more clearly dose-related sedative response in rodents than clonidine (Colpaert, 1986).

The sedative and hypothermic effects of α_2-agonists in rodents are thought to be mediated via stimulation of central presynaptic α_2-autoreceptors, which decreases NA release onto postsynaptic (probably α_1-) adrenoceptors (Zebrowska-Lupina *et al.*, 1977; Nomura *et al.*, 1980). This mechanism of action, however, is not universally accepted, as other investigators have suggested the involvement of postsynaptic α_2-adrenoceptors in the mediation of these responses (Spyraki & Fibiger, 1982). With regard to the slight attenuation of UK-14,304-induced hypothermia and hypoactivity seen 2 h after acute DMI in the present studies, two mechanisms could be responsible for this effect. A direct antagonist action of the drug at α_2-adrenoceptors or an indirect action via inhibition of NA re-uptake, which would increase levels of the neurotransmitter in the synaptic cleft. Since, as already mentioned, DMI is only a weak α_2-antagonist, but a potent inhibitor of NA re-uptake (Carlsson *et al.*, 1969), the latter mechanism seems the most likely explanation for the observed effect. Neither of these acute actions of DMI would seem to account for the marked attenuation of the responses to UK-14,304 seen 18–21 h after withdrawal from chronic drug treatment; the finding that after repeated twice daily administration for 14 days, DMI (15 mg kg^{-1}, orally) failed to inhibit significantly the onset of hypothermia induced by reserpine injected 12 h after the final dose of the uptake inhibitor, indicates that DMI was not present in effective concentrations at the time at which the responses to UK-14,304 were determined. This again implies that repeated administration of a NA uptake inhibitor induces an adaptive decrease in the sensitivity of central α_2-adrenoceptors, whether or not the receptors involved are located pre- or postsynaptically.

Other studies using biochemical and electrophysiological (McMillen *et al.*, 1980), behavioural (Spyraki & Fibiger, 1980; Heal *et al.*, 1983) and physiological (Gorka & Zacny, 1981) techniques have also demonstrated a decrease in the sensitivity of central α_2-adrenoceptors after long term NA re-uptake inhibition. Furthermore, several other types of antidepressant therapy, such as repeated ECT (Heal *et al.*, 1981) and chronic treatment with the monoamine oxidase inhibitor, clorgyline (Aulakh *et al.*, 1983), have also been reported to decrease the function of α_2-adrenoceptors.

In conclusion, the results of the present study suggest that repeated DMI administration reduces the sensitivity of the central α_2-adrenoceptors responsible for mediating the sedative and hypothermic actions of the α_2-agonist, UK-14,304 in the mouse. Furthermore, the attenuation of the thermogenic response to clonidine in reserpinized mice seen after this chronic treatment suggests that long term inhibition of NA uptake induces an adaptive decrease in the function of central postsynaptic α_2-adrenoceptors. Whether this action is shared by other types of chronic antidepressant therapy remains the subject of further research.

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Paf-induced release of spasmogens from guinea-pig lungs

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1 The injection of platelet activating factor (Paf; 250 ng), leukotriene B_4 (LTB_4; 50 ng) and leukotriene D_4 (LTD_4; 10 ng) elicited contractions of strips of guinea-pig trachea, bronchus and lung parenchyma.

2 When the effluent of perfused guinea-pig lungs was superfused over strips of guinea-pig trachea, bronchus and parenchyma, the intra-arterial injection of Paf (250 ng) caused the release of spasmogen(s) which contracted all three tissues.

3 The infusion of indomethacin ($10\,\mu g\,ml^{-1}$) into the pulmonary artery and over the assay tissues inhibited the responses of the tissues to the effluent of the lungs stimulated by Paf (250 ng) and LTB_4 (50 ng). However, treating only the assay tissues with indomethacin ($10\,\mu g\,ml^{-1}$) did not block the contractile responses to the effluent of the lungs stimulated with LTB_4 or Paf.
stimulated with Paf.

4 Pretreatment of the lungs with indomethacin ($10\,\mu g\,ml^{-1}$) or aspirin ($30\,\mu g\,ml^{-1}$) for 30 min, washing them out and suspending them over the assay tissues did not block the release of spasmogens elicited by Paf but appeared to inhibit the release of cyclo-oxygenase products.

5 The infusion of two lipoxygenase inhibitors, nordihydroguaiaretic acid (NDGA; $1\,\mu g\,ml^{-1}$) and L-655,240 ($1\,\mu g\,ml^{-1}$), into the pulmonary artery completely blocked the release of spasmogen(s) from the perfused lungs.

6 The slow reacting substance of anaphylaxis (SRS-A) antagonist, FPL-55712 ($10\,ng\,ml^{-1}$), did not block the responses of the tissues to the spasmogen(s) released by Paf.

7 The infusion of the Paf antagonist BN-52021 ($30\,\mu g\,ml^{-1}$) into the pulmonary artery completely abolished the release of spasmogen(s) induced by Paf.

8 These data suggest that a lipoxygenase product, possibly LTB_4, could be responsible for the spasmogenic activity released by the lungs following Paf stimulation. Cyclo-oxygenase products released following Paf stimulation appear to result from the initial LTB_4 generation.

Introduction

Experimental evidence suggests that platelet activating factor (Paf) is an important mediator of allergen-induced asthma (Page *et al.*, 1984; Vargaftig & Braquet, 1987). Intravenous injection of Paf induces a strong bronchoconstriction (Vargaftig *et al.*, 1980) which does not seem to be induced by a direct effect of Paf on bronchus (Jancar *et al.*, 1987). We have recently shown that Paf has no effect on preparations of guinea-pig lower bronchus whereas it induces a contraction of the upper bronchus which is followed by relaxations to subsequent injections of Paf. However, when Paf was injected into isolated perfused lungs, it stimulated the release of substance(s) causing contractions of bronchus, trachea and parenchyma strips (Jancar *et al.*, 1987). Intravenous injection of Paf into guinea-pigs was shown to induce a platelet-dependent bronchonstriction which is not mediated by cyclo-oxygenase metabolites (Vargaftig *et al.*, 1980; 1982) and is inhibited by several lipoxygenase inhibitors (Bonnet *et al.*, 1983). Aerosol administration of Paf in guinea-pigs was also shown to induce bronchoconstriction that is blocked by aspirin (Maridonneau-Parini *et al.*, 1985). In *in vitro* experiments on guinea-pig lungs

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perfused with cell-free solutions, the injection of Paf induced a bronchoconstriction that was platelet-independent and blocked by cyclo-oxygenase inhibitors. It was also shown that Paf induced a release of thromboxane A_2 (TxA_2) which preceded the increase in airway and pulmonary pressures (Lefort *et al.*, 1984). Thus, the effects of Paf in the lungs appear to depend on the route of administration, which probably reflects the stimulation of different cell types.

The present study represents an attempt to understand better the mechanism of action of Paf in the lung. Guinea-pig isolated lungs were perfused via the pulmonary artery and the effluent directed over strips of guinea-pig trachea, bronchus and parenchyma. After desensitization of the assay tissues to the direct effects of Paf, the lipid mediator was injected into the pulmonary artery circulation and the response of the tissues recorded. To investigate the nature of the spasmogens released, the lungs or the tissues were treated with aspirin and indomethacin, inhibitors of cyclo-oxygenase, nordihydroguaiaretic acid (NDGA), an inhibitor of lipoxygenases, L-655,240, an inhibitor of 5-lipoxygenase and an antagonist of thromboxane receptors, FPL-55712 and BN-52021, antagonists of the peptidoleukotrienes and Paf respectively, and a mixture of antagonists of histamine receptors, cholinoceptors and β-adrenoceptors.

Methods

Dunkin Hartley guinea-pigs weighing 300–400 g were killed by cervical dislocation. The thorax was cut open and the trachea, lungs and heart were removed and put in cold Krebs solution. Strips of lung parenchyma (3 × 3 × 30 mm) were cut along the edges of the lobes according to the technique described earlier (Sirois *et al.*, 1980). The trachea was removed, cut in spirals and divided into three equal segments. Strips of bronchus were prepared as described previously (Sirois *et al.*, 1987b). Briefly, a metal rod was inserted in the trachea and right bronchus. The parenchyma around the main bronchus was dissected and the right and left bronchi were then cut from the trachea. With a smaller metal rod used as support, the bronchi were further dissected free of parenchyma. The tissues were then cut in spirals of approximately 1.5 mm width and 1.5 cm long. Each bronchus was divided into two segments and the upper (external) part was used. It is referred to as GPB-EXT.

The tissues were placed in the organ baths of a cascade superfusion system and perfused with oxygenated (95% O_2 : 5% CO_2) Krebs solution (5 ml min^{-1}; 37°C). The agonists were given as bolus injections (10–50 μl) into the superfusion fluid and the responses were recorded with Grass FT03C isometric transducers coupled to a Grass polygraph.

The guinea-pig lungs were perfused as described previously (Engineer *et al.*, 1978). In brief, guinea-pigs were killed by cervical dislocation and the lungs were dissected and transferred to a perfusion apparatus where they were perfused with gassed Krebs solution via the pulmonary artery. The lungs were initially perfused at a rate of 10 ml min^{-1} for about 5 min to hasten blood clearance and then at 5 ml min^{-1}. The effluent of the lungs was directed over strips of guinea-pig trachea, bronchus and lung parenchyma.

Drugs used

Histamine hydrochloride, atropine sulphate and indomethacin were purchased from Sigma Chemical Co. (St. Louis, USA); Paf C_{16}(1-0-hexadecyl-2-0-acetyl-sn-glycero-3-phosphorylcholine) from Bachem (Switzerland); methysergide bimaleate (Sandoz Pharm., Montreal); propranolol hydrochloride (Ayerst Lab., Montreal); phenoxybenzamine hydrochloride (Smith Kline & French, Montreal); diphenhydramine hydrochloride (Parke Davis, Montreal); leukotriene D_4 methyl ester was kindly supplied by Dr S. Rakhit from Biomega (Montreal) and LTD_4 (free acid) by Dr J. Rokach (Merck Frosst Lab., Montreal). LTD_4 methyl ester was hydrolysed with a 5% solution of Na_2CO_3 for 1 h. Paf was dissolved in saline solution containing bovine serum albumin (0.25%), divided into aliquots and kept frozen at −20°C. Before each experiment, it was diluted to an appropriate concentration with Krebs solution and used immediately. Compounds BN-52021 and L-655,240 are 3-t-butyl-hexahydro-4,7b, 11-trihidroxy-8-methyl-9H-1,7a-epoxymethano-1H, 6aH-cyclopenta[c]furo[2,3-b]furo[3',2':3,4]cyclopenta[1,2-d]furan-5,9,12(4H)trione and (3-[1-(4-chlorobenzyl)-5-fluro-3-methyl-indol-2-yl]2,2-dimethylpropanoic acid) respectively (Braquet *et al.* 1987; Hall *et al.* 1987).

Results

Release of spasmogens from guinea-pig isolated lung by leukotrienes B_4, D_4 and Paf

Guinea-pig isolated lungs were perfused via pulmonary artery and the effluent directed over strips of guinea-pig trachea (GPT), upper external bronchus (GPB-EXT) and lung parenchyma (GPLPS). After a first injection of Paf (250 ng) over the assay tissues to record the initial responses, they were submitted to a continuous infusion of Paf (50 ng ml^{-1}) in order to desensitize them to the direct effect of this agonist. As shown in Figure 1, the first injection of Paf following the initiation of the

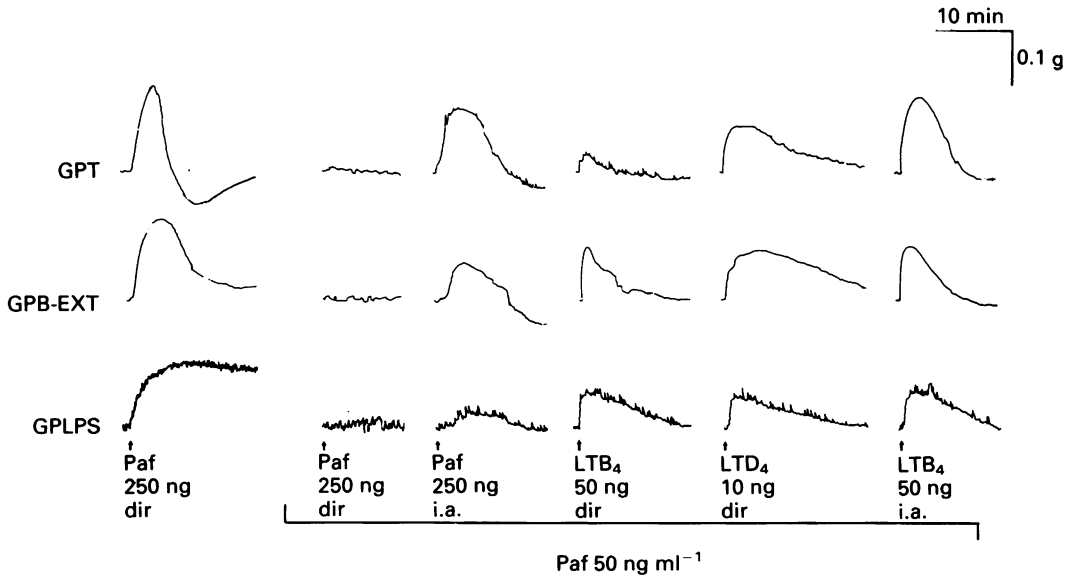


Figure 1 Release of spasmogens from perfused guinea-pig lungs stimulated by Paf. Paf (250 ng) was injected as a bolus several times either directly (dir) over strips of guinea-pig trachea (GPT), external bronchus (GPB-EXT) and lung parenchyma (GPLPS) or injected in the pulmonary circulation (i.a.) and the effluent was superfused over the tissues. The initial injection (dir) induced characteristic contractions of the three tissues. The continuous direct infusion of Paf (50 ng ml^{-1}) produced a desensitization of the isolated tissues as indicated by the absence of effect of the second direct injection of Paf. The injection of Paf (250 ng; third panel) into the pulmonary artery (i.a.) stimulated the release of spasmogens and the contraction of the isolated tissues. The fourth and fifth panels show the responses of the tissues to direct injections of leukotriene B_4 (LTB_4, 50 ng) and LTD_4 (10 ng). The last panel shows the effect of an intra-arterial injection of LTB_4 (50 ng). This experiment was repeated at least 3 times.

infusion did not elicit a response on any of the assay tissues. After these procedures, Paf (250 ng) and LTB_4 (50 ng) were injected into the pulmonary circulation (i.a.) and the responses of the assay tissues to the effluent of the stimulated lungs were recorded. Figure 1 shows that injections of Paf and LTB_4 into the lungs induced the release of substance(s) that contracted all three tissues. Direct injections (dir) of LTB_4 (50 ng) and LTD_4 (10 ng) over the assay tissues, made for control purposes, elicited contractions despite the desensitization to Paf.

Characterization of the spasmogenic activity present in the effluent of Paf-treated lungs

In a series of experiments, the assay tissues were continuously treated with a mixture of antagonists containing (μg ml^{-1}): methysergide (0.2), propranolol (3.0), phenoxybenzamine (0.1), atropine (0.1), and diphenhydramine (0.1). The lungs were suspended over the tissues and injected with 250 ng of Paf. These experiments were done to insure that the myotropic activity released from the lungs stimulated with Paf cannot be ascribed to histamine, 5-hydroxytryptamine, catecholamines or acetylcholine. The assay tissues treated with the mixture of inhibitors contracted to the effluent from Paf-stimulated lungs as the non-treated tissues (data not shown).

Effect of indomethacin and aspirin on Paf-stimulated release of spasmogens

In these experiments, the effect of indomethacin (10 μg ml^{-1}) was studied on the release of spasmogens by the lungs. As shown in Figure 2, the initial injection of Paf (250 ng) directly (dir) over the guinea-pig trachea, bronchus and parenchyma elicited strong contractions. For the remainder of the experiments, the assay tissues and the lungs received a continuous infusion of indomethacin (10 μg ml^{-1}); an infusion of Paf (50 ng ml^{-1}) over the tissues was also carried out to produce a complete desensitization. The subsequent injection of Paf (250 ng) into the lungs did not elicit contractions of assay tissues. The response of the tissues to the effluent from lungs stimulated with LTB_4 was also abolished (Figure 2). It can also be seen in this Figure that the

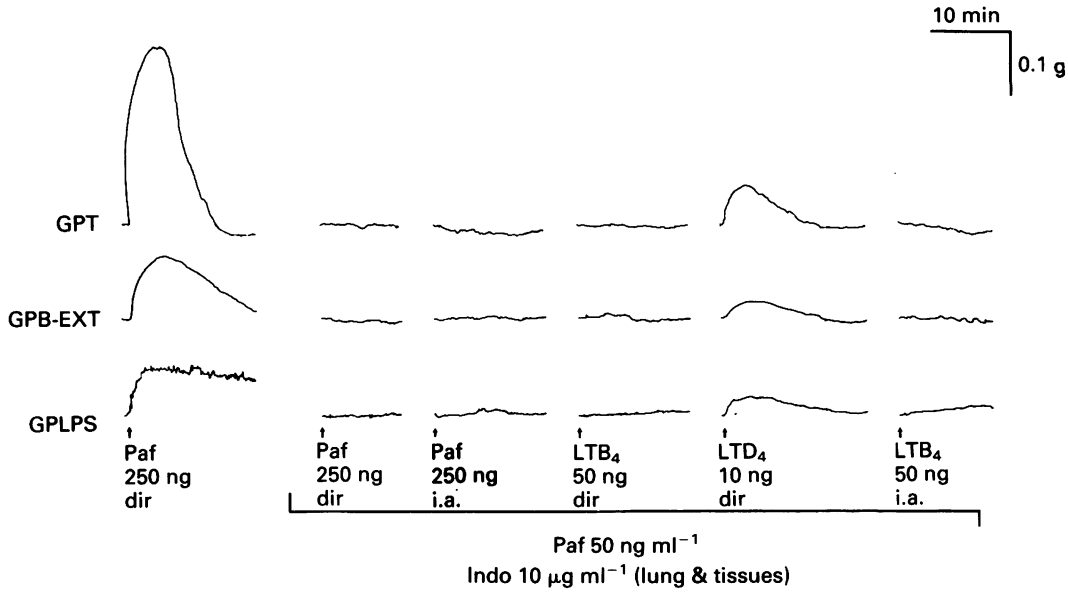


Figure 2 Release of spasmogens from perfused guinea-pig lungs stimulated by Paf and its inhibition by indomethacin. The injections of Paf were made as in Figure 1. Paf (50 ng ml^{-1}) was infused directly together with an infusion of indomethacin (Indo, i.a., 10 μg ml^{-1}) to block the release of prostaglandins and thromboxanes and the responses of the tissues to Paf. The injection of Paf (250 ng) in the pulmonary circulation (third panel) had no effect. The direct effect of leukotriene B_4 (LTB_4, 50 ng) was abolished because the response is mediated by the formation of cyclo-oxygenase products (fourth panel) whereas that to LTD_4 remained (fifth panel). The last panel shows that the intra-arterial injection of LTB_4 (50 ng) did not release prostaglandins and thromboxanes from the lungs. This is a representative experiment that was repeated at least three times.

contractions of the three tissues to the direct injection of LTB_4 were abolished by indomethacin. Contractions of the tissues to LTD_4 were reduced but not blocked by this treatment. It is not possible to conclude from this experiment whether release of mediator(s) from the lungs was inhibited by indomethacin, or whether the response of the tissues to the spasmogen released was blocked by this treatment. In an attempt to answer this question, two sets of experiments were done. First, indomethacin (10 μg ml^{-1}) was infused over the assay tissues only (Figure 3). The tissues were also perfused with Paf (50 ng ml^{-1}) to obtain desensitization. An intra-arterial injection of Paf (250 ng) elicited the release of spasmogens which were detected on the assay tissues. The tissues were no longer responsive to LTB_4 the action of which is mediated by prostaglandins and thromboxanes, but were stimulated by LTD_4. LTB_4 (50 ng) (i.a.) also stimulated the release of contractile substances from the perfused lungs.

In the other set of experiments, the lungs were infused with either indomethacin (10 μg ml^{-1}) or aspirin (30 μg ml^{-1}) and the effluent was not directed to the assay tissues. After 30 min, the infusion of cyclo-oxygenase inhibitors was stopped, the lungs were washed for 10–15 min and were then suspended over the assay tissues. Again Paf was infused over the tissues for the entire experiment to achieve complete desensitization. Following the injection of Paf into the pulmonary circulation, the effluent induced contractions of the assay tissues (Figure 4). Aspirin-like drugs did not block the release of the spasmogenic substance(s) from Paf-stimulated lungs. The tissues contracted to the direct injection of LTB_4. The intra-arterial injection of LTB_4 elicited contractions of the target tissues which were equivalent to those produced by the direct injection.

Effect of nordihydroguaiaretic acid (NDGA) on Paf-stimulated release of spasmogens

In an experimental design similar to that of the previous series of experiments, the perfused lungs were treated with NDGA, an inhibitor of lipoxygenases (1 μg ml^{-1}). As in the above experiments, the assay tissues were desensitized to Paf. Figure 5 shows that the injection of Paf in the pulmonary circulation did not release detectable amounts of spasmogens in the

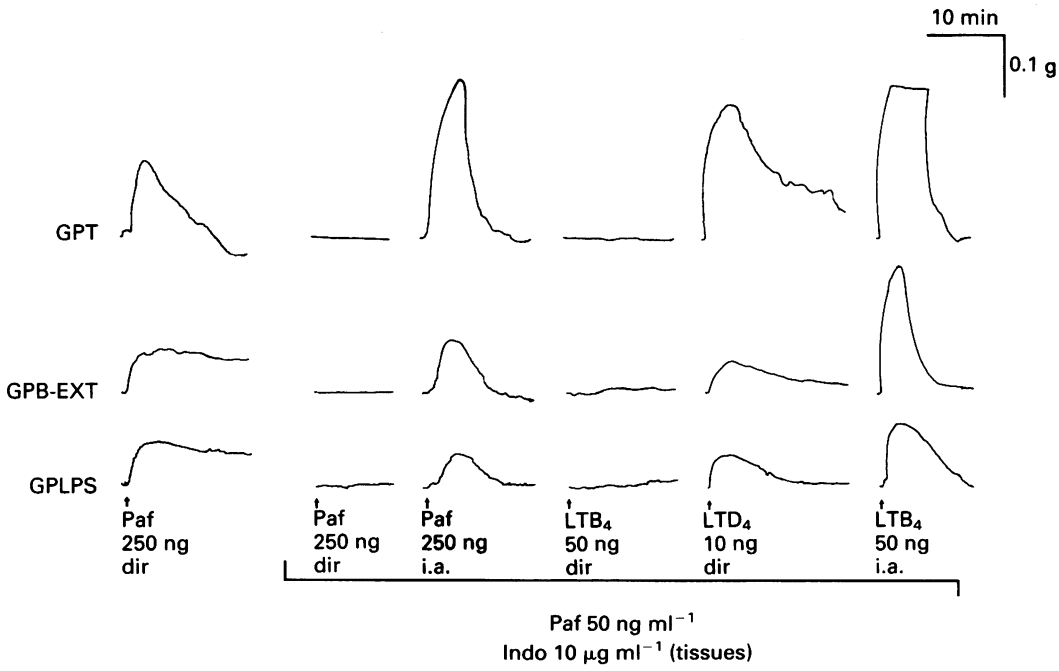


Figure 3 Release of spasmogens from perfused guinea-pig lungs stimulated by Paf and control effects of indomethacin on the target tissues. The injections of Paf were done as in Figure 1. A continuous direct infusion of Paf (50 ng ml^{-1}) was carried out together with an infusion of indomethacin (10 μg ml^{-1}) directly over assay tissues. The injection of Paf (250 ng) in the pulmonary circulation (third panel) stimulated the release of spasmogens and the contraction of the isolated tissues. The direct effects of leukotriene B_4 (LTB_4, 50 ng; fourth panel) were completely abolished whereas the direct effects of LTD_4 (10 ng; fifth panel) were not. The last panel shows that the intra-arterial injection of LTB_4 (50 ng) released cyclo-oxygenase products from the lungs. This is a representative experiment that was repeated at least 3 times.

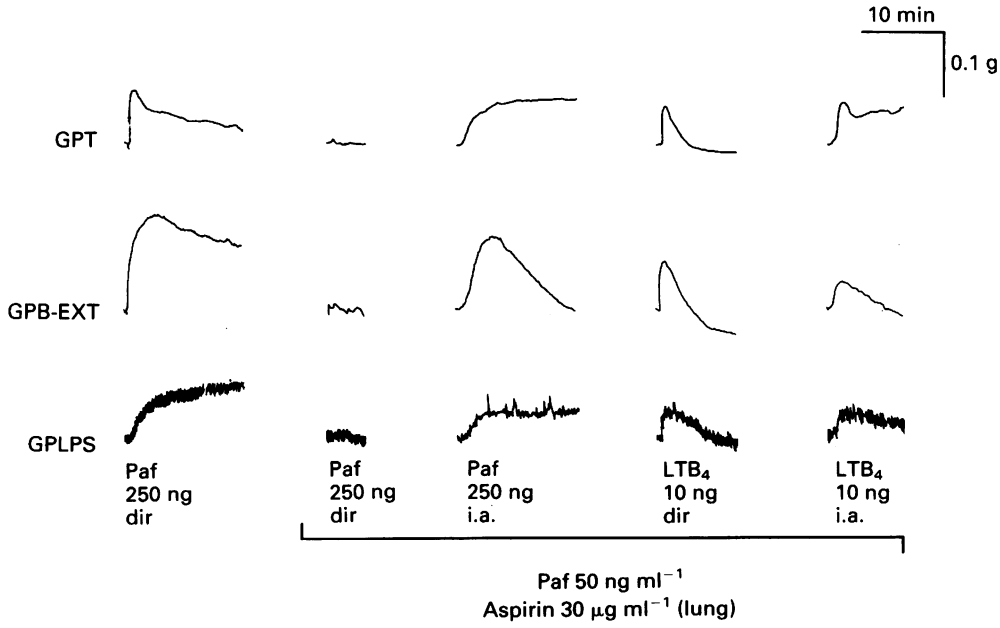


Figure 4 Release of spasmogens from perfused guinea-pig lungs and the effect of aspirin. The injections of Paf were made as in Figure 1. Paf (50 ng ml^{-1}) was infused continuously directly over the assay tissues. The lungs were pretreated for 30 min with aspirin (30 μg ml^{-1}) and the effluent was not directed over the assay tissues. The lungs were then washed for 10–15 min and suspended over the assay tissues. The injection of Paf (250 ng; third panel) in the pulmonary artery (i.a.) stimulated the release of spasmogens and the contraction of the isolated tissues. The fourth and fifth panels show the responses of the tissues to a direct (dir) and to an intra-arterial (i.a.) injections of leukotriene B_4 (LTB_4, 10 ng). This experiment was repeated four times.

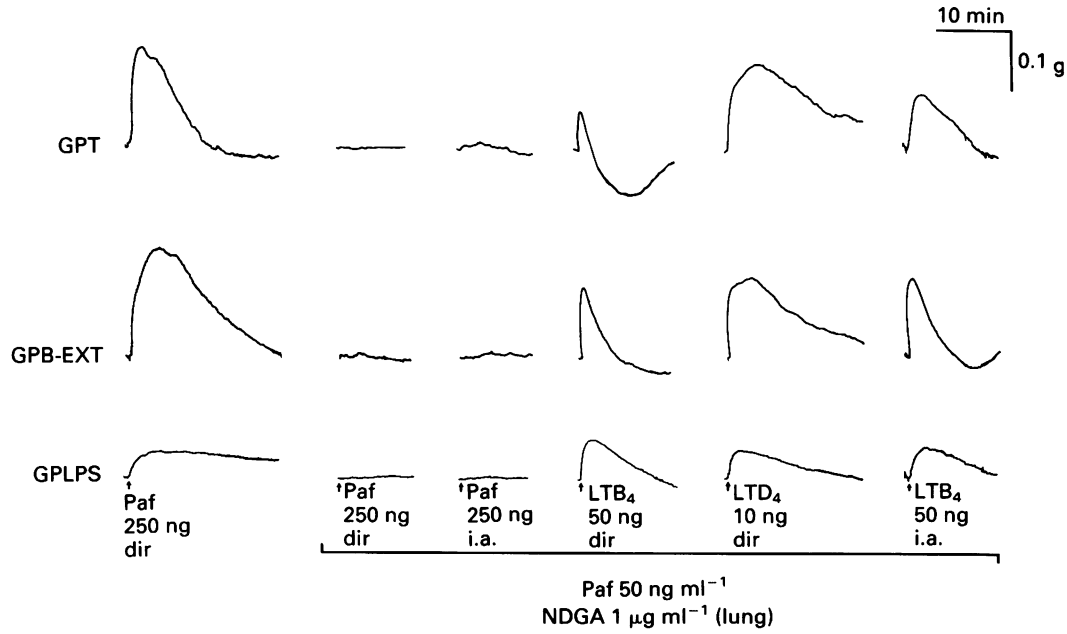


Figure 5 Effect of nordihydroguaiaretic acid (NDGA) on the release of spasmogens from perfused guinea-pig lungs stimulated by Paf. The injections of Paf were made as in Figure 1. Paf (50 ng ml^{-1}) was infused continuously together with an infusion of NDGA (1 μg ml^{-1}) into the pulmonary circulation to block the lipoxygenase. The injection of Paf (250 ng) into the pulmonary artery (i.a.; third panel) did not stimulate the release of spasmogens as shown by the absence of contraction of the target tissues. Direct injections of leukotriene B_4 (LTB_4, 50 ng) and LTD_4 (10 ng) elicited contractions of all three tissues (fourth and fifth panels respectively). The last panel shows that the intra-arterial injection of LTB_4 (50 ng) released cyclo-oxygenase products from the lungs. This is a representative experiment that was repeated at least 3 times.

presence of this drug. However, the tissues remained totally responsive to leukotrienes B_4 and D_4 and the lungs maintained their ability to release prostaglandins and thromboxanes following LTB_4 stimulation as shown by the marked contractions of the trachea, bronchus and parenchyma (last right hand side tracings).

Effect of compound L-655,240 on Paf-stimulated release of spasmogens

This set of experiments was designed as the above series. The compound L-655,240 a substance described as a thromboxane antagonist (Hall *et al.*, 1987) which also has definite inhibitory properties against lipoxygenase, was given into the pulmonary artery at a concentration of 1 μg ml^{-1}. As predicted from the results with NDGA, the injection of Paf into the pulmonary circulation did not lead to the release of spasmogens. As shown in Figure 6, injection of LTB_4 induced the release of substances which contracted the trachea, bronchus and parenchyma.

Effect of FPL-55712 on Paf-stimulated release of spasmogens

The next series of experiments was then planned as an initial attempt to characterize the lipoxygenase products released by lungs following Paf treatment. Control injections of Paf induced contractile responses of the guinea-pig trachea, bronchus and parenchyma. However, following the infusion of an antagonist of slow reacting substance of anaphylaxis (SRS-A) and LTD_4, FPL-55712 (10 ng ml^{-1}), together with Paf in order to prevent the responses of the tissues to Paf, the injection of Paf (as well as that of LTB_4) in the pulmonary artery induced the release of spasmogens (Figure 7).

Effect of compound BN-52021 on Paf-stimulated release of spasmogens

In this last set of experiments, the tissues were desensitized to Paf, and a Paf antagonist, BN-52021, was

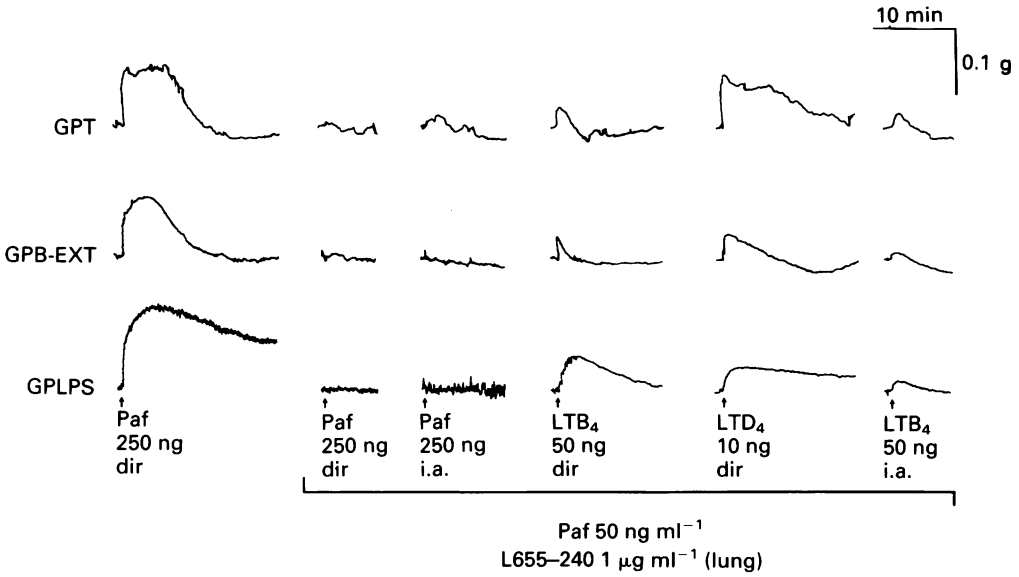


Figure 6 Effect of compound L-655,240 on the release of spasmogens from perfused guinea-pig lung stimulated by Paf. The injections of Paf were made as in Figure 1. Paf (50 ng ml^{-1}) was infused continuously together with an infusion of compound L-655,240 (1 μg mg^{-1}) in the pulmonary circulation to block the effects of thromboxane A_2 and the lipoxygenases. The injection of Paf (250 ng) into the pulmonary artery (i.a.; third panel) did not stimulate the release of spasmogens as shown by the absence of contraction of the target tissues. Direct injections of leukotriene B_4 (LTB_4, 50 ng) and LTD_4 (10 ng) elicited contractions of all three tissues (fourth and fifth panels respectively). The last panel shows that the intra-arterial injection of LTB_4 (50 ng) released spasmogens with reduced activity on the target tissues. This is a representative experiment that was repeated at least 3 times.

infused in the lung to block the receptors to Paf and study the specificity of the lung responses. As shown in Figure 8, the injection of Paf into the lungs did not generate the release of spasmogenic mediators. However, the lungs remained fully responsive to leukotrienes and released substances that contracted all three tissues.

Discussion

These results show that injection of Paf into the pulmonary circulation induces the release of substance(s) that contract strips of trachea, bronchus and parenchyma. These contractions cannot be attributed to Paf itself since the tissues were shown to be desensitized to the direct effects of Paf by its continuous infusion over the tissues. The contractions are not mediated by 5-hydroxytryptamine, catecholamines, acetylcholine or histamine since they were not affected by a mixture of antagonists to these substances. Pretreatment of the lungs with indomethacin or aspirin did not block the generation of the contractile substance(s), which indicates that the spasmogen is not a cyclo-oxygenase metabolite. The compound L-655,240 which is a thromboxane antagonist and a 5-lipoxygenase inhibitor (Hall *et al.*, 1987; Borgeat, personal communication) and NDGA, a lipoxygenase inhibitor blocked the release of the contractile substance(s). The peptido-leukotriene antagonist, FPL-55712, did not block the contractile response of the tissues to the spasmogen released. Thus a 5-lipoxygenase product, possibly LTB_4 could be the main spasmogen generated in the lungs following stimulation with Paf. This assumption is corroborated by the experiments where the lungs as well as the assay tissues were perfused with indomethacin. In this case, the contractions of the assay tissues were completely abolished. It is known that the contraction induced by LTB_4 in pulmonary tissues is mediated by cyclo-oxygenase products (Sirois *et al.*, 1982). Although the generation of LTB_4 in the lungs is not affected by indomethacin treatment, the effect of LTB_4 on the assay tissues should be blocked by indomethacin. An apparent contradiction appears when analysing the results obtained when the tissues but not the lungs were treated with indomethacin. This treatment did not affect the contractions of the assay tissues to the effluent from Paf-

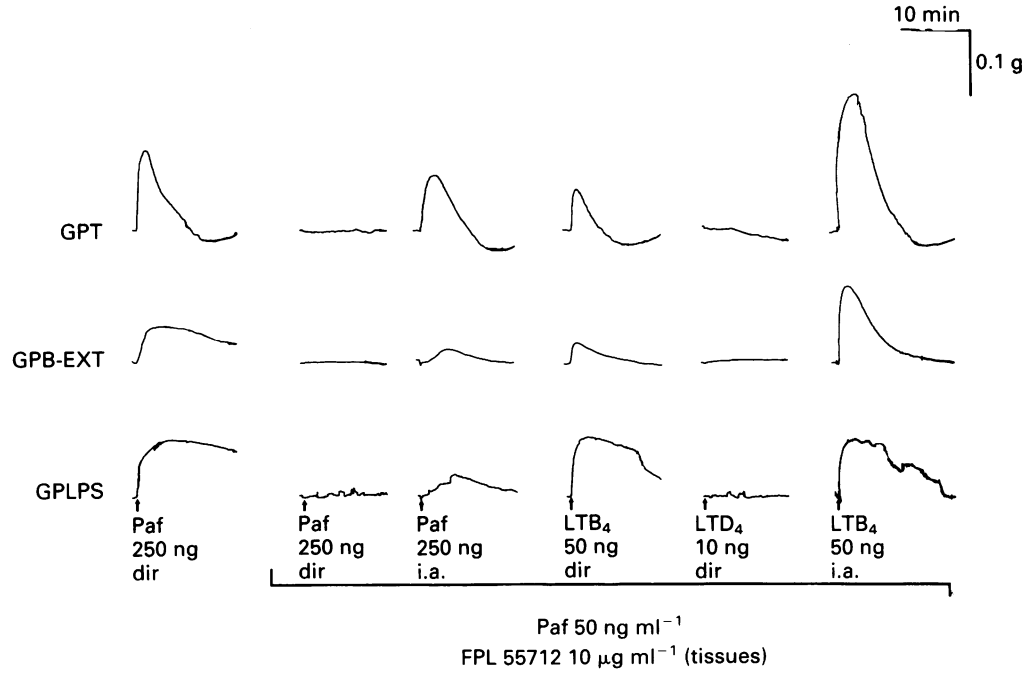


Figure 7 Effect of compound FPL-55712 on the release of spasmogens from perfused guinea-pig lungs stimulated by Paf. The injections of Paf were made as in Figure 1. Paf (50 ng ml^{-1}) was infused continuously together with compound FPL-55712 (10 μg ml^{-1}) directly over the assay tissues. The injection of Paf (250 ng) in the pulmonary artery (third panel) stimulated the release of spasmogens and the contraction of the isolated tissues. The direct injection of leukotriene B_4(LTB_4, 50 ng; fourth panel) elicited contractions of the tissue whereas that of LTD_4 (10 ng; fifth panel) was completely abolished by the selective leukotriene antagonist. The last panel shows that the intra-arterial injection of LTB_4 (50 ng) released cyclo-oxygenase products from the lungs. This is a representative experiment that was repeated at least 3 times.

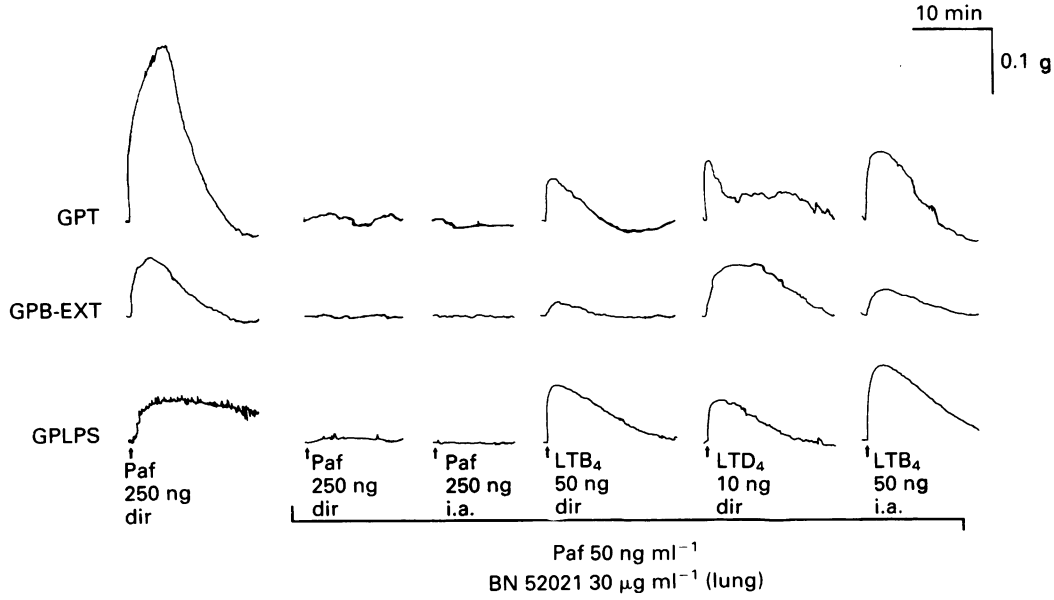


Figure 8 Effect of compound BN-52021 on the release of spasmogens from perfused guinea-pig lung stimulated by Paf. The injections of Paf were made as in Figure 1. Paf (50 ng ml^{-1}) was infused continuously together with an infusion of compound BN52021 (30 μg ml^{-1}) directly over the assay tissues. The third panel shows that the assay tissues did not react to the injection of Paf (250 ng) in the pulmonary artery. Direct injections of leukotriene B_4 (LTB_4, 50 ng) and LTD_4 (10 ng) elicited contractions of all three tissues (fourth and fifth panels respectively). The last panel shows that the intra-arterial injection of LTB_4 (50 ng) released cyclo-oxygenase products from the lungs. This is a representative experiment that was repeated at least 3 times.

stimulated lungs. In this case the contraction of the assay tissues cannot be attributed to LTB_4 since indomethacin abolished the response of the tissues to the direct injections of LTB_4. One possible explanation for these results is that Paf induces release of LTB_4 which in turn produces thromboxane, both steps occurring in the lungs. The thromboxane released in the lung effluent then induced a contraction of the assay tissues regardless of the presence of indomethacin.

Further evidence in support of this hypothesis is provided by the similar effects of LTB_4 and Paf injection into the pulmonary circulation and by the series of experiments showing that pretreatment of the lungs (not the tissues) with cyclo-oxygenase inhibitors aspirin (Figure 4) and indomethacin did not abolish the release of spasmogens. In accordance with this assumption are the data obtained by Lefort *et al.* (1984) who found that the bronchoconstriction induced by Paf injection into isolated perfused guinea-pig lungs was inhibited by aspirin. Furthermore, they have found that the bronchoconstriction was inhibited by NDGA and mepacrine but not by FPL-55712. These results support the hypothesis that generation of LTB_4 is a prerequisite for the effects of Paf in the lung. Indeed, Paf induces the release of considerable amounts of LTB_4 (Sirois *et al.*, 1987a) as well as thromboxane A_2 (Lefort *et al.*, 1984; Hamasaki *et al.*, 1984; Sirois *et al.*, 1987a) from isolated guinea-pig lungs and polymorphonuclear leukocytes (Chilton *et al.*, 1982; Lin *et al.*, 1982). Our results are also in agreement with those of Hamasaki *et al.* (1984) which showed that the increase of airway pressure in isolated perfused lungs of guinea-pig stimulated with Paf is inhibited by indomethacin.

The situation *in vivo* seems to be different. In this case, the bronchoconstriction induced by intravenous injection of Paf is not affected by cyclo-oxygenase inhibitors (Vargaftig *et al.*, 1980; 1982), although the bronchoconstriction induced by aerosol administration of Paf is inhibited by aspirin (Maridonneau-Parini, 1985). Since there are some discrepancies between the direct effects of Paf on guinea-pig bronchus and the bronchoconstriction induced by Paf *in vivo* (Jancar *et al.*, 1987), we believe that the *in vivo* action of Paf may represent an indirect effect via activation of platelets, neutrophils, vascular endothelial cells or other cell types. What cell type is the target for Paf-induced release of spasmogens in the lung is yet to be determined.

The different results obtained with various routes of administration of Paf can be explained by assuming that different spasmogens are released depending on the cell type that is first stimulated by Paf. Alveolar macrophages may be the first targets when Paf is administered in aerosol, whereas platelets and PMN leukocytes might be stimulated first when Paf is injected intravenously. In the isolated perfused lung model, none of these cells is likely to be involved although macrophages could be reached by diffusion.

It is also interesting to speculate about the biological significance of LTB_4 release in the lung. Morphological studies have shown a marked PMN leukocyte infiltration in the lungs following intravenous injection of Paf (Lellouch-Tubiana, 1985). Accumulation of these cells in the lung parenchyma and alveoli may be due to the secondary formation and to the effects of LTB_4 and may contribute to the tissue lesions in allergic respiratory diseases.

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Pharmacokinetics and dromotropic activity of ajmaline in rats with hyperthyroidism

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1 The pharmacokinetics and the dromotropic action (increased PQ interval) of intravenously administered adjmaline ($2\,mg\,kg^{-1}$) were studied in hyperthyroid rats with sinus tachycardia. The hyperthyroidism was induced by intraperitonal injection of 3,5,3'-triiodo-L-thyronine ($0.5\,mg\,kg^{-1}$) for 4 days.

2 The change in the ajmaline concentration in whole blood could be described by a biexponential equation. The steady state distribution volume of ajmaline decreased from $4.81\,l\,kg^{-1}$ in control rats to $3.80\,l\,kg^{-1}$ in hyperthyroid rats and the total body blood clearance was slightly higher in hyperthyroid rats than in control rats.

3 Ajmaline exhibited a saturable binding to rat plasma proteins, and one kind of binding site was found in the observed range of concentrations. The binding capacity was 2 fold higher in hyperthyroid rats than in control rats.

4 On the basis of the plasma unbound concentration, ajmaline exhibited an increased negative dromotropic activity in hyperthyroid rats compared with control rats.

5 A positive correlation was found between the pacing rate and the dromotropic action of ajmaline on atrioventricular conduction in isolated perfused hearts. There was no significant difference in the rate-dependence of the effect of ajmaline on the heart between control and hyperthyroid rats.

6 Our findings suggest that the increased dromotropic activity of ajmaline is mainly due to the increased heart rate in hyperthyroid rats.

Introduction

Most antiarrhythmic drugs have a relatively low therapeutic index and often produce intolerable side effects. Variability in the patients response to antiarrhythmics is a crucial consideration in designing dosage regimens, and the definition of the mechanisms of interindividual variability in drug response is of critical importance (Myerburg *et al.*, 1981; Follath *et al.*, 1983; Vozeh *et al.*, 1987).

Altered thyroid function may cause a significant variation in both the pharmacokinetics and the pharmacodynamics of antiarrhythmics, because thyroid hormones have an effect on fundamental molecular processes resulting in alteration of many physiological functions. Thyroid dysfunction affects the disposition of many drugs including propranolol and digoxin (Shenfield, 1981). Tachycardia is a common symptom associated with hyperthyroidism and may affect the antiarrhythmic activity of Class I drugs, because these drugs have been shown to depress the maximal upstroke velocity during phase 0 of the cardiac action potential in a frequency-dependent fashion (Heistracher, 1971; Hondeghem & Katzung, 1977). In addition, sudden cardiac death, ventricular arrhythmias, heart block and myocardial infarction may occur during thyrotoxicosis (Wei *et al.*, 1979).

This background prompted us to investigate the effects of thyroid dysfunction on the pharmacokinetics and the pharmacodynamics of an antiarrhythmic drug, by means of hyperthyroid model rats with sinus tachycardia. As a model drug, we selected a Class I antiarrhythmic drug ajmaline, the

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dromotropic action of which on atrioventricular conduction can be assessed by measuring PQ intervals on the electrocardiogram in rats (Hashimoto *et al.*, 1986). Because the pharmacological effects of ajmaline have been shown to be affected by plasma protein binding (Yasuhara *et al.*, 1987), the dromotropic activity in hyperthyroid rats was determined from the plasma unbound concentration. Furthermore, in rat isolated perfused hearts, we examined whether the change in the heart rate could affect the dromotropic activity of ajmaline, and to what extent the variations of the dromotropic activity of ajmaline in thyroid dysfunction could be accounted for by the frequency-dependent nature of the drug's action.

Methods

Induction of hyperthyroidism in rats

Male Wistar rats, each weighing 310–390 g, were used. Hyperthyroid (T_3) rats were produced by daily intraperitoneal injections of 3,5,3'-triiodo-L-thyronine (Sigma) 0.5 mg kg^{-1} in a solution volume of 1 ml kg^{-1} for 4 days (Gross & Lues, 1985). Animals that received the vehicle alone (4 mM NaOH in isotonic saline) served as a control. The experiments were begun 15 h after the last treatment.

Pharmacokinetics and pharmacological effect of ajmaline

The rats were anaesthetized with 50 mg kg^{-1} sodium pentobarbitone, administered as required. A catheter in the femoral artery was used for blood sampling and for measuring systemic arterial blood pressure by means of a capacitance transducer (Toyo Baldwin MPU-0.5-290-0-III). Another catheter was placed in the femoral vein for drug administration. The electrocardiogram (ECG) was recorded with bipolar standard limb leads. Arterial blood pressure, ECG and heart rate were monitored continuously by use of a polygraph (San-ei Model 366) and a recorder (San-ei Model 8K21-L). Body temperature was maintained with appropriate heating lamps.

Ajmaline (Gilurytmal, Nippon Chemiphar, 25 mg ml^{-1}) was diluted with isotonic saline, and 2 mg kg^{-1} was administered intravenously as a bolus injection of 1 ml kg^{-1}. Heparinized blood samples (0.15 ml) for the assay of ajmaline in whole blood were collected at 2, 5, 10, 30, 60, 90 and 120 min after the ajmaline injection. From the ECG recordings run at a paper speed of 250 mm s^{-1}, the PQ interval was measured before and after the injection of ajmaline, and the percentage increase of the PQ interval was calculated for measurement of the dromotropic action of the drug.

Plasma protein binding and erythrocyte partitioning of ajmaline

To calculate the plasma unbound concentration (Pf) from the whole blood concentration (Cb), the plasma protein binding and erythrocyte partitioning of ajmaline were determined *in vitro*. The heparinized blood was collected from four rats per group. Ajmaline was added to the blood to yield Cb from 0.1 to 1.0 μg ml^{-1}. After incubation for 15 min at 37°C, the haematocrit value (Ht) was measured by a Kubota Model KH-120 Hematocrit System (10,000 r.p.m., 5 min). After the plasma was separated by centrifugation, 0.1 ml of the plasma sample was taken for determiniation of the ajmaline concentration in plasma (Cp). Binding of ajmaline to plasma proteins was measured by the ultrafiltration technique by use of a Micropartition System (Amicon MPS-1). A 0.8 ml sample of plasma was placed in the ultrafiltration device equipped with a YMB membrane (Amicon) and centrifuged at 2000 *g* for 15 min at 20°C. About 80 μl of the ultrafiltrate was obtained for the determination of Pf. No significant adsorption of ajmaline was observed onto the ultrafiltration device (Yasuhara *et al.*, 1987).

Heart perfusion study

To analyse the dromotropic action of ajmaline in detail, the isolated perfused heart of the rat was subjected to artificial pacing. The anaesthetized rats were heparinized with 1000 iu kg^{-1} of sodium heparin. The rat isolated heart was perfused in a retrograde manner by the Langendorff technique in a non-recirculating fashion (Carmeliet & Zaman, 1979). Standard Krebs-Henseleit solution containing 11.1 mM D-glucose, aerated with 95% O_2 + 5% CO_2 and maintained at 31°C was used for the perfusion under a pressure of 130 mmHg. The hearts were stimulated *via* two stainless wire electrodes placed on the upper surface of the right auricle at 4 V and 1 ms duration, by means of an electronic stimulator (San-ei Model 3F46) and an isolator (San-ei Model 5361). Two steel wire electrodes fixed to the stainless steel aortic cannula and the apex of the ventricle were used to record the ECG. At any pacing rate, the ECG was equilibrated within 30 pacing stimuli, whether ajmaline was present or not. The equilibrated atrioventricular (AV) intervals at each pacing rate were measured before and after 30 min of the perfusion with ajmaline 0.326 μg ml^{-1}, and the percentage increase of the AV interval was calculated.

Assay of ajmaline

A specific reverse phase high performance liquid chromatographic (h.p.l.c.) method described pre-

Table 1 Comparison of the characteristics between 3,5,3′-triiodo-L-thyronine-treated (T_3) rats and control rats

	Control ($n = 4$)	T_3 ($n = 5$)
Body weight		
before treatment (g)	345 ± 7	336 ± 7
after 4 days treatment (g)	353 ± 6	316 ± 9**
gain (g)	8 ± 6	−20 ± 4***
Heart weight ($g\,kg^{-1}$)	3.06 ± 0.14	4.20 ± 0.11†
Haemodynamics		
heart rate (min^{-1})	374 ± 21	537 ± 17†
mean blood pressure (mmHg)	133 ± 9	132 ± 5
PQ interval (ms)	39.3 ± 0.8	35.9 ± 1.2

Values are expressed as mean ± s.e.mean for n animals.
** $P < 0.025$; *** $P < 0.01$ and † $P < 0.005$.

viously (Hori *et al.*, 1984) was used to quantitate ajmaline. The assay for Cb and Cp involved extraction of 0.1 ml sample and 1.0 ml glycine buffer (pH 10.0, 0.1 M, saturated with sodium chloride) with 5 ml of diethylether, and re-extraction of the organic phase with 0.2 ml of 0.85% phosphoric acid solution. A 50 μl sample of the water phase or the ultrafiltrate for the determination of Pf was subjected to h.p.l.c.

Data analysis

The disposition of ajmaline after intravenous administration was analysed by a biexponential equation: $Cb(t) = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t}$ for Cb at time t (Hashimoto *et al.*, 1986). The total body blood clearance (Cl_t) and the steady state distribution volume (Vd_{ss}) were calculated by the following equation: $Cl_t = dose/(A/\alpha + B/\beta)$ and $Vd_{ss} = Cl_t^2(A/\alpha^2 + B/\beta^2)/dose$ (Gibaldi & Perrier, 1982).

The plasma protein binding of ajmaline was analysed by the following equation, assuming one class of binding site in plasma: $Cp = Pf + N \cdot K \cdot Pf/(1 + K \cdot Pf)$, where N and K are the maximal binding capacity and association constant, respectively. The erythrocyte partitioning of ajmaline was studied by analysing the relationship of Pf to the drug concentration in blood cells (Cr), which was derived from the equation: $Cr = \{Cb - Cp(1 - Ht)\}/Ht$. Assuming a Freundlich type equation (Martin *et al.*, 1969), Cr was related to Pf in the following equation: $Cr = a \cdot Pf^b$, where a and b are constants. Accordingly, Pf could be estimated from any Cb by an iterative method after the values of N, K, a, b and Ht were determined (Yasuhara *et al.*, 1987).

The parameters were determined by least squares regression analysis using a digital computer. Values are expressed as mean ± s.e.mean. Statistical significance of difference between mean values was calculated by a non-paired t test provided that the variances were similar. If this was not the case, Mann-Whitney's U-test was applied. P values of less than 0.05 (two-tailed) were considered to be significantly different.

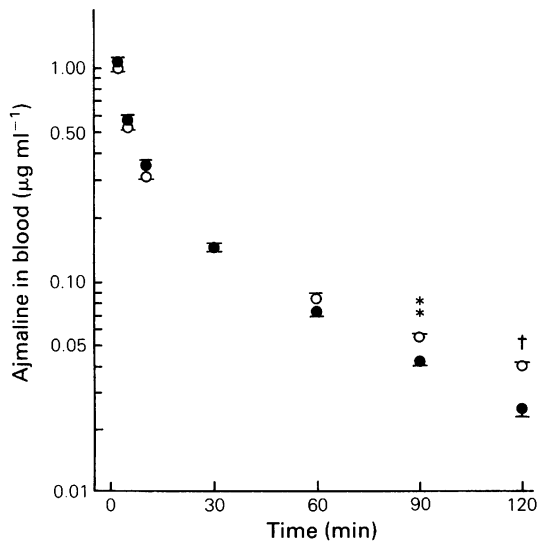


Figure 1 Time course of ajmaline concentration in whole blood in T_3 (●, $n = 5$) and control (○, $n = 4$) rats following i.v. injection of ajmaline $2\,mg\,kg^{-1}$. The vertical bar represents s.e.mean.
** $P < 0.025$ and † $P < 0.005$.

Results

Table 1 shows the effects of 3,5,3′-triiodo-L-thyronine-induced hyperthyroidism on various characteristics of rats. The body weight of the animals decreased slightly after 4 days of treatment with 3,5,3′-triiodo-L-thyronine. The heart weight normalized by the body weight was significantly higher in T_3 rats than in control rats. The heart rate determined under pentobarbitone anaesthesia was significantly

Table 2 Pharmacokinetic parameters of ajmaline at an intravenous dose of 2 mg kg^{-1} in hyperthyroid (T_3) and control rats

	Control ($n = 4$)	T_3 ($n = 5$)
A (μg ml^{-1})	1.32 ± 0.12	1.31 ± 0.07
B (μg ml^{-1})	0.245 ± 0.008	0.302 ± 0.013**
α (min^{-1})	0.280 ± 0.013	0.279 ± 0.008
β (min^{-1})	0.0165 ± 0.0005	0.0222 ± 0.0011†
Cl_t (ml min^{-1} kg^{-1})	102 ± 1	109 ± 2**
Vd_{ss} (l kg^{-1})	4.81 ± 0.07	3.80 ± 0.25***

Parameters were calculated from the biexponential equation by non-linear iterative least squares method using a digital computer. A and B are the extrapolated intercepts on time zero, and α and β are the rapid and slow disposition rate constant, respectively. Cl_t is the total body blood clearance and Vd_{ss} is the steady state distribution volume. Values are expressed as mean ± s.e.mean for n animals.
** $P < 0.025$; *** $P < 0.01$ and † $P < 0.005$.

higher in T_3 rats than in control rats, but the mean arterial blood pressure was similar in the two groups of rats. There was no significant difference in the PQ interval between T_3 and control rats.

Figure 1 shows the blood concentrations of ajmaline following i.v. injection (2 mg kg^{-1}) in T_3 and control rats. Cb values in T_3 rats were significantly lower than those in control rats at 90 and 120 min after the dosing. Cb data were fitted to the biexponential equation, and Table 2 shows the estimated pharmacokinetic parameters. B, β and Cl_t were slightly increased, and Vd_{ss} was decreased in T_3 rats compared with the control rats.

In T_3 and control rats the heart rate transiently decreased just after the administration of ajmaline (2 mg kg^{-1}); the heart rate in T_3 rats was higher than that in control rats throughout the experiments (Figure 2). The mean arterial blood pressure was not affected by i.v. administration of ajmaline (2 mg kg^{-1}) in either T_3 or control rats, and its change at 2 min after dosing was 3 ± 4 mmHg ($n = 5$) and −5 ± 3 mmHg ($n = 4$) in T_3 and control rats, respectively. Following injection of ajmaline (2 mg kg^{-1}), both T_3 and control rats showed rapid prolongation of PQ intervals, and the percentage increases of the PQ interval at 2 min after dosing were 43.7 ± 3.9% ($n = 5$) in T_3 rats and were significantly ($P < 0.025$) greater than the values of 34.2 ± 1.3% ($n = 4$) in control rats. Thereafter, the PQ interval decreased gradually and returned to the pre-dose levels by 120 min.

To determine the pharmacological effect of ajmaline from the unbound drug concentration in blood, the relationship of Pf to Cb in T_3 and control rats was studied in four separate experiments (Figure 3).

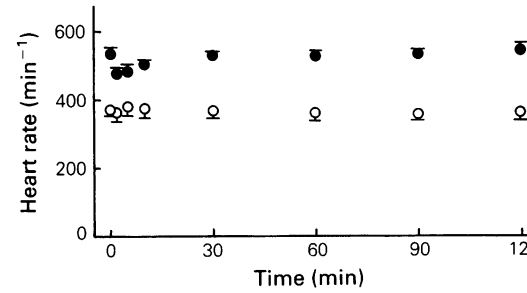


Figure 2 Time course of heart rate in T_3 (●, $n = 5$) and control (○, $n = 4$) rats after the i.v. injection of ajmaline 2 mg kg^{-1}. The vertical bar represents s.e.mean.

Pf was significantly lower in T_3 rats than in control rats, over the concentration-range studied. The plasma protein binding and erythrocyte partitioning of ajmaline were also measured in order to describe the relationship of Pf to Cb quantitatively. As shown in Table 3, the decreased unbound fraction of ajmaline in the blood of T_3 rats was mainly due to the increased binding capacities of T_3 rat plasma. The simulated curves in Figure 3, which were drawn to fit the mean values of plasms protein binding parameters, erythrocyte partitioning parameters and haematocrit value in each group, were highly correlated with the observed data.

The effect of hyperthyroidism on the negative dromotroic activity of ajmaline was assessed by

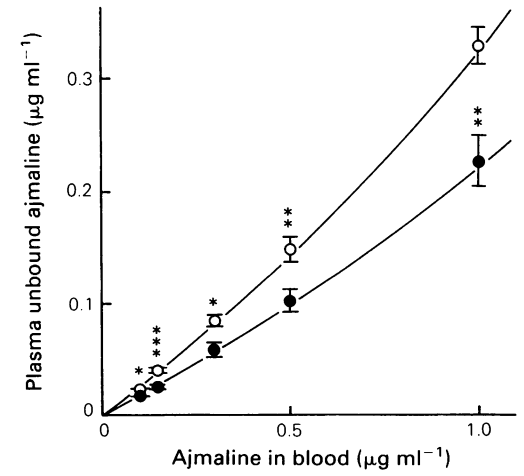


Figure 3 Relationship between whole blood and plasma unbound concentration of ajmaline in T_3 (●, $n = 4$) and control (○, $n = 4$) blood. The vertical bar represents s.e.mean. The solid curves were drawn to fit the mean parameters in each group, as given in Table 1.
* $P < 0.05$; ** $P < 0.025$ and *** $P < 0.01$.

Table 3 Protein binding and erythrocyte partitioning parameters of ajmaline in hyperthyroid (T_3) and control rats

	Control ($n = 4$)	T_3 ($n = 4$)
N ($\mu g\,ml^{-1}$)	1.72 ± 0.23	3.48 ± 0.61*
K ($ml\,\mu g^{-1}$)	2.40 ± 0.08	2.45 ± 0.23
a	2.79 ± 0.17	2.12 ± 0.06***
b	1.05 ± 0.06	1.05 ± 0.05
Ht	0.414 ± 0.009	0.438 ± 0.003*

The drug concentration data in plasma for individual experiments were fitted to the equation $Cp = Pf + N \cdot K \cdot Pf/(1 + K \cdot Pf)$ for the plasma concentration, Cp at the plasma unbound concentration, Pf. The blood cell concentrations (Cr) were analysed by the following equation: $Cr = a \cdot Pf^b$. Ht is the haematocrit value. Values are expressed as mean ± s.e.mean for n experiments.
* $P < 0.05$ and *** $P < 0.01$.

analysing the relationship of the percentage increase of the PQ interval to Pf. Figure 4 shows the response to Pf on a semi-logarithmic scale, indicating the difference in the negative dromotropic activity between the two groups of rats. In fact, the relationship between Pf-response could be analysed by linear regression in individual rats ($P < 0.005$). The slopes were $197.5 \pm 31.6\%\,ml\,\mu g^{-1}$ ($n = 5$) in T_3 rats, and were significantly greater than the values of $110.4 \pm 8.9\%\,ml\,\mu g^{-1}$ ($n = 4$) in control rats ($P < 0.025$). The intercepts were very small: $0.9 \pm 0.2\%$ and $0.0 \pm 0.5\%$ in T_3 and control rats, respectively. These results indicated the increase in

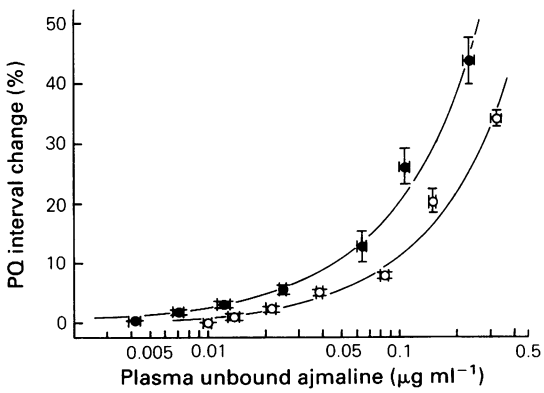


Figure 4 Relationship between plasma unbound concentration and PQ interval change in T_3 (●, $n = 5$) and control (○, $n = 4$) rats following i.v. injection of ajmaline $2\,mg\,kg^{-1}$. The vertical bars represent s.e.mean. The solid curves were drawn by the following equations: $R = 110.4\,Pf$ for control rats and $R = 197.5\,Pf + 0.9$ for T_3 rats, where R is response and Pf is the plasma unbound concentration.

Table 4 Rate-dependent dromotropic activity of ajmaline ($1\,\mu M$, $0.326\,\mu g\,ml^{-1}$) for atrioventricular (AV) conduction in isolated hyperthyroid (T_3) and control rat hearts

Pacing rate (min^{-1})	*Change in AV interval* (% increase) *Control* ($n = 5$)	T_3 ($n = 5$)
150	16	
200	20, 22	
250	25 ± 1	25 ± 1
300	28 ± 1	28 ± 1
350	32 ± 2	31 ± 2
400	34	35 ± 2
450		33, 40

Values are expressed as mean ± s.e.mean for n hearts. If this is not the case, individual values are quoted.

the negative dromotropic activity of ajmaline in T_3 rats compared with control rats.

The mechanism of the increased dromotropic activity of ajmaline in hyperthyroidism was investigated by means of isolated perfused rat hearts. The AV intervals in isolated heart preparations in the absence of ajmaline were similar in T_3 and control rats: 65.0 ± 2.5 ms and 62.2 ± 1.5 ms at the pacing rate of $250\,min^{-1}$, and 71.8 ± 3.5 ms and 69.8 ± 2.5 ms at the pacing rate of $350\,min^{-1}$ in T_3 ($n = 5$) and control ($n = 5$) rats, respectively. Ajmaline ($0.326\,\mu g\,ml^{-1}$) produced a rate-dependent dromotropic effect over the range of the pacing rate, to which the perfused heart could respond; 250–$450\,min^{-1}$ and 150–$400\,min^{-1}$ in T_3 and control rat hearts, respectively. Table 4 shows the percentage increases of the AV interval for each pacing rate in T_3 and control hearts. The relationship between the pacing rate and the percentage increase of the AV interval could be analysed by the linear regression for individual rat hearts ($P < 0.05$). The slope was $0.070 \pm 0.010\%$ min and $0.080 \pm 0.009\%$ min, and the intercept was $6.8 \pm 2.6\%$ and $4.3 \pm 2.1\%$ in T_3 ($n = 5$) and control ($n = 5$) rat hearts, respectively. Thus, there was no significant difference in the rate-dependence of the ajmaline effect between hearts obtained from T_3 and control rats. These results suggested that the increased dromotropic activity of ajmaline was mainly due to the increased heart rate associated with the hyperthyroidism.

Discussion

The present study demonstrated that hyperthyroidism could affect both the pharmacokinetics and the pharmacodynamics of ajmaline. Thyroid hormones are said to have an effect on fundamental molecular processes resulting in alteration of many

physiological functions including cardiac output, blood flow rate in tissues and metabolic activity in the liver (Shenfield, 1981). In the present study T_3 rats had sinus tachycardia and cardiac hypertrophy, which suggested accelerated heart function.

T_3 rats had smaller Vd_{ss} and slightly higher Cl_t of ajmaline than the control rats. The plasma protein binding of ajmaline *in vitro* was increased for T_3 rats compared with control rats. It has been suggested that drug distribution in the body depends on plasma protein binding, and increased binding can lead to a decrease in the distribution volume of the drug which can be distributed to the tissues (Tozer, 1981). Accordingly, the smaller distribution volume in T_3 rats is likely to be due to the lower unbound fraction of ajmaline in the blood. On the other hand, the total clearance of ajmaline was increased in T_3 rats in spite of the lower unbound fraction. This was probably related to the mechanism of ajmaline clearance. Ajmaline is poorly excreted in the urine (Kleinsorge & Gaida, 1961; Iven, 1977) and avidly extracted by the liver (Yamada *et al.*, 1986). Moreover, we have shown that the total clearance of ajmaline is decreased in rats with acute myocardial ischaemia compared with sham-operated control rats (Hashimoto *et al.*, 1986). These findings suggested that the elimination process of ajmaline is not much restricted either by the unbound fraction in the blood or by the metabolic activity in the liver, but is largely dependent on the hepatic blood flow rate (Wilkinson & Shand, 1975). Accordingly, the principal cause for the increased total blood clearance in T_3 rats may be the increased hepatic blood flow rate in hyperthyroidism (Wells *et al.*, 1983).

In the present study, the capacity of plasma protein binding of ajmaline, a weak basic drug with a pKa of 8.3 (Petter, 1974), was increased in T_3 rats compared with control rats. We do not have a clear explanation for the mechanism of this increased binding. The effect of hyperthyroidism on the plasma protein binding of propranolol, another basic drug, has been studied by several groups, but the results are controversial. Feely *et al.* (1981) have reported that the protein binding of propranolol is lower in hyperthyroidism than in the euthyroid state, and have implied the possibility of hyperthyroidism-induced changes in the concentration of albumin or α_1-acid glycoprotein, and the presence of binding displacement in the plasma of hyperthyroid patients. On the contrary, Wells *et al.* (1983) have reported that the plasma unbound fraction of propranolol is slightly lower in thyrotoxic patients than in euthyroid patients although not significantly.

We have shown that the negative dromotropic action of ajmaline for the AV conduction system can be quantitatively assessed by measuring the PQ interval in rats (Hashimoto *et al.*, 1986) and dogs (Yasuhara *et al.*, 1987). On the other hand, changes in the extent of the plasma protein binding have been shown to affect the responses of some antiarrhythmic drugs, including lignocaine (Rick *et al.*, 1987) and disopyramide (Huang & Øie, 1982). We have also shown that the dromotropic action of ajmaline correlates with unbound drug concentrations better than with total plasma concentrations in dogs (Yasuhara *et al.*, 1987). Accordingly, in the present study, we determined the dromotropic activity of ajmaline from the unbound drug concentrations, and found it to be significantly increased in T_3 rats.

The isolated heart perfusion study showed that ajmaline could decrease the AV conduction rate in a frequency-dependent fashion (Table 4). A positive linear correlation was observed between the pacing rate and the prolongation of the AV interval by ajmaline, and there was no difference in the pacing rate-dependence between control and T_3 rats. This indicated that both groups of rats had similar cardiac sensitivity to the dromotropic action of ajmaline at the same heart rate. Since T_3 rats had a much higher heart rate than the control rats, ajmaline could produce a higher dromotropic effect in T_3 rats than in the control rats at the same unbound drug concentration in the blood.

In conclusion, these results suggest that hyperthyroidism has pronounced effects on both the pharmacokinetics and pharmacodynamics of ajmaline, and that the heart rate is an important determining factor for the negative dromotropic effect of ajmaline.

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Influence of the endothelium on contractile effects of 5-hydroxytryptamine and selective 5-HT agonists in canine basilar artery

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1 We have investigated the influence of endothelial damage on the cerebrovascular reactivity to 5-hydroxytryptamine (5-HT) and some selective 5-HT agonists in canine basilar artery.

2 5-HT, α-methyl 5-HT, GR 43175 (3-[2-dimethyl amino] ethyl-N-methyl-1H-indole-5-methane sulphonamide) and 5-carboxamidotryptamine (5-CT) produced concentration-dependent contractions of untreated dog basilar artery with a functional endothelium. Following endothelial damage by perfusion with Triton X-100 (0.1%), which abolished the relaxant response to substance P, the maximum contractile effect of 5-HT, α-methyl 5-HT, GR 43175 and 5-CT was markedly enhanced although there was little change in the EC_{50} values. Endothelial damage did not modify the vasoconstrictor effect of the thromboxane agonist, U46619, or potassium chloride.

3 Neither 5-HT nor 5-CT caused relaxation of untreated canine basilar arteries contracted with prostaglandin $F_{2\alpha}$, U46619, uridine triphosphate or potassium chloride.

4 These results suggest that canine basilar artery spontaneously releases endothelium-derived relaxing factor which can attenuate the vasoconstrictor effect of 5-HT and selective 5-HT agonists. This effect appeared to be specific since the vasoconstrictor response to U46619 was not modified.

5 These results demonstrate that the cerebrovascular endothelium can markedly influence the reactivity of the vascular smooth muscle of canine basilar artery to 5-HT and 5-HT_1-like receptor agonists. However we could find no evidence that 5-HT receptor activation stimulates endothelial cell function as it does in some other blood vessels.

Introduction

Over recent years it has been realised that the vascular endothelium has an important influence on blood vessel tone via release of 'endothelium-derived relaxing factor' (EDRF). Thus in many isolated blood vessels, the response to a variety of agonists is dependent on the integrity of the endothelium (Furchgott, 1983). This can reflect interaction of an agonist with specific receptors located on the endothelium cell to cause EDRF release (Furchgott, 1983). However, it has recently been established that certain blood vessels release EDRF spontaneously (Martin *et al.*, 1986). 5-Hydroxytryptamine (5-HT) is a highly potent spasmogen in isolated cerebral arteries (Muller-Schweinitzer & Engel, 1983; Peroutka *et al.*, 1986). In canine and primate basilar artery, the contractile effects of 5-HT appear to be mediated through stimulation of a mixed population of 5-HT_1-like and 5-HT_2 receptors (Connor *et al.*, 1987). However, in those experiments no attempt was made to remove the endothelium and, indeed, studies in our laboratories have demonstrated that such vessels retain a functional endothelium (Connor & Feniuk, 1987). We have therefore investigated the influence of the cerebrovascular endothelium on 5-HT-induced contractions of dog isolated basilar artery. A preliminary account of these findings has been presented to the British Pharmacological Society (Connor & Feniuk, 1988).

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Methods

Preparation of artery segments

Beagle dogs (7–10 kg, either sex) were killed with pentobarbitone sodium (100 mg kg^{-1}, i.v.) and basilar arteries were removed and stored overnight in modified Krebs solution (Apperley *et al.*, 1976) at 4°C. A segment of artery was perfused intraluminally with Triton X-100 (0.1%, 0.5 ml min^{-1} for 1 min) to abolish endothelial function as described in detail by

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Connor & Feniuk (1987); control preparations were taken from an adjacent segment of unperfused artery.

Ring segments (3–4 mm) of untreated or Triton X-100-perfused basilar artery were placed in separate 10 ml organ baths containing modified Krebs solution (Apperley *et al.*, 1976) at 37°C, bubbled with 95% O_2, 5% CO_2. The segments were suspended between two L-shaped steel wire supports (0.2 mm diameter) inserted into the lumen to record isometric tension changes. Preparations were maintained at an initial resting tension of 200–300 mg for 1 h and then tension was increased to 1 g. Preparations were allowed to equilibrate for a further hour and then the response to a submaximal concentration of potassium chloride (30 mM) was determined in each tissue.

Assessment of endothelial integrity

At least 30 min later, after frequent washing, the functional integrity of the endothelium was assessed in each preparation by examining the response to the endothelium-dependent vasodilator agent substance P (1–10 nM) (Furchgott, 1983) after vascular tone had been increased by a submaximal concentration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 1 μM). Untreated preparations consistently responded to substance P with marked concentration-related relaxations (about 100% reduction in $PGF_{2\alpha}$ tone), whilst in Triton X-100-perfused segments, substance P caused little or no relaxation (less than 15% reduction in $PGF_{2\alpha}$ tone) and, in many preparations, caused small increases in tone (Connor & Feniuk, 1987). Occasionally, a preparation that had been perfused with Triton X-100 would still respond to substance P with relaxation, suggesting that some endothelial function remained; these preparations (less than 10% of total) were rejected.

Determination of agonist potency

Agonists were administered to the bathing fluid of paired untreated and Triton X-100-perfused preparations according to a cumulative concentration schedule, allowing sufficient time for the effects of each concentration to become fully established before adding the next concentration, until a maximum response was obtained or when relaxation started to occur. Contractions were expressed as % response obtained to the initial challenge with potassium chloride (30 mM). No more than 2 agonists were examined in any one preparation.

Determination of antagonist potency

Concentration-effect curves for 5-HT were constructed in two untreated or Triton X-perfused segments. Antagonist was added to one bath, whilst the second preparation served as control to assess any spontaneous change in tissue sensitivity to 5-HT. Thirty minutes equilibration time was allowed for contact of the antagonist with the tissue before the agonist concentration-effect curve was redetermined. Results were expressed in terms of % maximum response to 5-HT obtained in the first curve. In experiments in which the antagonist caused a parallel rightward displacement of the agonist concentration-effect curve, the agonist concentration-ratio (CR) was calculated at the level of 50% maximum 5-HT response. Corrections were made for any spontaneous changes in sensitivity to 5-HT. In some experiments (see below) ketanserin (1 μM) was continually present in the Krebs solution.

Histology

Histological examination was performed either via scanning electron microscopy (EM) or light microscopy (haematoxylin and eosin staining on serial transverse sections) on untreated and Triton X-100-perfused dog basilar artery segments to assess the integrity of the endothelium.

Statistical analysis

Significance was calculated by a paired Student's t test; $P < 0.05$ was considered as statistically significant.

Drugs

The following drugs and compounds were used in this study: 5-hydroxytryptamine creatinine sulphate (Sigma), ketanserin (Janssen), methiothepin maleate (Roche), methylene blue (Sigma), phenoxybenzamine hydrochloride (SKF), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, dinoprost tromethamine, Upjohn), substance P (Bachem), Triton X-100 (Sigma) and uridine triphosphate (Sigma).

5-Carboxamidotryptamine (5-CT), (±)-α-methyl 5-hydroxytryptamine (α-Me 5-HT), GR 43175 (3-[2-dimethyl amino]ethyl-N-methyl-1H-indole-5-methanesulphonamide) and U46619 (11,9-epoxymethano PGH_2) were synthesized by members of the Chemistry Research Department, Glaxo Group Research Ltd., Ware.

Ketanserin was initially dissolved in 0.1 M tartaric acid and U46619 was initially dissolved in 1% sodium bicarbonate; dilutions were made in distilled water. Dilutions of $PGF_{2\alpha}$ were made in 0.9% saline. All other drugs were dissolved and diluted in distilled water.

Results

Responses to potassium chloride

The initial submaximal challenge concentration of potassium chloride (30 mM) produced similar contractile responses in paired untreated and Triton X-100-perfused dog basilar artery (1.68 ± 0.12 g and 1.58 ± 0.10 g, respectively, $n = 20$). Subsequent contractile responses to agonists were expressed as a % of the response to potassium chloride.

Effects of 5-HT and selective 5-HT agonists

After confirming that Triton X-100 had either abolished or markedly attenuated substance P-induced relaxation (see Methods), the contractile effects of 5-HT or selective 5-HT agonists were examined, and compared to responses obtained in paired untreated preparations with a functional endothelium. 5-HT produced contractions of Triton X-100-perfused artery segments which were much greater in magnitude than responses obtained in corresponding untreated controls; the maximum contraction obtained for 5-HT appeared to be approximately doubled by Triton X-100 perfusion (Figure 1, Table 1). However, there was no change in sensitivity of the tissue to 5-HT since EC_{50} values were similar in untreated and Triton X-100 perfused preparations (Table 1). Interestingly in some experiments, the nature of the 5-HT-induced contraction seemed to be modified by perfusion with Triton X-100. In untreated preparations, contractions tended to be rapid in onset but transiently maintained whilst, in contrast, preparations that had been perfused with Triton X-100 responded to 5-HT consistently with slower, more maintained increases in tension. A representative trace which illustrates this difference is shown in Figure 2 (a and b). In different experiments, the influence of methylene blue (10 μM) on 5-HT-induced contractions of preparations with a functional endothelium was examined. Although methylene blue itself caused a marked, sustained

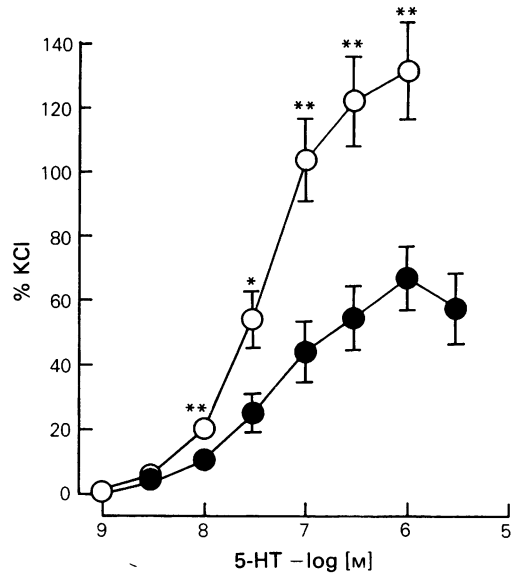


Figure 1 Contractile effects of 5-HT in control (●) and Triton X-100 perfused (○) canine basilar artery ($n = 7$). Responses are expressed in terms of the contraction obtained to 30 mM KCl; values are means (s.e.mean shown by vertical bars) from n experiments. * $P < 0.05$, ** $P < 0.01$ (paired Student's t test) compared to controls.

Table 1 EC_{50} values and maximum responses produced for 5-hydroxytryptamine (5-HT) and selective 5-HT agonists in control (untreated) and Triton X-100-perfused canine basilar artery

	Control		*Triton-X-perfused*	
Agonist	EC_{50} (nM)	*Max response* (% KCl)	EC_{50} (nM)	*Max response* (% KCl)
5-HT	62 (30–126)	67 ± 10	40 (30–54)	130 ± 15**
α-Me 5-HT	320 (150–670)	72 ± 3	310 (160–630)	140 ± 16*
GR 43175	94 (19–462)	26 ± 5	107 (19–588)	66 ± 3**
5-CT	4.2 (2.6–6.8)	20 ± 7	4.5 (3.9–5.1)	41 ± 7**

Values are geometric mean (95% confidence limits) or arithmetic mean ± s.e.mean, $n = 4$–7. * $P < 0.05$; ** $P < 0.01$ significantly different from control (paired Student's t test). Responses are expressed in terms of the contraction obtained to 30 mM KCl.

α-Me 5-HT = (±)-α-methyl 5-hydroxytryptamine; GR 43175 = 3-[2-dimethyl amino]ethyl-N-methyl-1H-indole-5-methane sulphonamide; 5-CT = 5-carboxamidotryptamine.

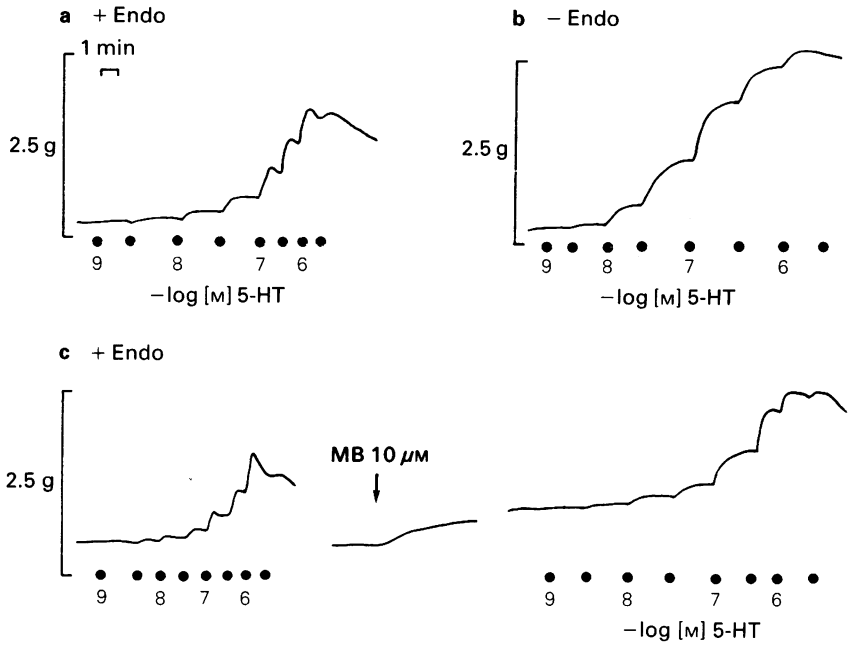


Figure 2 Representative tracing to illustrate the effects of 5-hydroxytryptamine (5-HT) in (a) untreated, endothelium-intact, and (b) Triton X-100-perfused canine basilar artery. Responses are time matched from paired preparations. In (c) the effects of 5-HT are shown before and 30 min after methylene blue, (MB, 10 μM), in untreated canine basilar artery. Note the contractile effect of methylene blue alone. 5-HT was added to the bath at 0.5 log unit increments at points indicated; figures indicate cumulative concentration (−log M).

contraction of dog basilar artery (0.79 ± 0.16 g, $n = 4$), which made an analysis of its effects on 5-HT more complex, 5-HT contractions, in the presence of methylene blue, appeared to be larger and more sustained (Figure 2c).

The selective 5-HT agonists, α-methyl 5-HT, GR 43175 and 5-CT each caused contraction of control dog basilar artery segments. These agonists differed markedly in the magnitude of contraction produced with a rank order of α-methyl 5-HT > GR 43175 > 5-CT. However, the effects of each agonist appeared to be greatly enhanced by Triton X-100 perfusion (see Table 1) and, furthermore, responses appeared to be enhanced by a similar extent (about 100%). There was, however, no evidence for a change in sensitivity of the tissue, since the respective agonist EC_{50} values were similar in untreated and Triton X-100-perfused tissues (Table 1). The concentration-effect curves for α-methyl 5-HT, GR 43175 and 5-CT in untreated and treated dog basilar artery are shown in Figure 3.

In separate experiments, attempts were made to demonstrate a relaxant effect of 5-HT or 5-CT in endothelium-intact dog basilar artery. Tone was increased with submaximal concentrations of a variety of spasmogens, (e.g. U46619 (10–30 nM), $PGF_{2\alpha}$ (1–3 μM), uridine triphosphate (10–100 μM), potassium chloride (20–30 mM)) but 5-HT and 5-CT caused only further contractions. Furthermore, even in the presence of antagonists to block contraction (ketanserin 1 μM or phenoxybenzamine 30 μM), no relaxation to either 5-HT or 5-CT could be revealed (results not shown).

Effects of antagonists

The contractile effects of 5-HT were antagonized by ketanserin (1 μM) to a similar degree in untreated and Triton X-100-treated dog basilar artery; there was little shift in the curve but a depression of the maximum response (Figure 4a and b). Furthermore, the antagonist potency of methiothepin (0.1 μM, in the continued presence of ketanserin 1 μM) did not appear to be modified by Triton X-100; calculated CRs were 33 (20–49) and 39 (29–65) (geometric mean (range) $n = 3$) in untreated and Triton X-100-treated dog basilar artery, respectively (Figure 4c and d).

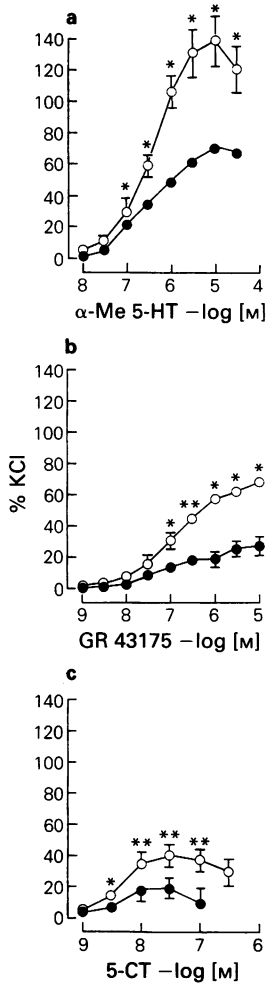


Figure 3 Effects of selective 5-hydroxytryptamine (5-HT) agonists in control (●) and Triton X-100-perfused (○) canine basilar artery; (a) α-methyl 5-HT, (b) GR 43175 (3-[2-dimethyl amino] ethyl-N-methyl-1H-indole-5-methane sulphonamide) and (c) 5-carboxamidotryptamine (5-CT). Responses are expressed in terms of the contraction obtained to 30 mM KCl. Values are means (s.e.mean shown by vertical bars) from 4 experiments. * $P < 0.05$, ** $P < 0.01$ (paired Student's t test) compared to controls.

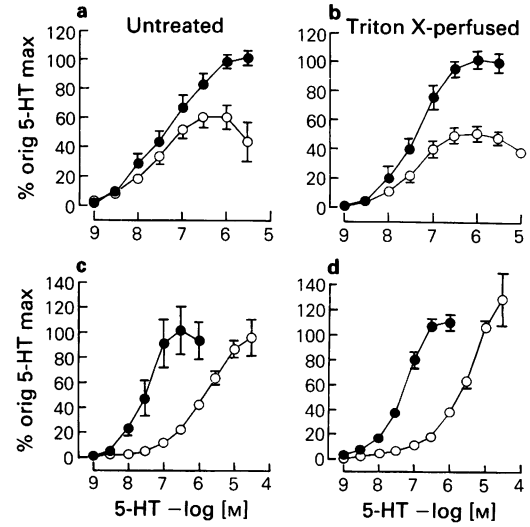


Figure 4 Antagonist effect of ketanserin, 1 μM (a,b, $n = 5$–7) or methiothepin, 0.1 μM (in the presence of ketanserin 1 μM; c,d, $n = 3$) against 5-HT-induced contraction of endothelium-intact (untreated) or endothelium denuded (Triton X-perfused) canine basilar artery. Responses shown are the 2nd 5-HT control curve (●) and 5-HT in the presence of antagonist (○) expressed in terms of the maximum response for the 1st 5-HT curve in each tissue. Values shown are means (with s.e.mean shown by vertical bars) from n experiments.

Effects of other agonists

The contractile effects of some other agonists were also examined in untreated and Triton X-100-perfused dog basilar artery. $PGF_{2\alpha}$ produced larger responses in preparations without a functional endothelium although this difference did not attain statistical significance (Figure 5a); the EC_{50} value was 2(0.3–11) and 0.6(0.4–0.7) μM and the maximum response was 126 ± 14 and 205 ± 44% of the contraction obtained to 30 mM KCl for untreated and Triton X-perfused preparations respectively ($n = 3$). The effects of the thromboxane A_2-mimetic, U46619, were unchanged (Figure 5b); the EC_{50} value was 4(2–6) and 3(2–6) nM and the maximum response was 151 ± 22 and 156 ± 12% of the contraction obtained to 30 mM KCl respectively ($n = 4$, values are means (95% confidence limits) or means ± s.e.mean). Potassium chloride also produced largely similar contractions of untreated and Triton X-100-perfused preparations (Figure 5c).

Histology

Using scanning EM, untreated artery segments were seen to have a relatively intact layer of endothelial cells over the intimal surface. In marked contrast, examination of artery segments that had been perfused with Triton X-100 showed large areas from which endothelial cells were absent. Remaining endothelial cells were damaged or fragmented.

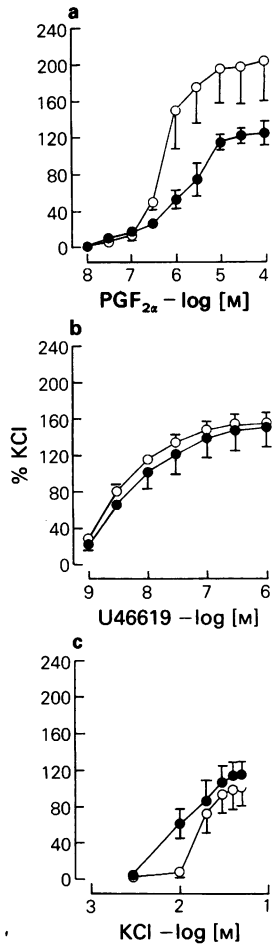


Figure 5 Contractile effects of (a) prostaplandin $F_{2\alpha}$ ($PGF_{2\alpha}$) ($n = 3$), (b) U46619 ($n = 4$) and (c) potassium chloride (KCl, $n = 4$) in untreated (●) and Triton X-100-perfused (○) canine basilar artery. Responses are expressed in terms of the contraction obtained to 30 mM KCl. Values are means (with s.e.mean shown by vertical bars) from n observations.

Similar evidence for the ability of Triton X-100 perfusion to cause loss of endothelial cells was obtained from light microscopy examination of serial transverse sections of control and Triton X-perfused basilar artery segments (see Connor & Feniuk, 1987).

Discussion

These experiments were performed in order to determine whether the endothelium can modify the contractile effects of 5-HT and selective 5-HT agonists in canine basilar artery. It is now well established that, in many isolated blood vessels, the endothelium can have an important influence on responses produced by a variety of agonists. This is attributed to the ability of the endothelium to produce EDRF, which acts directly on the vascular smooth muscle to cause relaxation and therefore offsets any direct contractile effect of the agonist (Furchgott, 1983). Release of EDRF can be produced through the action of an agonist at specific receptors thought to be located on the endothelial cell membrane. More recently it has become evident that, in certain blood vessels, there is a continuous basal release of EDRF from the endothelium. This exerts a depressant influence on the ability of a wide variety of spasmogens to increase tone in the vessel (Martin *et al.*, 1986).

Removal of the endothelium from isolated blood vessels is usually performed by gentle mechanical abrasion. However, in small vessels such as cerebral arteries, this technique can also cause some degree of smooth muscle damage. Therefore in the present studies, the endothelium of canine basilar artery was removed by a method described by Verrechia *et al.* (1986) in which the vessel was perfused with Triton X-100 (0.1%). The integrity of the endothelium was assessed pharmacologically, by examining the relaxant effects of the endothelium-dependent vasodilator substance P (Furchgott, 1983), and histologically. We have previously found that Triton X-100-perfusion of canine basilar artery will abolish the relaxant effect of substance P, whilst relaxations produced by sodium nitroprusside are unchanged (Connor & Feniuk, 1987). This indicates that the capacity of the vascular smooth muscle to relax is not impaired by this procedure. In the present studies, we have compared the effects of 5-HT in canine basilar artery with an intact endothelium, with responses obtained in Triton X-100-perfused basilar artery which lacked a functional endothelium. In these preparations, potassium chloride (30 mM) produced similar responses, suggesting that Triton X-100 had not caused smooth muscle damage. Triton X-100 perfusion markedly enhanced the magnitude of contractions produced by 5-HT. However, since the EC_{50} value for 5-HT was similar in untreated and Triton X-100-perfused preparations, it appeared that damage to the endothelium did not modify the sensitivity of the cerebrovascular smooth muscle to 5-HT. Interestingly, in some experiments there was a clear difference in the nature of the 5-HT-induced contractions in endothelium-intact compared to the endothelium-damaged preparations. In untreated preparations, the effects of 5-HT were rapid in onset and transiently maintained, whilst in contrast, after Triton X-100 perfusion, 5-HT produced slower, more maintained contractions (see Figure 2). These results are

in contrast to a previous study in cat cerebral arteries in which, after rubbing to remove the endothelium, responses to 5-HT appeared unchanged (Young *et al.*, 1986). However, in those experiments, contractions produced by potassium chloride were reduced in denuded preparations, compared to unrubbed preparations, suggesting that the rubbing procedure had caused some degree of smooth muscle damage. A recent report by Garland (1987) is in agreement with our findings: 5-HT-induced contractions of rabbit basilar artery were increased by removal of the endothelium although 5-HT-induced depolarization was unchanged.

A subclassification of 5-HT receptors into 5-HT_1-like, 5-HT_2 and 5-HT_3 receptor types has recently been proposed (Bradley *et al.*, 1986a). It is now apparent that 5-HT_1-like receptors are a heterogeneous group and can be further subdivided (Humphrey *et al.*, 1988). There is a lack of agreement in the literature on the 5-HT receptor type which mediates contraction of cerebral arteries (Muller-Schweinitzer & Engel, 1983; Bradley *et al.*, 1986b; Peroutka *et al.*, 1986; Connor *et al.*, 1987); this is probably due, at least in part, to the involvement of a mixed population of 5-HT_1-like and 5-HT_2 receptors. In the present studies, to assess the influence of endothelial damage on cerebrovascular reactivity, the effects of α-methyl 5-HT, 5-CT and GR 43175 were compared in endothelium-intact and Triton X-100-perfused canine basilar artery. These 5-HT receptor agonists show very different selectivities for the various 5-HT receptor subtypes (Richardson *et al.*, 1985; Bradley *et al.*, 1986a; Humphrey *et al.*, 1988). However, despite these differences each agonist, like 5-HT, produced significantly larger contractions in preparations without an intact endothelium, with no accompanying change in their respective EC_{50} values. The maximum response produced by each of the selective agonists and 5-HT was increased by a similar extent ($\simeq 100\%$). Therefore, there was no indication that selectivity for a particular 5-HT receptor subtype resulted in a greater, or lesser, degree of enhancement. Furthermore, analysis of the influence of either the 5-HT_2 antagonist ketanserin or the 5-HT_1 antagonist methiothepin (in the presence of a high concentration of ketanserin) on 5-HT-induced contractions of canine basilar artery revealed that endothelial damage did not modify the potency of either antagonist.

A possible explanation for these findings is that 5-HT and selective 5-HT agonists can stimulate the release of EDRF from the endothelium of canine basilar artery, through an action at a specific 5-HT receptor. In a few peripheral isolated blood vessels, such as dog and pig coronary artery (Cocks & Angus, 1983), rabbit jugular vein (Leff *et al.*, 1987), and chick jugular vein (Imaizumi *et al.*, 1984), 5-HT has been reported to stimulate release of EDRF through an atypical 5-HT (non 5-HT_2) receptor. However in dog basilar artery, either alone or in the presence of ketanserin or phenoxybenzamine to prevent the contractile effects, we could not reveal a relaxant effect for 5-HT or 5-CT when tone in the preparation was increased. This would suggest that 5-HT does not directly stimulate release of EDRF through a specific 5-HT receptor, although the possibility that the same receptors mediate contraction and relaxation cannot be discounted. Further evidence against there being an endothelium-dependent 5-HT receptor which mediates relaxation in this tissue comes from the finding that endothelium removal potentiates the effects of 5-HT and a variety of 5-HT receptor agonists showing varying degrees of selectivity for different 5-HT receptor subtypes by a remarkably similar extent. If a specific 5-HT receptor were involved, the selective agonists might have been expected to be differentially affected by removal of the endothelium.

5-HT can stimulate prostacyclin synthesis in smooth muscle cells (Coughlin *et al.*, 1984). Since prostacyclin is a potent dilator agent of cerebral arteries (Boullin, 1980), it could be hypothesized that our findings might be explained by a 5-HT-induced release of prostacyclin from the cerebrovascular endothelium. However, this is unlikely since flurbiprofen, at a concentration (0.5 μM) which inhibits prostaglandin synthesis (Nozu, 1978), did not enhance the contractile effects of 5-HT in endothelium-intact canine basilar artery. Indeed, although in the presence of flurbiprofen, there was no shift in the concentration-effect curve to 5-HT (at EC_{50} level), there appeared to be a small reduction (20% compared to 1st curve) in the maximum contraction obtained (our own unpublished observations).

It could be argued that our findings are due to some effect of Triton X-100 on the cerebrovascular smooth muscle which was unrelated to endothelium removal. Responses to $PGF_{2\alpha}$ were also smaller in the untreated preparations. However, in contrast, the thromboxane-A_2-mimetic, U46619, and potassium chloride produced contractions which were similar in untreated and Triton X-perfused preparations, making such a suggestion seem unlikely.

A more probable explanation for our findings is that there is a spontaneous release of EDRF in dog basilar artery, which attenuates contractions produced by certain spasmogens. Although we have not investigated why the cerebrovascular endothelium appears able to reduce responses to some spasmogens but not others, it is interesting that Martin *et al.* (1986) reported that the endothelium of rat aorta can differentially modify the effects of various spasmogens, probably via spontaneous release of EDRF.

They found that for partial agonists, the magnitude of the response was increased and for full agonists, tissue sensitivity was increased, by endothelium removal. Our findings may suggest that the influence of endothelial removal on the contractile response to a given agonist is dependent upon its relative intrinsic efficacy. Thus for agonists with a high intrinsic efficacy, (U46619), endothelial removal has little influence, whilst for agonists with a low intrinsic efficacy, the contractile response is markedly enhanced. However, unlike Martin *et al.* (1986), we were unable to detect an increase in tissue sensitivity to agonists with high intrinsic efficacy following endothelial removal. The reason for this difference is unclear. A possible receptor-mediated release of EDRF by agonists with low intrinsic efficacy has already been discounted (see above).

Although we have not provided direct evidence for 'EDRF' being responsible for attenuating influence of the endothelium, this is the most likely candidate. Our attempts to characterize this attenuating factor as 'EDRF' by the use of haemoglobin (Martin *et al.*, 1986) were hampered by the fact that haemoglobin caused considerable foaming in the tissue baths and also caused contraction of the isolated cerebral arteries (Connor & Feniuk, 1987). However, our preliminary studies using methylene blue lend some support to this proposal. Methylene blue alone caused marked contraction of the cerebral artery, which might reflect blockade of spontaneous release of EDRF. Indeed, this concentration of methylene blue (10 μM) has been shown previously to block endothelium-dependent relaxation (Martin *et al.*, 1985). Furthermore, it is noteworthy that the effects of 5-HT in the presence of methylene blue appeared to be remarkably similar to the effects of 5-HT in endothelium-denuded dog basilar artery (compare Figure 2b and 2c).

In conclusion, these results suggest that the endothelium can have an important influence on the responsiveness of isolated canine basilar artery to a variety of spasmogens. Of particular interest is the finding that 5-HT caused contractions of the isolated cerebral artery which were markedly depressed in the presence of a functional endothelium, possibly through spontaneous release of EDRF. Whether or not the endothelium of human cerebral blood vessels has the ability to attenuate contractions to spasmogens, like 5-HT remains to be determined. However our findings raise the possibility that in conditions where there is damage to the cerebrovascular endothelium, either in the form of a reduced capacity for EDRF release or actual loss of endothelial cells, the vasoconstrictor effects of endogenous 5-HT on cerebral blood vessels could be markedly enhanced. This could have important implications in cerebrovascular disorders such as cerebral vasospasm or transient ischaemic attacks, in which diminished blood flow to certain brain areas is a consequence of cerebral artery constriction, and where 5-HT, along with other spasmogens, may be involved.

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The influence of ouabain on twitch potentiation by anticholinesterases in the phrenic nerve-diaphragm muscles of mice

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1 (+)-Tubocurarine, hexamethonium, atropine, ouabain, and removal of potassium from the bathing medium were examined for their effects on indirectly evoked twitches (IT) of mouse phrenic nerve-diaphragm muscles in the presence or absence of neostigmine.

2 Neostigmine increased the amplitude of IT. The twitch potentiation was reduced by (+)-tubocurarine at low concentrations that had no inhibitory effect on normal IT. Hexamethonium (10–100 μM), but not atropine (0.1–1 μM), partially inhibited the twitch potentiation. Neither hexamethonium nor atropine had an inhibitory effect on IT in the absence of neostigmine.

3 Ouabain (5 μM) abolished the twitch potentiation by neostigmine while having no inhibitory effect on directly evoked twitches in the presence of neostigmine and (+)-tubocurarine together.

4 The potentiating effect of neostigmine was less in a potassium-free bathing solution. The inhibitory effect of ouabain disappeared in this solution.

5 Reinclusion of KCl at 2.5 mM restored both the potentiating effect of neostigmine and the antagonistic effect of ouabain. This reinclusion did not potentiate IT in the absence of neostigmine.

6 An interaction resembling that between ouabain and neostigmine was obtained between ouabain and physostigmine or paraoxon.

7 Both endplate potentials (e.p.ps) and miniature e.p.ps increased in terms of their amplitude and duration in the presence of neostigmine. Ouabain did not reduce the enhanced endplate responses.

8 These results indicate that the potentiation of IT by anticholinesterases may occur via nicotinic receptors which are sensitive to both (+)-tubocurarine and hexamethonium, and that the interaction between anticholinesterases and ouabain depends on the presence of K^+. It appears that the mechanisms of twitch potentiation are dependent on the ionic gradients maintained by Na^+-K^+-ATPase.

Introduction

Anticholinesterases increase the peak tension of the twitch response to neural stimulation, the so-called twitch potentiation (Clark & Hobbiger, 1983). This potentiation is probably due to an inhibition of acetylcholinesterase (Masland & Wigton, 1940). The inhibition of acetylcholinesterase makes possible the prolonged action of the transmitter in the synaptic cleft of the neuromuscular junction (Masland & Wigton, 1940). Four hypotheses have been proposed to explain the twitch potentiation which follows the inhibition of acetylcholinesterase. First, the prolonged action of acetylcholine at a nerve terminal might result in repetitive antidromic action potentials in the nerve terminal, which would be conducted to the other terminals of the same neurone and would be associated with the twitch potentiation (Masland & Wigton, 1940). Second, the excess transmitter might cause increases in the amplitude and duration of endplate response, giving rise to repetitive muscle action potentials (Eccles *et al.*, 1942). Third, the twitch potentiation might be the direct result of the excess acetylcholine in the synaptic cleft, independent of the repetitive antidromic firings, following the inhibition of acetylcholinesterase (Clark & Hobbiger, 1983). Fourth, apart from the inhibition of acetylcholinesterase, anticholinesterases might

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directly produce the antidromic action potentials by acting on nerve terminals to cause the twitch potentiation (Werner & Kuperman, 1963; Riker & Okamoto, 1969). Thus, it is uncertain how twitch potentiation occurs in the presence of anticholinesterases.

The present study was carried out to examine the effects of different types of agents on both the twitch response and the endplate responses in the presence and absence of anticholinesterases. It is suggested that ionic gradients maintained by Na^+-K^+-ATPase activity are important for the mechanisms of potentiation of the twitch but not of the endplate potential in the presence of anticholinesterases.

Methods

Media and preparation of tissue

All experiments were performed on the isolated left hemidiaphragm preparation of male mice of the ddy strain with a body weight of 28–33 g (8 to 12-weeks-old). The preparation was soaked in Krebs-Ringer solution of the following composition (mM): NaCl 136, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 15 and glucose 11. A potassium-free bathing solution (K-free bathing solution) was prepared by replacing 5 mM KCl with equimolar NaCl. A solution containing 0.8 mM Ca and 5 mM Mg (low Ca bathing solution) was also prepared for the measurements of endplate potentials (e.p.ps). The solution was bubbled with a mixture of 95% O_2 and 5% CO_2 and kept at pH 7.3 and 36°C except where otherwise stated. The preparation was equilibrated in the relevant bathing solution for at least 30 min before addition of any agents. The temperature of the solution in the bath was monitored by a thermister (Shibaura Electric Co., Model MGA-II) and held constant by means of an external water jacket and a thermoregulatory device (Taiyo, Thermominder Mini 80).

Tension recordings

One end of the site of insertion into the rib cage of the left hemidiaphragm with its phrenic nerve was fixed and the other central tendon was attached to a force displacement transducer (Nihon Kohden, TB-612T). The phrenic nerve was stimulated through a circular electrode connected to an electronic square pulse generator (Nihon Kohden, SEN-3201) with a constant voltage output. Muscle layers were stimulated directly in the presence of 2.5 or 10 μM (+)-tubocurarine. All stimuli were supramaximal (4 V or more) and of 0.07 ms duration at 0.1 Hz. The resulting isometric muscle twitches were recorded on a penwriting recorder (Nihon Kohden, WI-641G).

Electrophysiology

Intracellular recordings were made with glass microcapillary electrodes filled with 3 M KCl and of 6–10 MΩ resistance. The electrode was inserted into fibres near endplate regions. The signals were led through a high-impedance unity-gain preamplifier (Nihon Kohden, MEZ-8201), displayed on an oscilloscope (Nihon Kohden, VC-10) and stored on magnetic tape (Nihon Kohden, RMG-5204).

Miniature e.p.ps (m.e.p.ps) of 0.1 mV or larger amplitude were counted by a computer (Nihon Kohden, DAB-1100). M.e.p.ps were recorded for successive periods of 1 min after exposure to a given solution and the mean frequency of m.e.p.ps (s^{-1}) was calculated. Only data from junctions at which frequency of m.e.p.ps in the control solution was relatively stable for some time were included in the experimental results. E.p.ps at all the endplates, were evoked by stimulation of the phrenic nerve with 256 square pulses of 0.1 ms duration and supramaximal voltage at 2 Hz from a suction electrode in the low Ca bathing solution. The quantal content (m) was estimated by the method of failures from

$$m = \log_e(N/N_0),$$

where N = number of trials; N_0 = number of failures (Crawford, 1974).

Student's t test were used for statistical analysis and a probability of less than 0.05 was deemed statistically significant.

Drugs used were: (+)-tubocurarine chloride, neostigmine bromide, physostigmine sulphate, hexamethonium chloride, atropine sulphate (Wako Pure Chemicals); paraoxon (Sigma); ouabain (Merck).

Results

Interactions between anticholinesterases and anticholinoceptors

Twitch response to stimulation of the phrenic nerve was abolished by 2.5 μM (+)-tubocurarine, a result which demonstrates the indirect origin of the response (indirectly-evoked twitches, IT). In the presence of 10 μM (+)-tubocurarine, stimulation of the muscle layer produced directly-evoked twitches (DT). Neostigmine (Figure 1), physostigmine (see Figure 7) and paraoxon (data not shown) increased the amplitude of IT but not of DT. The effect of neostigmine was dependent on its concentration; it was effective at 50 nM and produced a maximum effect at 200 nM (228.7 $\pm$ 14.0% of control before the addition of neostigmine, mean $\pm$ s.e.mean, n = 10) or at higher concentrations. These agents frequently caused repetitive fasciculations of twitches between

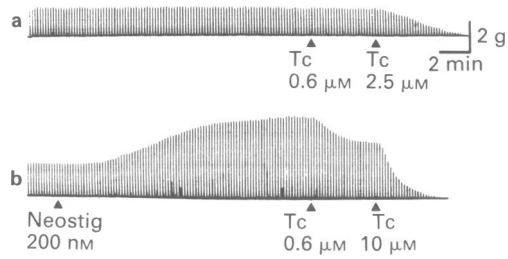


Figure 1 Effectiveness of a low concentration (0.6 μM) of (+)-tubocurarine on twitch potentiation by neostigmine in the mouse phrenic nerve-diaphragm preparations. Phrenic nerve was stimulated by 0.07 ms pulses of supramaximal voltage at 0.1 Hz. Tc: (+)-tubocurarine; Neostig: neostigmine.

the neuronal stimulations. The spontaneous twitches were abolished by (+)-tubocurarine at concentrations lower than those which were effective in abolishing IT in the absence of neostigmine.

The inhibitory effect of (+)-tubocurarine on IT depended on its concentration. Inhibitory concentration for half maximal effect (IC_{50}) was 1.25 μM (95% confidence limit, 1.13–1.39 μM, $n = 12$) in the absence of neostigmine. In the presence of neostigmine, 0.6 μM (+)-tubocurarine appreciably inhibited IT, whereas it had little effect on IT in the absence of neostigmine (Figures 1 and 2). Figure 2 shows the effectiveness of very low concentrations of (+)-tubocurarine in reducing IT in the presence of neostigmine. Hexamethonium, at 10 to 100 μM, had little effect on IT in the standard bathing medium. However, hexamethonium significantly inhibited IT

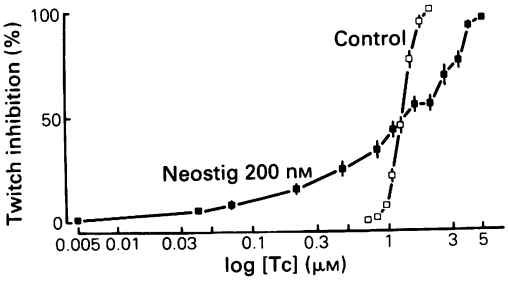


Figure 2 Concentration-effect curve to (+)-tubocurarine of indirectly evoked twitches in the presence (■) and absence (□) of neostigmine (200 nM). (+)-Tubocurarine was added cumulatively at intervals of 4 min, because this period was long enough to allow the effect to reach a steady state. Ordinate scale: percentage inhibition of twitches in terms of the twitch just prior to the first addition of (+)-tubocurarine. Abscissa scale: log μM concentration of (+)-tubocurarine. Vertical bars show s.e.mean of results from $n = 12$ preparations. Tc: (+)-tubocurarine; Neostig: neostigmine.

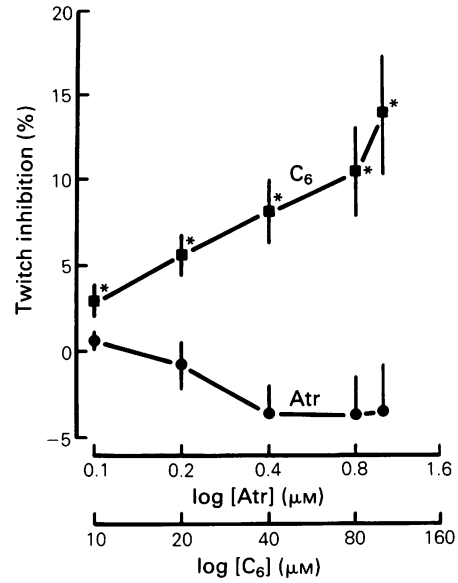


Figure 3 Effects of hexamethonium and atropine on twitch potentiation by neostigmine. Phrenic nerve was activated by 0.07 ms pulses of supramaximal voltage at 0.1 Hz in the presence of 200 nM neostigmine. Cholinoceptor blockers were added cumulatively at intervals of 4 min. Percentage inhibitions of twitches are presented. Hexamethonium and atropine in concentrations used here had no inhibitory effects on indirectly-evoked twitches in the absence of neostigmine. Vertical bars show s.e.mean of results from $n = 8$ preparations. * $P < 0.05$ by Student's t tests. C_6: hexamethonium; Atr: atropine.

potentiated by neostigmine in a concentration-dependent manner; thus neostigmine made IT sensitive to hexamethonium (Figure 3). Atropine, at 0.1 to 1 μM, had no such inhibitory effect on IT in the presence or in the absence of neostigmine. The spontaneous twitches evoked by neostigmine also

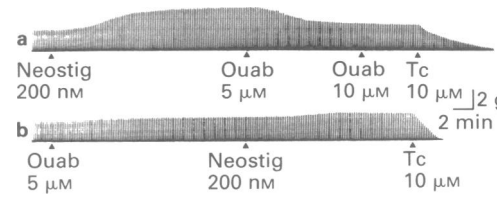


Figure 4 An interaction between ouabain and neostigmine on indirectly-evoked twitches. Phrenic nerve was stimulated with 0.07 ms pulses of supramaximal voltage at 1 Hz. Ouabain (5 μM) was added before (b) or after (a) the application of 200 nM neostigmine. Supplemental ouabain (10 μM) in trace (a) was barely effective. Tc: (+)-tubocurarine; Ouab: ouabain; Neostig: neostigmine.

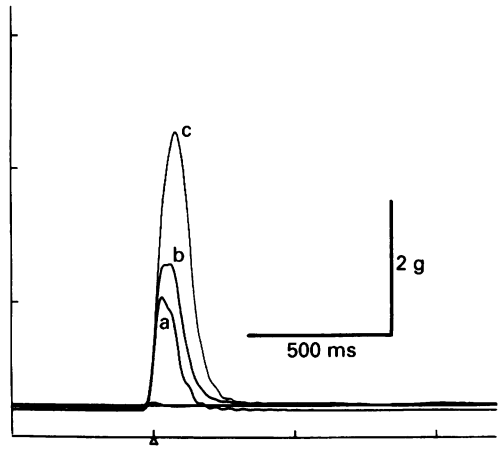


Figure 5 Interaction between neostigmine and ouabain on twitch curves induced indirectly in a mouse phrenic nerve-diaphragm: (a) control; (b) 20 min after addition of 200 nM neostigmine; (c) 20 min after addition of 5 μM ouabain to (b). Each tracing was averaged over 8 trials.

disappeared in the presence of hexamethonium, but not of atropine.

Interaction between anticholinesterases and ouabain or removal of potassium

Ouabain, at 5 or 10 μM, had no inhibitory effect on IT by itself; rather, it actually increased the amplitude of IT (see Figure 4b) and of DT, thus producing

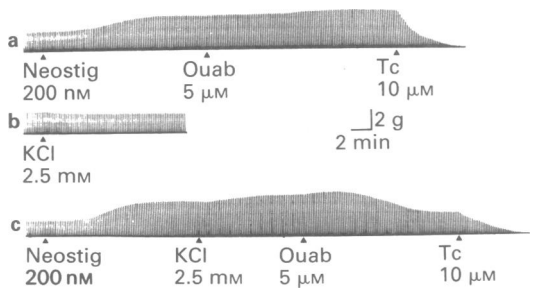


Figure 6 (a) Potentiating effect of neostigmine (200 nM) on indirectly evoked twitches in a K-free bathing solution and the absence of an antagonistic effect of 5 μM ouabain on this type of twitch potentiation. (b) No potentiating effect of reinclusion of 2.5 mM KCl on indirectly evoked twitches in the K-free bathing solution. (c) Restoration by the reinclusion of KCl both of the potentiating effect of neostigmine and of the antagonistic effect of ouabain. Phrenic nerve was stimulated with 0.07 ms pulses of supramaximal voltage at 0.1 Hz. Tc: (+)-tubocurarine; Neostig: neostigmine; Ouab: ouabain.

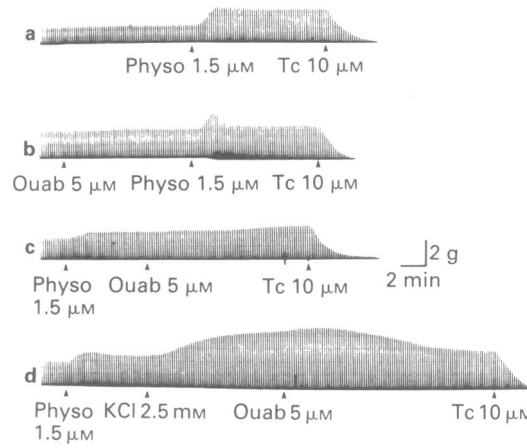


Figure 7 (a and b) Effects of physostigmine (1.5 μM) on indirectly evoked twitches in a standard bathing solution with or without ouabain (5 μM). (c) Partial twitch potentiation by physostigmine (1.5 μM) in a K-free bathing solution and the absence of an effect of ouabain (5 μM) on this type of twitch potentiation. (d) Restoration of potentiating effect of physostigmine and of antagonistic effect of ouabain by reinclusion of 2.5 mM KCl. Phrenic nerve was stimulated by 0.07 ms pulses of supramaximal voltage at 0.1 Hz. Tc: (+)-tubocurarine; Physo: physostigmine; Ouab: ouabain.

an inotropic effect. Only when IT were increased by neostigmine did ouabain (5 μM) reduce them to a control level (Figure 4). Ouabain occasionally caused a transiently inotropic effect on IT before inhibiting them. Pretreatment with ouabain in the standard solution was effective in inhibiting the potentiation of twitches by neostigmine. Figure 5 shows the effect of 200 nM neostigmine combined with and without 5 μM ouabain on twitch curves. Neostigmine enhanced both the amplitude and duration of IT. Ouabain reduced both these parameters. In the K-free bathing solution, a potentiating effect of neostigmine appeared, but it was less potent ($166.0 \pm 13.5\%$ of control, $n = 8$, compared to $228.7 \pm 14.0\%$ of control in the standard solution) (Figure 6). Such an effect of neostigmine was insensitive to pre- and post-application of ouabain to this solution. The KCl concentration of the solution containing neostigmine was raised to 2.5 mM; this reinclusion of KCl restored the fully potentiating effect of neostigmine ($293.0 \pm 17.4\%$, $n = 8$) (Figure 6). The antagonistic effect of the post-application of ouabain was also reproduced after this reinclusion of KCl. In the absence of neostigmine, the reinclusion of KCl had no potentiating effect on the original amplitude of IT. Such an antagonism of ouabain on the twitch potentiation was observed with other anticholinesterases, such as physostigmine (Figure 7) and

paraoxon (data not shown). The potentiating effect of physostigmine was also less powerful and was not antagonized by ouabain in the K-free solution. The spontaneous twitches induced by anticholinesterases were measured in the presence of ouabain.

Endplate responses

The frequency, amplitude and duration of m.e.p.ps and the quantal content of e.p.ps were measured in a low Ca bathing solution. The effects of neostigmine on these parameters were examined in the presence or absence of ouabain. Typical results are shown in Figures 8 and 9. It is apparent that ouabain does not inhibit the effects of neostigmine on these parameters. Neostigmine (200 nM) greatly enhanced both the amplitude and duration of m.e.p.ps and e.p.ps, but caused no appreciable changes in the frequency of m.e.p.ps and in the quantal content. In the presence of neostigmine, ouabain (5 μM) increased the frequency of m.e.p.ps and the quantal content but did not decrease either the amplitude or the duration of both m.e.p.ps and e.p.ps. In the same bathing solution, but without neostigmine, ouabain increased the frequency of m.e.p.ps and the quantal content by itself (data not shown). Thus, neostigmine seemed to augment the endplate response to the transmitter, in terms of their amplitude and duration, but did not appreciably augment the release of transmitter; ouabain did not change the altered sensitivity of the endplate to the transmitter in the presence of neostigmine.

Discussion

The anticholinesterases used here caused twitch potentiation in response to neural stimulation in the mouse phrenic nerve-diaphragm preparations, as has been observed by many other investigators (Chang

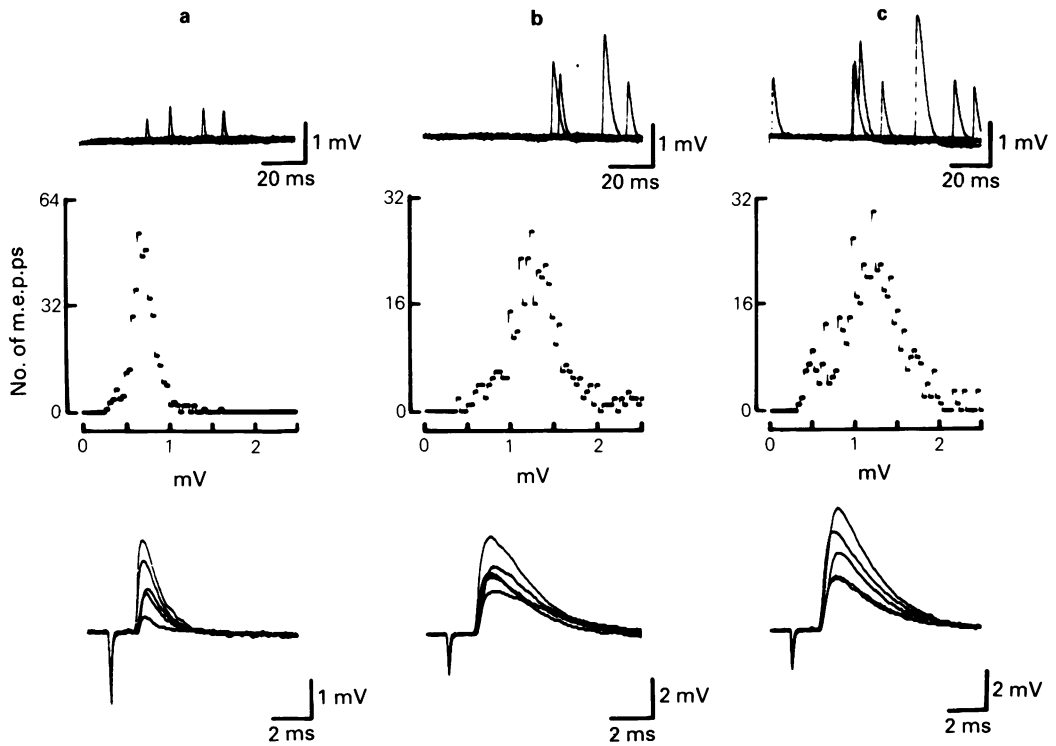


Figure 8 Typical results of the effects of neostigmine with or without ouabain on endplate potential changes. All the measurements were recorded from the same endplate in 0.8 mM Ca^{2+}, 5 mM Mg^{2+} bathing solution. (a) Control, (b) 15–25 min after addition of neostigmine (200 nM); (c) 15–25 min after addition of ouabain (5 μM) to the solution which contained neostigmine. Upper panels: m.e.p.ps (superimposed tracings of 10 sweeps). Middle panels: histograms of amplitude for m.e.p.ps, 391 counts per 180 s for control, 375 counts per 180 s for neostigmine-treatment, 483 counts per 180 s for neostigmine-ouabain-treatment. Bottom panels: e.p.ps (superimposed tracings of 5 sweeps).

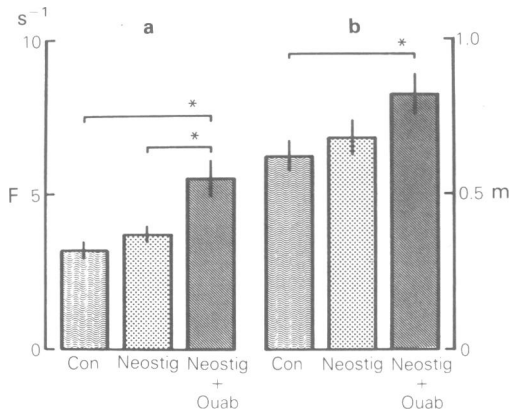


Figure 9 Summary of the effects of neostigmine (200 nM) with or without ouabain (5 μM) on changes in endplate potentials in mouse phrenic nerve-diaphragm preparations in 0.8 mM Ca^{2+}, 5 mM Mg^{2+} bathing solution. Control values were measured within 10 min after equilibration for 30 min in the low Ca^{2+} solution. Values in the presence of agents were recorded within 10 min after a 20 min incubation period. Ouabain was added 30 min after pretreatment with neostigmine. Vertical bars show s.e.mean of results from 33 to 40 endplates of 21 preparations. * $P < 0.05$. F: frequency of m.e.p.ps (s^{-1}). m: quantal content calculated from e.p.ps evoked by 256 pulses of 0.1 ms duration, supramaximal voltage at 2 Hz. Neostig: neostigmine; Ouab: ouabain.

et al., 1985; see Hobbiger, 1976). The twitch potentiation by neostigmine was inhibited by agents which block nicotinic receptors, namely (+)-tubocurarine and hexamethonium, at concentrations of these inhibitors lower than those that affect the twitches of normal muscle. Similar observations have been obtained in the rat and mouse striated muscles with pancuronium and hexamethonium (Clark *et al.*, 1983; 1984). The latter authors explained these observations in terms of the reduced safety margin for initiation of additional action potentials by prolonged endplate potentials. Further, a third class of nicotinic receptors has been proposed to be active during post-tetanic twitch potentiation (Waldenlind, 1978). This type of receptor was also sensitive both to (+)-tubocurarine and to hexamethonium at concentrations lower than those that affect the normal twitches.

Repetitive fasciculations of twitches in the presence of anticholinesterases have been considered to result from the effect of transmitter released repetitively at the motor nerve terminal, that is to say, via receptors at the neuromuscular junction (Masland & Wigton, 1940; Eccles *et al.*, 1942; Werner & Kuperman, 1963; Riker & Okamoto, 1969). In the present experiments, spontaneous twitches occurred in the presence of neostigmine and were blocked by (+)-tubocurarine and hexamethonium at concentrations lower than those that reduced the amplitude of control IT. Thus, the post-tetanic twitch potentiation (Waldenlind, 1978) and the neostigmine-induced repetitive twitches presented here seem to have a similar pharmacological property.

Twitch potentiation by anticholinesterases was blocked by ouabain and reduced partially in a K-free bathing solution. The antagonistic effect of ouabain disappeared in the K-free bathing solution. Reinclusion of K^+ restored the potentiating effect of neostigmine and the antagonistic effect of ouabain. These results indicate that the interaction between anticholinesterases and ouabain depends on K^+. Ouabain, in common with the removal of K^+ from the bathing solution, has been shown to inhibit Na^+-K^+-ATPase (Clausen, 1986). Thus, it is possible that the potentiating effect of anticholinesterases may depend on the ionic gradients maintained by the activity of this enzyme.

Neostigmine increased the amplitude and duration of m.e.p.ps and e.p.ps but not the frequency of m.e.p.ps or the quantal content of e.p.ps, thus demonstrating its postsynaptic potentiating effect. Such a postsynaptic effect of anticholinesterases is caused by excess transmitter in the synaptic cleft (Eccles *et al.*, 1942). Ouabain did not antagonize this postsynaptic potentiating effect; rather it actually enhanced presynaptic events such as the frequency of m.e.p.ps and quantal content of e.p.ps, even in the presence of neostigmine. However, as mentioned above the twitch potentiation induced by anticholinesterases disappeared in the presence of ouabain. Thus, it is suggested that the ouabain-induced inhibition of the twitch potentiation by anticholinesterases results from inhibition of the mechanisms which lead to repetitive muscle action potentials.

The mechanism by which ouabain and K-free solution reduce the twitch potentiation induced by anticholinesterases is obscure. This twitch potentiation may involve at least two mechanisms: (1) repetitive muscle action potentials arising from a single prolonged endplate potential, and (2) repetitive firing in nerve terminals leading to multiple endplate potentials. Inhibition of Na^+-K^+-ATPase by ouabain and by K-free solution would cause a rise in cytosolic Na^+. The rise in cytosolic Na^+ might inhibit the generation of an action potential by altered gradients for Na^+, thus causing an inhibition of the excitation of muscle fibres. Alternatively, this in turn might permit increased cytosolic Ca^{2+} by reduced extrusion of Ca^{2+} through Na^+/Ca^{2+} exchange (see Langer, 1982). The increase in cytosolic Ca^{2+} is well known to activate cellular responses such as secretion and contraction. However, ouabain and K-free solution reduced the twitch potentiation induced by

anticholinesterases. Thus, changes in transmitter release and/or altered excitability of pre- or postsynaptic membranes, arising from possible altered gradients for Na^+ and Ca^{2+} might underlie the effects of ouabain. K-free solution would exert additional effects on membrane potential as hyperpolarization of muscle fibres (Akaike, 1982).

In summary, the twitch potentiation by anticholinesterases may occur partly via nicotinic receptors which are sensitive to both (+)-tubocurarine and hexamethonium at concentrations lower than those which inhibit normal muscle twitches. The enhancement of the twitch disappeared in the presence of ouabain. The interaction between neostigmine and ouabain depends on the external concentration of K^+, so that a possible role can be proposed for the activity of Na^+-K^+-ATPase in maintaining the necessary ionic gradients.

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E-Type prostaglandins but not iloprost inhibit platelet activating factor-induced generation of leukotriene B_4 by human polymorphonuclear leukocytes

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1 Platelet-activating factor (Paf) was found to stimulate leukotriene B_4 release from human polymorphonuclear leukocytes (PMN). This stimulation was considerably enhanced in the presence of small amounts of exogenous arachidonic acid.

2 Prostaglandin E_2 (PGE_2) and PGE_1 (0.1–10 μM) inhibited this reaction dose-dependently. No inhibition was seen with iloprost at these concentrations.

3 The data suggest that E prostaglandins are potent inhibitors of Paf-induced leukotriene generation by human PMNs and thus may serve as local factors controlling Paf-mediated inflammatory reactions.

Introduction

In response to appropriate stimuli, platelet-activating factor (Paf) (Benveniste, 1974) is formed and released by a variety of cells including polymorphonuclear leukocytes (PMN) (Lotner *et al.*, 1980). This lipid is a potent stimulus of leukocyte activation and appears to serve as a mediator of local changes in smooth muscle tone and late inflammatory reactions. In human PMN, Paf stimulates the formation of reactive oxygen species, secretion of lysosomal enzymes (Shaw *et al.*, 1981), and generation of leukotriene B_4 (LTB_4) (Lin *et al.*, 1982). It has been suggested that PMN activation caused by receptor-mediated mechanisms such as stimulation by the chemotactic peptide formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), is under the control of prostaglandins since prostaglandin E (PGE) modulates PMN responses to different stimuli *in vitro* (O'Flaherty *et al.*, 1979; Fantone & Kinnes, 1983; Ham *et al.*, 1983; Gryglewski *et al.*, 1987). Additionally, it has been shown that PGE but not prostacyclin attenuates the carrageenin-induced inflammatory reactions *in vivo* (Bonta & Parnham, 1979; Parnham *et al.*, 1979). Recently, we have demonstrated that the E-type prostaglandins at low micromolar concentrations inhibit the Paf- and FMLP-induced release of superoxide anions and β-glucuronidase from human PMN under conditions when PGI_2 and iloprost failed to do so (Schrör & Hecker, 1986; 1987). In this study we demonstrate that this inhibitory action of PGE is associated with significant suppression of LTB_4 generation even in the presence of exogenous arachidonic acid, whereas no inhibition is seen with iloprost. A preliminary communication of some of these results has been presented to the British Pharmacological Society (Ney *et al.*, 1988).

Methods

Preparation and incubation of human PMN

Human PMN were prepared from fresh human blood according to standard laboratory procedures (Böyum, 1968) including dextran sedimentation, Ficoll-Paque gradient centrifugation and hypotonic lysis of the remaining erythrocytes. The final suspension contained more than 95% PMN and their viability was at least 95% (trypan blue exclusion test).

PMN 5×10^7 were suspended in Hank's balanced salt solution (HBSS), pH 7.4, and incubated with arachidonic acid (AA) (10 μM), PGE_1, PGE_2, iloprost, or the appropriate vehicles for 10 min at 37°C. The total ethanol content per sample was always less than 0.1% in order to avoid direct actions of the solvent. Afterwards the PMN were stimulated by Paf (3 μM), FMLP (10 μM) or calimycin (A 23187, 10 μM) for another 10 min at 37°C. Then the samples were centrifuged (1100 g; 10 min; 4°C) and the resulting supernatants were analysed for LTB_4 and its ω-hydroxy and ω-carboxy metabolites.

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Table 1 Effect of various stimuli and exogenous arachidonic acid (AA) (10 μM) on leukotriene B_4 (LTB_4) synthesis by human polymorphonuclear leukocytes (PMN)

		Product (ng per 5×10^7 cells)		
Stimulus	n	*LTB_4*	*ω-oxidized LTB_4*	*LTB_4 + ω-oxidized LTB_4*
AA	4–5	10 ± 3	15 ± 5	24 ± 7
Paf (3 μM)	3–5	17 ± 9	11 ± 5	21 ± 8
FMLP (10 μM)	5	5 ± 2	10 ± 3	15 ± 6
Paf (3 μM) + AA	12	96 ± 16	115 ± 22	211 ± 32
FMLP (10 μM) + AA	12	72 ± 19	96 ± 22	170 ± 36
Calimycin (10 μM)	13	1174 ± 115	780 ± 94	1974 ± 184

PMN were preincubated with AA (10 μM) for 10 min at 37°C and the appropriate stimulus was added. Incubation was continued for another 10 min at 37°C and the reaction was stopped by centrifugation (see Methods for details). Results are expressed as ng per 5×10^7 cells (mean ± s.e.mean of *n* observations).
Paf = platelet activating factor; FMLP = formyl-L-methionyl-L-leucyl-L-phenylalanine.

Extraction and h.p.l.c. analysis

The supernatants were acidified with HCl to pH 3 and 200 ng PGB_2 was added as an internal standard. Extraction of eicosanoids was performed according to Powell (1982) using a Baker-10 SPE system equipped with 3 ml octadecyl (C_{18}) SPE-columns (Baker Chemikalien Groß-Gerau, F.R.G.). After evaporation of the solvent in a stream of nitrogen, the sediment was redissolved in 250 μl water:acetonitrile, 66:34 (v/v); 100 μl of this solution were subjected to h.p.l.c. analysis. Analyses were performed with a L-5000 low pressure mixing unit fitted with a model 655A-12 pump and a model 655A-22 variable wavelength u.v.-monitor (set at 280 nm) (Merck-Hitachi, Darmstadt, F.R.G.). Separations were carried out on a LiChrosorb C_{18} 5 μm material reversed phase column (4 mm × 250 mm; E. Merck, Darmstadt, F.R.G.). The various compounds were eluted at a flow rate of 1 ml min^{-1} with water (solvent A) and acetonitrile (solvent B) each containing 0.05% acetic acid as follows: step 1: time 0 to 5.5 min, 66% A/34% B (isocratic); step 2: time 5.5 to 6 min, 66% A/34% B to 54% A/46% B; step 3: time 6 to 19 min, 54% A/46% B (isocratic); step 4: time 19 to 19.5 min, 54% A/46% B to 42% A/58% B; step 5: time 19.5 to 50 min, 42% A/58% B (isocratic). Thereafter, the column was rinsed with solvent B for at least 10 min. Retention times of 20-COOH-LTB_4, 20-OH-LTB_4, PGB_2, and LTB_4 were 5.4, 5.8, 16.6, and 19.6 min, respectively. The identity of the extracted compounds was confirmed by comparing the retention times in h.p.l.c. and the recorded u.v.-spectra with that of authentic synthetic substances. The surface areas of the peaks were calculated with a D-2000 chromato-integrator (Merck-Hitachi, Darmstadt, F.R.G.) and compared to the PGB_2 internal standard after correction for differences in absorption coefficients (ε) of the different compounds. The ε-values used for calculations were 26,800 and 39,500 for PGB_2 and leukotrienes, respectively (Borgeat & Samuelsson, 1979).

Materials

The following were used (suppliers in parentheses): Paf, PGB_2 and Calimycin (A 23187) (Sigma Chemie GmbH, Deisenhofen, F.R.G.); PGE_1 and PGE_2 (Upjohn Co., Kalamazoo, MI, U.S.A.); dextran T 500 and Ficoll-Paque (Pharmacia, Freiburg, F.R.G.); arachidonic acid (Nu-Chek-Prep Inc., Elysian, MN, U.S.A.); LTB_4, 20-hydroxy-LTB_4 and 20-carboxy-LTB_4 (Paesel GmbH, Frankfurt, F.R.G.); and FMLP (Calbiochem GmbH, Frankfurt, F.R.G.). Iloprost was generously provided by Schering AG (Berlin/Bergkamen, F.R.G.). All other substances and solvents were from E. Merck (Darmstadt, F.R.G.) and of the highest purity available.

Statistics

Results are expressed as the mean ± s.e.mean of the number of experiments (*n*). Statistical significance of differences between means was analysed by the two-tailed *t* test. *P* levels of <0.05 were considered significant.

Results

Incubation of human PMN with arachidonic acid (10 μM) or Paf (3 μM) alone yielded only barely

detectable amounts of LTB_4 and its ω-oxidized metabolites resulting in the release of 24 ± 7 ng and 21 ± 8 ng per 5×10^7 cells (mean ± s.e.mean; $n = 5$). In the presence of exogenous AA (10 μM), subsequent Paf challenge increased the LTB_4 release severalfold. Increasing concentrations of Paf caused an increase in LTB_4 production over the dose range 0.1–3 μM although there was no significant difference between the amounts of LTB_4 produced by the single stimulus concentrations used (data not shown; $n = 6$). At 10 μM the stimulated release of LTB_4 and its metabolites declined. Therefore, to avoid any cytotoxic effects which can be exerted by Paf at higher micromolar concentrations (see Pinckard *et al.*, 1988) and to retain comparability with earlier results on Paf-stimulated β-glucuronidase release and superoxide anion generation, all experiments were conducted with a Paf concentration of 3 μM. This amount increased the release of LTB_4 and its ω-oxidized metabolites about 10 fold (96 ± 16 ng; mean ± s.e.mean) (Table 1), which was considerably more than with either Paf or AA alone. The total amount of Paf stimulated LTB_4 and its ω-oxidized metabolites in the presence of 10 μM AA ranged between 62 ng and 429 ng per 5×10^7 PMN in 12 different cell preparations averaging 211 ± 32 ng per 5×10^7 PMN (mean ± s.e.mean). In comparison with Paf, the calcium-ionophore calimycin was a much more potent activator of the arachidonic acid cascade of human PMN. At a concentration of 10 μM it elicited a release of about 2 μg of LTB_4 and its ω-oxidized metabolites (Table 1).

Treatment of PMN for 10 min with PGE_1 and PGE_2 (10 μM) prior to Paf stimulation yielded no measurable LTB_4 formation above the detection limit (5 ng). Paf stimulation (3 μM) in the presence of AA (10 μM) was significantly suppressed by PGE_1 and PGE_2. The chromatograms shown in Figure 1 clearly demonstrate that both the release of LTB_4 and its metabolites is reduced by PGE_1 (10 μM) as compared to control, whereas iloprost, a chemically stable PGI_2 analogue, is ineffective at the same concentration.

The inhibitory effect of PGE-compounds appears to be dose-dependent between 0.1 and 10 μM (Figure 2). Treatment of human PMN with increasing amounts of PGE_2 or PGE_1 leads to an increased inhibition of the release of LTB_4 and its ω-oxidized metabolites. The two compounds are equieffective on a molar basis. About 50% inhibition was obtained at 10 μM PGE ($P < 0.05$). Iloprost at concentrations of 1 and 10 μM exhibited no significant inhibition of release ($131 \pm 19\%$ and $98 \pm 19\%$, respectively; mean ± s.e.mean; $n = 4$) in comparison to control (854 ± 221 ng; mean ± s.e.mean; 100%; $n = 4$).

Figure 3 shows the effect of PGE_1, PGE_2 and iloprost at the maximum concentration used (10 μM

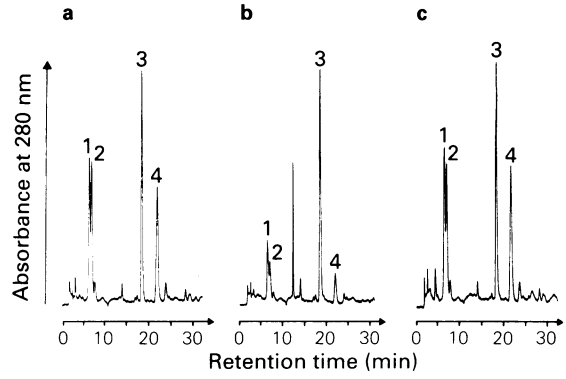


Figure 1 H.p.l.c.-analysis of supernatants, prepared from platelet-activating factor (Paf)-(3 μM) stimulated PMN (5×10^7 cells) pretreated for 10 min with buffer (a), prostaglandin E_1 (PGE_1) (10 μM) (b) or iloprost (10 μM) (c) in presence of 10 μM arachidonic acid. PGB_2 (200 ng) was used as internal standard. The numbers denote 20-COOH-leukotriene B_4 (LTB_4) (1), 20-OH-LTB_4 (2), PGB_2 (3), and LTB_4 (4).

each) on the release of LTB_4 and its ω-oxidized metabolites from Paf-stimulated human PMN. Both the release of LTB_4 as well as that of its metabolites is blocked to a comparable extent. Only 69 ± 33 ng

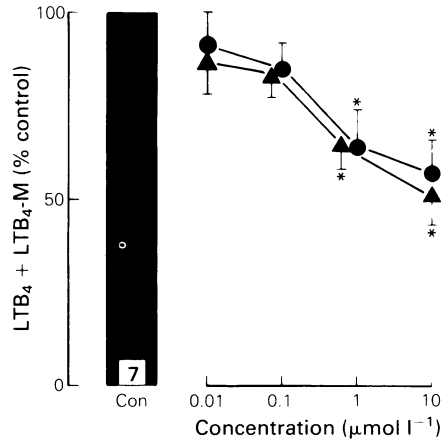


Figure 2 Dose-dependent inhibition of platelet activating factor (Paf) (3 μM) stimulated leukotriene B_4 (LTB_4) by prostaglandin E_2 (PGE_2) (▲) and PGE_1 (●). PMN 5×10^7 were incubated with the indicated concentrations of prostaglandins and arachidonic acid (10 μM) for 10 min prior to stimulation by Paf. The release of LTB_4 and its ω-oxidized metabolites in the absence (Con) (100%, equivalent to 723 ± 149 ng; mean ± s.e.mean) and presence of prostaglandins was used to calculate %release of control. Each point refers to the mean of seven different experiments; bars show s.e.mean. Asterisks denote significant suppression ($P < 0.05$).

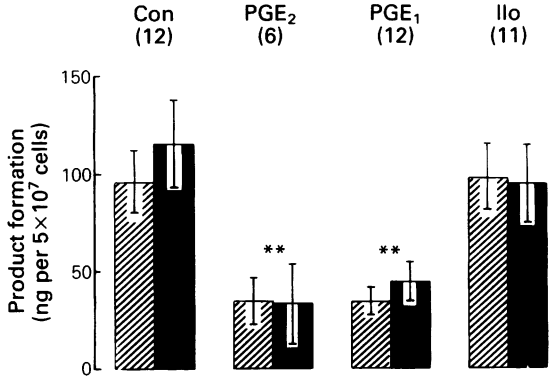


Figure 3 Effects of prostaglandin E_1 (PGE_1), PGE_2 and iloprost (Ilo) on generation of leukotriene B_4 (LTB_4) (hatched column) and its ω-oxidized metabolites (closed column) in plateler activating factor (Paf) stimulated PMN as compared to buffer treated controls (Con). PMN 5×10^7 were incubated with the prostaglandins (10 μM) and arachidonic acid (10 μM) for 10 min prior to stimulation by Paf (3 μM). Compared to control, PGE_1 and PGE_2 inhibited generation of LTB_4 and subsequent metabolite formation by 60–70% ($P < 0.01$) while no change was seen with iloprost ($P > 0.05$). Each column refers to the mean of the number of experiments indicated in parentheses; bars show s.e.mean.

and 81 ± 17 ng (mean $\pm$ s.e.mean; $n = 6$–12) were liberated in the presence of PGE_2 and PGE_1, respectively ($P < 0.01$). On the contrary, iloprost failed to inhibit significantly the Paf-stimulated LTB_4 release (Figures 1 and 3).

Similar results were obtained when the chemotactic peptide FMLP was used as the agonist. Human PMN stimulated under control conditions (10 μM FMLP, 10 μM AA) released 170 ± 36 ng (mean $\pm$ s.e.mean; $n = 12$) of LTB_4 and its ω-oxidized metabolites (Table 1). Pretreatment of PMN with PGE_1 (10 μM) before FMLP stimulation significantly reduced this release to 65 ± 17 ng (mean $\pm$ s.e.mean; $n = 12$; $P < 0.05$), this was equivalent to an inhibition by about 60% of control. Again iloprost (10 μM) was ineffective (182 ± 39 ng; mean $\pm$ s.e.mean; $n = 11$) (Table 2).

In contrast, no inhibition with any prostaglandin was seen, if the ionophore calimycin (10 μM) was used as stimulating agent (Table 3).

Discussion

Paf (3 μM) alone induced only minor release of LTB_4 and its ω-oxidized metabolites from human PMN. This is in accordance with observations of Lin *et al.* (1982) who used neutrophils labelled with radioactive AA to measure LTB_4 release. In the presence of small amounts of AA (10 μM), this release was enhanced about ten fold (Table 1). Recently, similar results have been reported for other receptor-mediated stimuli like FMLP, C5a (Clancy *et al.*, 1983; Haines *et al.*, 1987) and aggregated immunoglobulin G (Smith *et al.*, 1986). In contrast to Haines *et al.* (1987), we found that addition of 10 μM AA was sufficient to induce a significant increase in the production of LTB_4 and its metabolites by Paf and FMLP stimulated human PMN. One major variation in the experimental design may account for this difference. We stimulated the cells with a ten fold higher FMLP concentration (10 μM) so addition of a minor amount of arachidonic acid, 10 μM compared to 50 μM used by Haines *et al.* (1987), was sufficient to induce LTB_4 release. It has previously been suggested (Clancy *et al.*, 1983) that membrane phospholipases fail to liberate AA from phospholipids

Table 2 Inhibition of leukotriene B_4 (LTB_4) release from formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 10 μM; arachidonic acid (AA) 10 μM) stimulated human polymorphonuclear leukocytes (PMN) by prostaglandins

		Product (ng per 5×10^7 cells)		
Drugs	n	*LTB_4*	*ω-oxidized LTB_4*	*LTB_4 + ω-oxidized LTB_4*
None	12	72 ± 19	96 ± 22	170 ± 36
PGE_1 (10 μM)	12	31 ± 10*	36 ± 9*	65 ± 17*
Iloprost (10 μM)	11	74 ± 20	106 ± 23	182 ± 39

* Statistically different ($P < 0.05$) from control (no inhibitor).
PMN were preincubated with AA (10 μM) and prostaglandins or vehicle for 10 min at 37°C and FMLP (10 μM) was added. Incubation was continued for another 10 min at 37°C and the reaction was stopped by centrifugation. (see Methods for details). Results are expressed as ng per 5×10^7 cells (mean $\pm$ s.e.mean of *n* observations).

Table 3 Action of prostaglandins (10 μM) on calimycin (10 μM)-induced release of leukotriene B_4 (LTB_4) and its ω-oxidized metabolites from human polymorphonuclear leukocytes (PMN)

		Product (ng per 5×10^7 cells)	
Drug	n	*Without drug* (control)	*With drug*
PGE_2	3	1222 ± 67	1247 ± 99
PGE_1	5	1447 ± 180	1489 ± 159
Iloprost	4	1471 ± 231	1358 ± 332

PMN were preincubated with prostaglandins or vehicle for 10 min at 37°C before stimulation with calimycin for another 10 min at 37°C. After centrifugation, LTB_4 and its metabolites were quantified by h.p.l.c. (see Methods for details). Results are expressed as ng per 5×10^7 cells (mean $\pm$ s.e.mean of n observations).

under the conditions mentioned above. However, Chilton *et al.* (1982) using Paf as an agonist, demonstrated that this compound had a small but reproducible effect on the release of incorporated radiolabelled AA from rabbit neutrophils. This is in accordance with our results (Table 1). On the other hand, AA alone induced LTB_4 release to the same small extent as Paf. Thus, besides the necessity of phospholipase activation, there seems to be a separate requirement for activation of 5-lipoxygenase. As demonstrated here for the first time, only the combination of the two substances, Paf and AA, leads to a marked increase in LTB_4 release by human PMN. The data extend the 'TB_4 paradox' (Haines *et al.*, 1987) to another receptor-mediated PMN stimulus, Paf. However, our data cannot rule out the possibility that Paf, FMLP and AA induce an increase in intracellular LTB_4 levels as has been reported for unopsonized zymosan (Williams *et al.*, 1985). Since only the fraction of LTB_4 released from stimulated PMN is considered to be relevant for activation of further cells in the surroundings, we quantified the amounts of LTB_4 in the supernatants.

LTB_4 is known to be one of the most potent chemotactic agents for PMN (Smith *et al.*, 1980) and may amplify their tissue destructive potential by attracting further PMN to the inflamed area. Therefore, an important question is, how this activation is controlled in order to limit the action of PMN-derived noxious compounds to the area of generation.

A major new finding in this study is the potency of both, PGE_1 and PGE_2 for inhibition of Paf-induced LTB_4 release by human PMN while the synthetic prostacyclin analogue iloprost fails to do so. This inhibitory action of the PGEs is not due to a more rapid metabolism of LTB_4 via the 20-hydroxylase pathway since the occurrence of the ω-oxidized metabolites is depressed to a comparable extent. The results described here confirm earlier observations on Paf-induced superoxide and β-glucuronidase release by human PMN (Schrör & Hecker, 1986) and suggest an overall suppression of Paf-stimulated increase in PMN activity by PGE.

It is proposed that Paf and FMLP act via specific receptors at the PMN plasma membrane (Williams *et al.*, 1977; Valone & Goetzl, 1983). Thus, the data presented here demonstrate the potent inhibition of receptor-mediated LTB_4 release by E-type prostaglandins from human PMN. Furthermore, they confirm earlier observations on the inhibition of receptor-mediated LTB_4 release, superoxide anion formation, and degranulation from human PMN (Ham *et al.*, 1983; Schrör & Hecker, 1986; Gryglewski *et al.*, 1987).

PGEs but not PGI_2 or iloprost have been shown to increase the cyclic AMP level in human PMN stimulated by Paf or FMLP about three to four fold at concentrations which inhibit superoxide anion generation and lysosomal enzyme release (Schrör & Hecker, 1986; 1987). Thus, PGEs seem to interact with the signal transduction through the plasma membrane to intracellular target sites. However, they obviously do not inhibit transformation of liberated AA into LTB_4 if this receptor step is bypassed for example by direct elevation of cellular free calcium levels with the ionophore calimycin. Ham *et al.* (1983) suggested that the inhibitory potency of a variety of prostaglandins on PMN parallels their affinity for PGE-receptors in rat kidney. Our data are consistent with this hypothesis and extend it to the human PMN suggesting that the inhibitory prostaglandin-receptor on human PMN also belongs to the E-type. It is important to note that the inhibitory action of PGEs on Paf-induced LTB_4 release is also found in the presence of exogenous AA. According to a recent study (Tou, 1987), exogenous AA is rapidly taken up by PMN and reacylated into their phospholipids. Thus, inhibition of reacylation, inhibition of receptor-mediated AA release, or inhibition of activation of 5-lipoxygenase may occur with PGEs.

Protein kinase C (PKC) may serve as another intracellular target of PGE action. This enzyme is

mobilized by Paf, although the effects were only moderate and were observed only in the presence of cytochalasin B (O'Flaherty & Nishihira, 1987). De Chaffoy de Courcelles *et al.* (1987) reported that direct activation of PKC in human platelets was antagonized by PGE_1. Whether PKC is the primary target of the inhibitory PGE action in human PMN needs to be confirmed by further experiments. In any case, PGEs could be important factors for the local control of LTB_4 formation if exogenous AA is available from non-leukocyte sources, such as injured tissues in the course of an inflammatory process.

Paf is a pathophysiologically relevant mediator of inflammation and activates leukocytes *in vivo* (see Braquet *et al.*, 1987). It has been shown that PGE_1 and PGE_2 inhibit the late phase of carrageenin-induced inflammatory reactions, i.e. processes which involve the active contribution of leukocytes (Bonta & Parnham, 1979; Parnham *et al.*, 1979). In their experiments, PGI_2 failed to show any depressing activity (Parnham *et al.*, 1979). These findings together with our data would imply that the anti-inflammatory action of PGE-type compounds may result in part from inhibition of Paf-induced LTB_4 release as well as depression of superoxide anion generation and liberation of tissue destructive enzymes. Since PGE_2 is synthesized by many cell types involved in the inflammatory reaction, it may serve as an important control mechanism to limit excessive PMN activation.

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Muscarinic agonists and potassium currents in guinea-pig myenteric neurones

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1 Intracellular electrophysiological recordings were obtained from single neurones of the guinea-pig myenteric plexus *in vitro*. Using single electrode voltage clamp techniques, four distinct potassium currents were described and the effects of muscarinic agonists on these currents were studied.

2 A calcium-dependent potassium current (gK_{Ca}) was present in AH neurones at rest, and was much increased following a brief depolarization (50 ms, to 0 mV). Muscarinic agonists reduced both the resting current and the current evoked by depolarization. Pirenzepine competitively antagonized the suppression by muscarine of the calcium-dependent potassium current (or after-hyperpolarization) following an action potential. The dissociation equilibrium constant for pirenzepine was about 10 nM.

3 The conductance of AH neurones increased two to three fold when they were hyperpolarized negative to −90 mV. This inward rectification was blocked by extracellular caesium (2 mM) or rubidium (2 mM), but not by tetraethylammonium (TEA, 40 mM), 4-aminopyridine (100 μM) or cobalt (2 mM). The inward rectification was unaffected by muscarinic agonists.

4 When AH neurones were depolarized from very negative holding potentials (< −80 mV) a brief outward current was recorded with a duration of about 200 ms. This transient or A current was completely blocked by 4-aminopyridine (100 μM) but was not affected by tetrodotoxin (300 nM), TEA (40 mM) or cobalt (2 mM). Muscarinic agonists did not affect the A current.

5 In S neurones, and in AH neurones in calcium-free solutions, the potassium conductance (in TEA and caesium) behaved according to constant field assumptions. This background conductance was suppressed by muscarinic agonists.

6 It is concluded that the depolarization by muscarinic agonists of myenteric AH neurones is due to a suppression of both a calcium-dependent potassium conductance and a background potassium conductance. Muscarinic depolarization of S neurones results only from suppression of the background potassium conductance. Effects on both conductances result from M_1-receptor activation. Inward rectifying and transient outward (A) potassium currents are unaffected.

Introduction

Acetylcholine acts on muscarinic receptors as an excitatory synaptic transmitter in a number of different tissues. The first demonstration was made in the superior cervical ganglion (Eccles & Libet, 1961) but the most complete analysis has involved the bullfrog sympathetic ganglia (see Kuba & Koketsu, 1978). In bullfrog sympathetic neurones, muscarinic synaptic potentials were found to be due to a decrease in the potassium conductance of the membrane (Weight & Votava, 1970). Subsequent studies showed that the depolarization evoked by muscarinic agonists was due to both inhibition of a time- and voltage-dependent potassium current called the M current (Brown & Adams, 1980; Adams & Brown, 1982) and inhibition of a calcium-activated potassium current (Tokimasa, 1984; 1985).

There is good evidence that synaptically released acetylcholine acts at muscarinic receptors to excite neurones in the enteric nervous system (North & Tokimasa, 1982), sympathetic ganglia (Libet, 1979), and the hippocampus (Cole & Nicoll, 1984); therefore, a precise description of the potassium conductance or conductances affected by muscarinic stimulation is functionally relevant. It is particularly

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important to know whether muscarine is effective only when the cell is strongly depolarized (e.g. M current inhibition), effective when the cell is firing action potentials (e.g. calcium-dependent potassium currents), or is effective even when the cell is at rest or hyperpolarized (e.g. background potassium conductance). Were more than one type of potassium conductance to be affected, then the possibility might exist that they are affected selectively by agonists at distinct muscarinic receptor subtypes. This could provide the opportunity for selective blockade of some, but not all, of the muscarinic actions of acetylcholine on a given cell.

We therefore considered it useful to examine the action of muscarine on identified potassium conductances in the myenteric neurones, and to determine the receptor subtype involved in any of the effects observed. This approach necessitated first a more complete description than heretofore of the potassium conductances in the myenteric neurones. The present paper describes some properties of four potassium currents observed in myenteric neurones and the effects of acetylcholine (ACh) or other muscarinic agonists upon them. A preliminary account of some of the present study has appeared in abstract form (Tokimasa & North, 1987).

Methods

Adult male guinea-pigs (250–350 g) were heavily stunned and bled from the neck. A segment of ileum obtained between 10–30 cm from the ileocecal junction was quickly removed and placed in Krebs solution of the following composition (mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11. The solution was gassed with 95% O_2/5% CO_2. A small piece (1–2 cm) of intestine was cut open along the mesenteric border and pinned flat on Sylgard in a recording chamber with mucosal surface up. Individual myenteric ganglia were exposed by successively stripping away the mucosa, submucosa and circular muscle layers. A single ganglion was immobilized by pinning through the closely adjacent longitudinal muscle using small stainless steel pins (10 μm diameter). The ganglion was visualized using differential interference contrast optics (200 × magnification). Intracellular recordings were made with glass microelectrodes filled with 2 M KCl (tip resistance 15–80 MΩ) using either current clamp with an active bridge circuit or single electrode voltage clamp with a sample and hold circuit (Axoclamp 2, 3 kHz switching frequency, 50–50 duty cycle). Myenteric plexus preparations were superfused (flow rate 2 ml min^{-1}) with warm Krebs solution (36–37°C at the site of recording). Drugs were applied either by superfusion with Krebs solution containing a known concentration of drug or by local application using either pressure ejection or iontophoresis from a pipette positioned near the impaled neurone (for details see Nishi & North, 1973; North *et al.*, 1985).

Myenteric neurones were classified using the criteria of Nishi & North (1973) and Hirst *et al.* (1974). Neurones responding to focal stimulation of nerve strands entering the ganglion with a fast excitatory postsynaptic potential (e.p.s.p.) were termed S cells. Neurones which produced a long lasting (more than 2 s) after-hyperpolarization following a single action potential, and which lacked fast e.p.s.ps, were classified as AH neurones.

Membrane currents were measured in two ways. The membrane potential was commanded to different levels for 200–500 ms (hyperpolarized or depolarized) and the resulting 'instantaneous' (after voltage control was achieved, about 5 ms) and steady-state currents were measured. The second procedure used slow ramp commands (1 mV s^{-1}) from resting potential to construct a steady-state current-voltage (I/V) curve. This provided the same membrane currents as those at the termination of a 500 ms command pulse. During all voltage clamp studies the headstage amplifier potential was monitored continuously with a separate oscilloscope.

All drugs were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.) except pirenzepine which was a gift of Karl Thomae (Germany). Results are presented as the mean ± s.e.mean.

Results

Calcium-sensitive potassium current

In AH neurones, there is an outward potassium current at rest that is dependent on a persistent inward calcium current: the same potassium current is also responsible for the long lasting after-hyperpolarization that follows a single spike (Hirst *et al.*, 1985; North & Tokimasa, 1987). After-currents were evoked by applying step depolarizing voltage commands (typically from a holding potential of −60 mV to 0 mV, see North & Tokimasa, 1987).

Oxotremorine (300 nM) produced an inward shift in holding current in some cells and had no effect in others (mean inward current was 30 ± 16 pA, $n = 6$). Oxotremorine decreased the amplitude of the after-current (Figure 1a): the rate of decline of the after-current was accelerated by oxotremorine. Higher concentrations of oxotremorine produced larger inward currents, further suppressed the peak amplitude of the after-current and accelerated its rate of

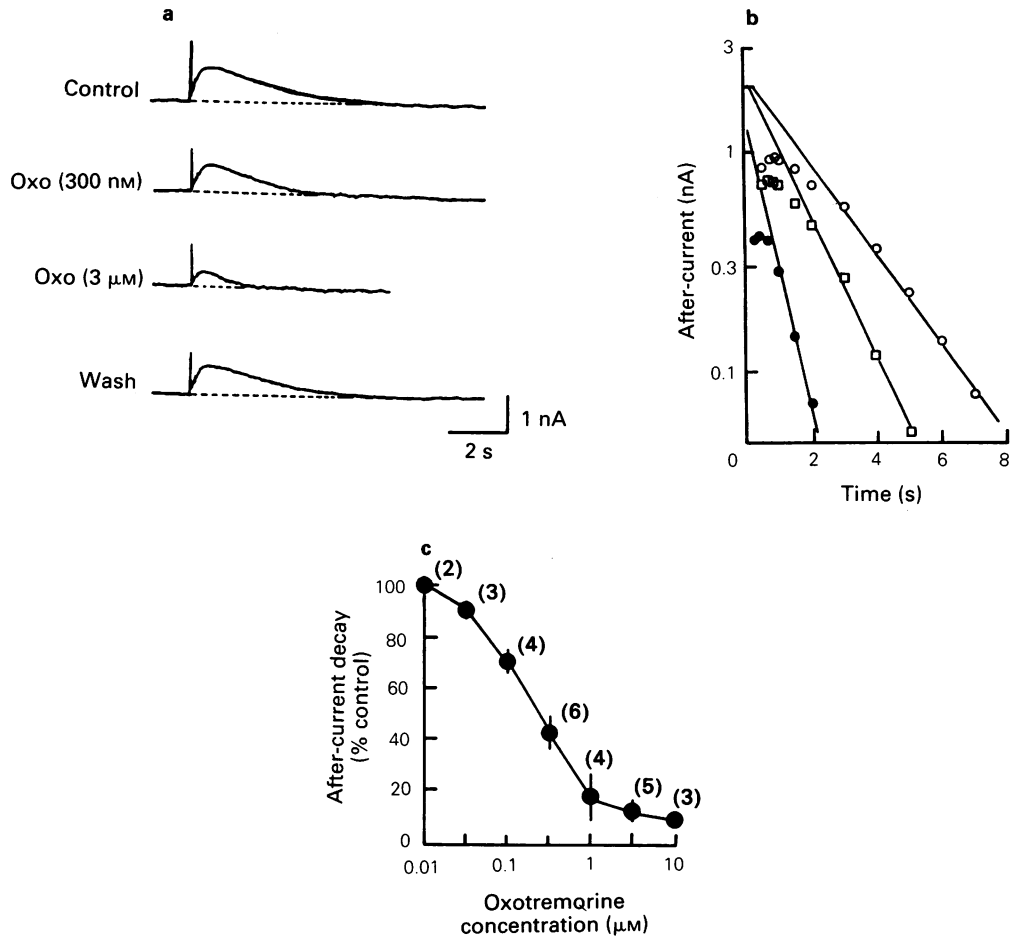


Figure 1 Oxotremorine reduces the calcium-dependent potassium current in AH neurones. (a) Outward currents were evoked by a depolarizing step from −60 to 0 mV of 50 ms duration (time indicated by brief upward deflections on current traces). The outward current peaked at about 1 nA and lasted for 5 s. Oxotremorine (Oxo, 300 nM and 3 μM shown) reduced the amplitude and duration of the outward current. (b) Graphical representation of results from (a). Amplitude of outward current (nA, log scale) is plotted as a function of time after the onset of the brief depolarizing voltage command from −60 to 0 mV. (○) Control; (□) oxotremorine 300 nM; (●) oxotremorine 3 μM. The lines were fitted by eye to the decay phase of the current. (c) The results of similar experiments from other cells (number indicated beside point). The time constant of decay of the calcium-dependent outward current in the presence of various concentrations of oxotremorine is expressed relative to its value before application of oxotremorine in the same cell.

decline (Figure 1b). Figure 1c shows the concentration-response curve for the increase in the rate of after-current decay obtained from all cells tested.

The depression of the after-current by muscarinic agonists could result from a decrease in the amount of calcium entering the cell during depolarization. This was tested in two ways. First, calcium currents were measured directly using microelectrodes containing caesium chloride to reduce potassium currents. Oxotremorine (20 μM) produced less than a 10% reduction in inward calcium current (Figure 2b). The second test was to apply ACh after the termination of the depolarizing command (Figure 2c–g). Small amounts of ACh applied by iontophoresis, which had little or no effect on resting membrane conductance, when applied close to the peak of the after-current (about 500 ms after the depolarizing step used to evoke calcium entry) significantly reduced the duration of the after-current. This effect

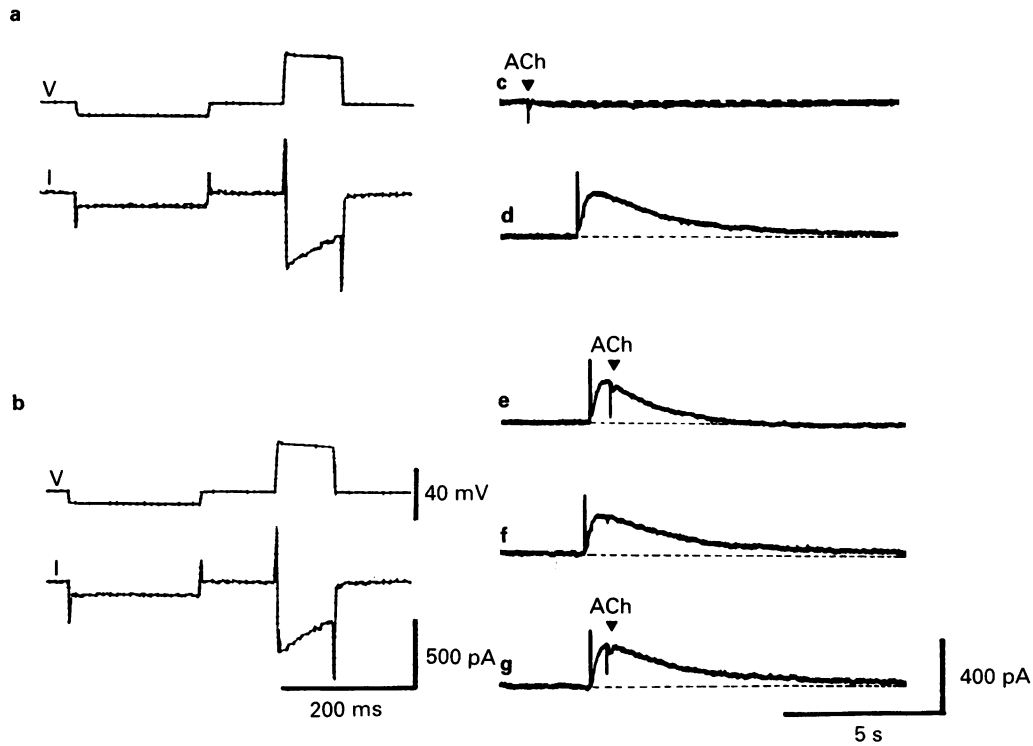


Figure 2 Muscarinic inhibition of calcium-dependent outward current probably does not result from inhibition of calcium entry. (a) and (b) Membrane currents evoked by hyperpolarizing (to −62 mV) and depolarizing (to −7 mV) voltage steps from a holding potential of −50 mV in the same AH neurone. Recording electrode contained caesium chloride. (a) Control. (b) After addition of muscarine (20 μM). There was no change in holding current at −50 mV and no change in the inward current evoked by a step depolarization. (c)–(g) A different AH neurone, potassium chloride in recording electrode. Acetylcholine (ACh) applied after the calcium entry depressed the calcium-dependent potassium current. Holding potential −65 mV; tetrodotoxin (TTX, 100 nM) present throughout. (c) Iontophoretic application of ACh (50 nA for 25 ms, triangle) caused a very small inward current. In (d)–(g) a depolarizing voltage command (to 0 mV for 50 ms) was used to evoke the calcium-sensitive potassium current. (d) Control. (e) ACh applied close to the peak of the calcium-dependent potassium current caused a significant shortening of the after-current. (f) and (g) The same experiment as in (d) and (e) but after adding hyoscine (1 μM) to the solution. ACh was no longer effective.

was not observed in the presence of hyoscine (1 μM).

When muscarine was applied by superfusion, the concentration causing half-maximal suppression of after-hyperpolarization (EC_{50}) in the absence of pirenzepine was approximately 300 nM. This value was progressively increased in the presence of increasing concentrations of pirenzepine. Pirenzepine blocked both the reduction of the peak amplitude of the after-hyperpolarization and also the reduction of its duration (Figure 3a). In the neurone illustrated in Figure 3, Schild analysis indicated that the dissociation equilibrium constant (K_D) of pirenzepine was 10 nM (Figure 3b). In seven other neurones the estimated K_D was always less than 100 nM (range 10–100 nM), although in these cases only one or two pirenzepine concentrations were tested against a full concentration-response curve for muscarine.

Background potassium current

In the voltage range close to rest, the resistance of the membrane to the passage of ionic current was greater when the current was inward than when it was outward. The rectification was observed in control solutions in S neurones and in solutions containing 0 mM calcium/12 mM magnesium/2 mM cobalt/10 mM tetraethylammonium (TEA)/2 mM caesium in AH neurones. The rectification occurred

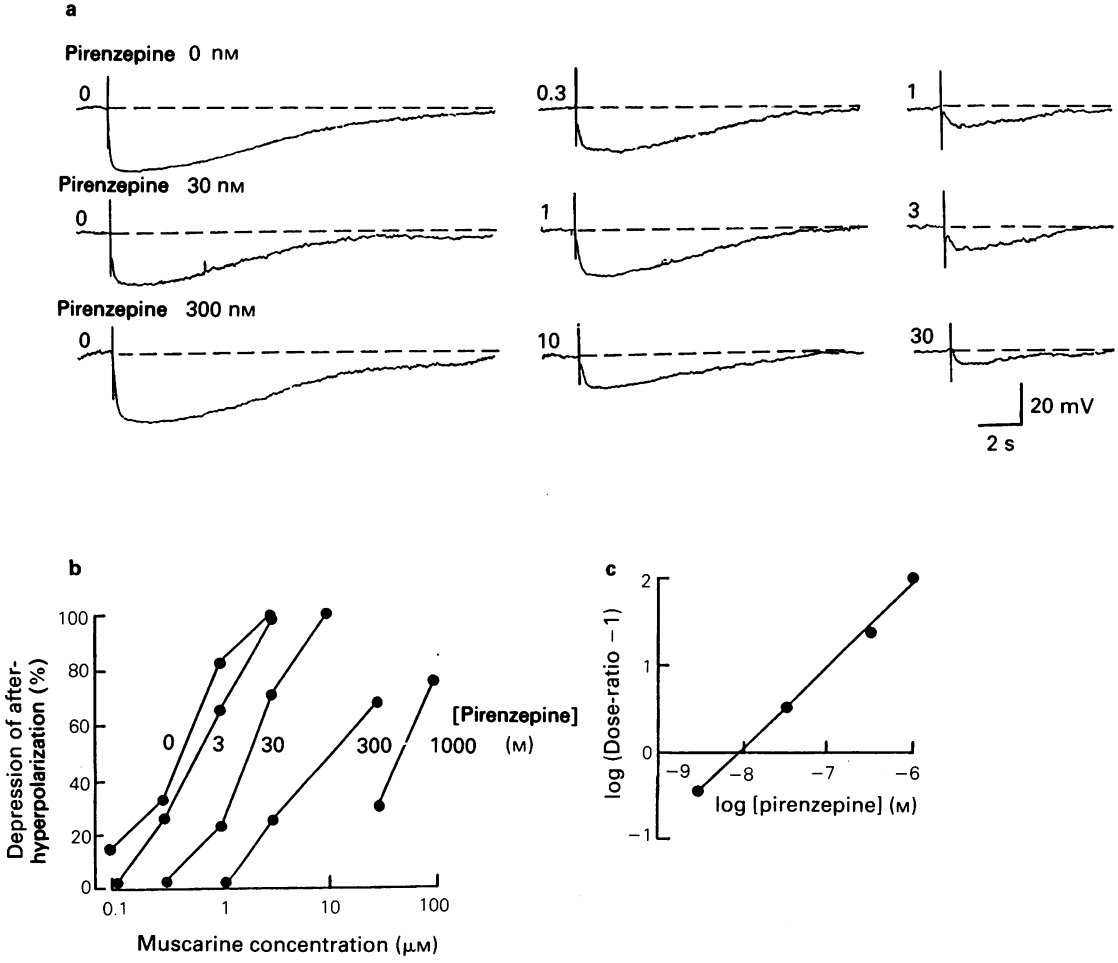


Figure 3 Muscarinic inhibition of the calcium-dependent potassium current involves the M_1-receptor subtype. (a) A single action potential was evoked in an AH nuerone by a depolarizing current pulse. Muscarine and pirenzepine were applied by superfusion. Top three traces show resulting after-hyperpolarization in the absence and in the presence of muscarine (0.3 and 1 μM). Second row of three traces show similar records in the presence of pirenzepine (30 nM), and the lowest row in the presence of pirenzepine (300 nM). In each case, the concentration of muscarine present is indicated beside the trace (μM). (b) Suppression of the peak amplitude of the after-hyperpolarization caused by various concentrations of muscarine. Muscarine was applied on 17 occasions during the course of recording from this neurone, first in the absence and then in the presence of 3, 30, 300 and 1000 nM pirenzepine. (c) Schild plot of data shown in (b); line (fitted by eye) has a slope of 0.97 and gives a pA_2 of 8.0.

within the resolution of the voltage clamp (5 ms) and was thus called instantaneous.

Membrane potassium current can be calculated from the following equation:

$$I_K = \{-P_K E_m F^2/RT\}\{([K_i] - [K_o] \exp(-E_m F/RT))/(\exp(-E_m F/RT) - 1)\}$$

where E_m is the membrane potential, $[K_i]$ and $[K_o]$ represent the potassium ion concentration inside and outside the cell respectively, P_K is membrane permeability to potassium ($cm\,s^{-1}$) and the other symbols have their usual meanings. $[K_i]$ was assumed to be 163 mM which gives a reversal for the after-hyperpolarization following an action potential at the observed value of −94 mV when $[K_o]$ is 4.7 mM. P_K was assumed to be independent of membrane potential and ion concentrations. Membrane current was calculated in arbitrary units of P_K and a value of P_K was chosen to give the best fit for the

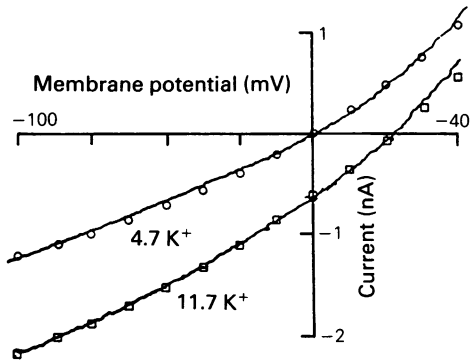


Figure 4 Characterization of background potassium current in an S neurone. Continuous lines are membrane currents observed during slow ramp voltage commands from −100 to −40 mV, first in 4.7 and then in 11.7 mM potassium. (○) and (□) Membrane currents predicted from constant field theory (see text). Superfusing solution contained tetraethylammonium (10 mM), tetrodotoxin (300 nM), cobalt (2 mM) and caesium (2 mM).

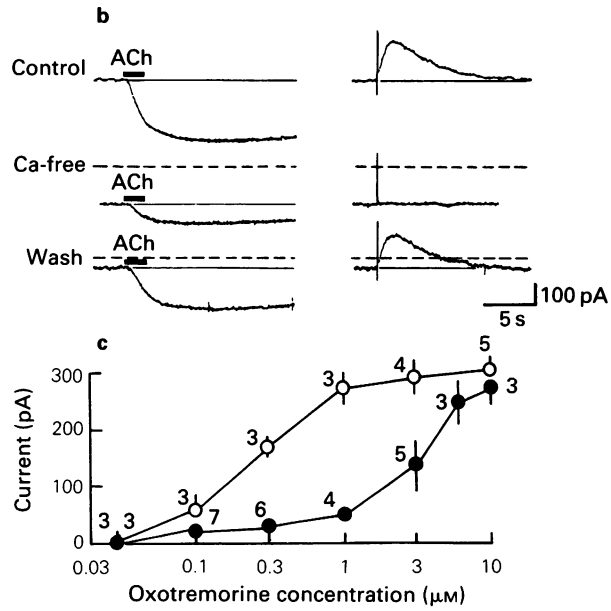
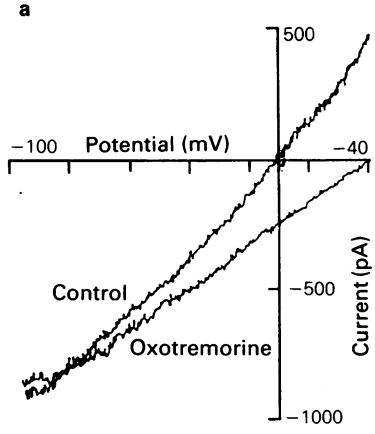


Figure 5 Muscarine reduces background potassium current. (a) Recording from an S neurone. Current-voltage (I/V) relations were obtained by slow ramp depolarizing voltage commands from −100 to −40 mV, first in the absence and then in the presence of oxotremorine (3 μM). Oxotremorine caused an inward current of about 250 pA at the resting potential (−55 mV); this reversed to an outward current at about −90 mV. (b) Recording from an AH neurone. Each pair of traces shows (left side) the effect of applying ACh by iontophoresis and (right side) the calcium-dependent potassium current followed by a brief depolarizing voltage step (see Figure 2 legend). Top traces, control. Middle traces, during superfusion with calcium-free solution. The calcium-free solution caused an inward shift in holding current, and the effect of ACh was reduced proportionately. Right side shows complete abolition of calcium-dependent outward current following depolarizing command pulse. Lowest traces, superfusion again with the normal solution, containing calcium. There was almost full recovery of the holding current, the effect of ACh and of the calcium-dependent potassium current. Hexamethonium (200 μM) was present throughout. Holding potential −60 mV. (c) S neurones were more sensitive to oxotremorine than AH neurones. The peak inward current caused by superfusion with various concentrations of oxotremorine in S (○) and AH (●) cells is shown. Number of neurones tested is indicated beside each point. Vertical bars represent s.e.mean.

chord conductance between −60 and −65, for $[K_o] = 4.7$ mM. The close agreement between the observed and predicted membrane currents (Figure 4) implies that the residual potassium conductance between −40 and −100 mV shows the properties that are based on constant field assumptions.

Application of ACh to S neurones either by iontophoresis or superfusion produced an inward current that was associated with a decrease in background conductance. Hyperpolarization of the membrane resulted in a decrease in amplitude of the ACh current. The ACh current became outward at −90 mV (90.0 ± 3.0, mean ± s.e.mean, $n = 8$). In a solution containing 23.5 mM potassium the reversal potential was −62.8 ± 2.9 mV ($n = 4$). The effect of



oxotremorine on the steady state I/V relation was determined by passing a slow (1.0 mV s^{-1}) ramp voltage command and measuring the resulting membrane current (Figure 5a). Oxotremorine (applied by superfusion) caused an inward current at the resting potential of −55 mV, that became smaller with

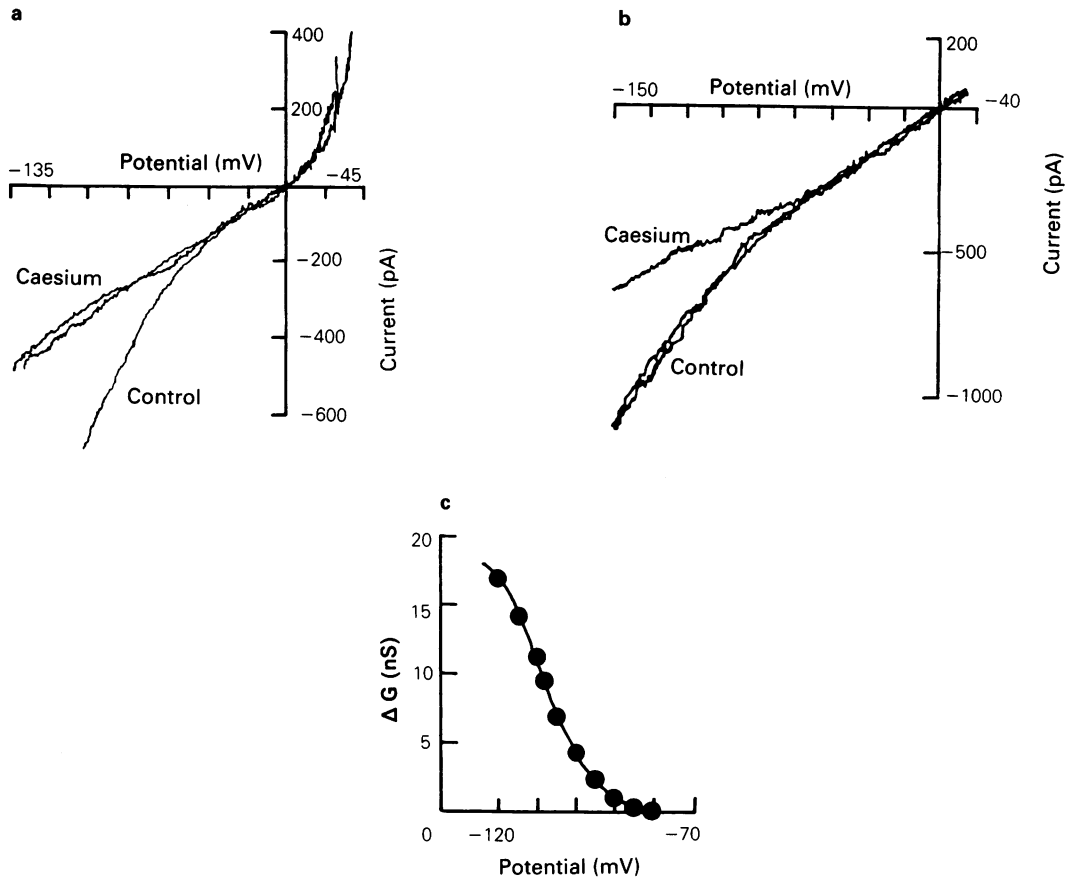


Figure 6 Inward rectifier potassium current. (a) Recording from an AH neurone. Membrane currents were measured during slow ramp voltage command. Figure shows one control recording, and two (almost superimposed) records after adding caesium (2 mM) to the superfusing solution. The effects of caesium were fully reversible in 5 min. (b) A similar experiment in another AH neurone in which the recording electrode contained caesium chloride (2 mM). Two control records are shown, and a single record in the presence of extracellular caesium (2 mM). Control I/V plots were made 30 min after impalement; at this time the outward calcium-dependent potassium current that follows a depolarizing step command (see Figure 2 legend) was completely blocked (not shown). (c) Inward rectifier potassium conductance. Graph shows membrane conductance blocked by extracellular caesium (2 mM) (ΔG) from a neurone such as that shown in (a). Inward rectifier current (●) was measured as the difference between the membrane current before and after adding extracellular caesium. The solid line is $\Delta G = \Delta G_{max}/[1 + \exp(V_m - V)/k]$ where V is the membrane potential. The best fit (by least squares method) values for V_m and k were -108 and 6 mV. ΔG_{max} was 19.1 nS.

membrane hyperpolarization. The inward current produced by oxotremorine, like that produced by ACh, resulted from a reduction in the background conductance. The mean reversal potential for the oxotremorine current calculated from the I/V curves was -89.0 ± 4.0 mV ($n = 4$).

In AH neurones, ACh and other muscarinic agonists produced inward currents which became smaller but did not disappear entirely when the superfusing solution was changed to one that was calcium-free (see above). The reduction in the amplitude of the inward current produced by muscarinic agonists and that caused by changing to a calcium-free solution was equal to the shift in holding current caused by the calcium-free solution. For example, Figure 5b shows an experiment in which the first application of ACh caused an inward current that peaked at 270 pA. The calcium-free solution then caused an inward shift in holding current of about 170 pA due to blockade of the persistent calcium-sensitive pot-

assium current (North & Tokimasa, 1987). The residual response to ACh in calcium-free solution had the same properties as that described in S neurones. The current reversed polarity at -91.5 ± 2.3 mV ($n = 3$), and was associated with a decrease in membrane conductance.

The inward currents in both S and AH neurones produced by superfusion of oxotremorine or muscarine were concentration-dependent, but S cells were approximately 10 times more sensitive to muscarinic agonists than AH neurones (Figure 5c). Concentrations of oxotremorine which produced inward currents in S cells started at 100 nM with peak currents occurring after superfusion with 1–3 μM; in AH neurones concentrations of oxotremorine which produced similar currents ranged between 1–10 μM. The sensitivity of S neurones to the effects of muscarinic agonists when membrane currents were measured was similar to that previously found for membrane depolarization in S neurones of guinea-pig submucous (North *et al.*, 1985) and myenteric plexuses (Morita *et al.*, 1982).

Inward rectifier potassium current

The conductance of the membrane of most (80%) AH neurones increased about three fold when the membrane was hyperpolarized to potentials more negative than −90 mV (see Figure 6c). In about 20% of AH neurones conductance did not change with hyperpolarization up to −120 mV. Slope conductance at −70 mV was 9.2 ± 1.7 nS, and at −120 mV was 26.0 ± 4.0 nS ($n = 9$). Inward rectification in AH cells was not affected by concentrations of tetraethylammonium (TEA) up to 40 mM but was blocked completely by extracellular caesium (2 mM, Figure 6a). Extracellular rubidium (2 mM) also blocked the inward rectification. Inward rectification was not affected by changing the superfusing solution to one which contained no calcium, high magnesium (12 mM) and cobalt (2 mM), nor was it altered by 4-aminopyridine (1 mM); it was observed during recordings with electrodes filled with caesium chloride (Figure 6b).

The inward rectifier current was defined by subtracting the steady-state membrane current from that in the presence of caesium (Figure 6). The inward rectifier current was zero at −85 mV (and at more positive potentials) and then increased with hyperpolarization. The inward current at −115 mV was 347 ± 50 pA ($n = 5$). The potential at which inward rectification began was estimated visually from the divergence of the I/V plots before and after addition of caesium (Figure 6a or b). In control solutions this potential was -90.9 ± 1.5 mV ($n = 7$).

Muscarinic agonists did not affect inward rectification in those cells which showed this current; conversely, addition of caesium (2 mM) to the superfusing solution (which completely blocked inward rectification) did not alter the currents induced by muscarine.

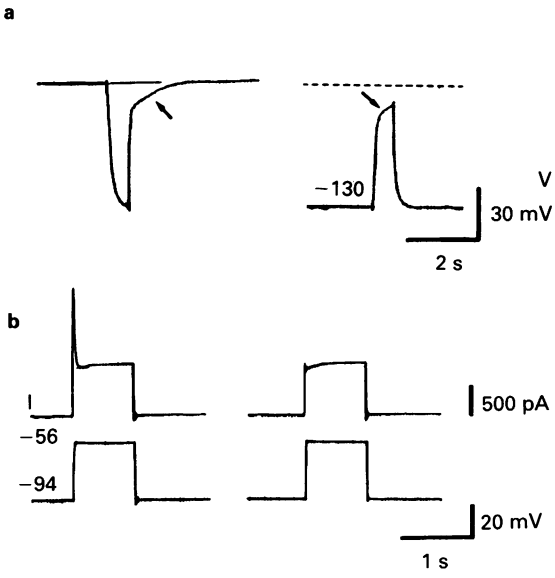


Figure 7 Transient or A potassium current in an AH neurone. (a) Effect of A current on time course of electrotonic potentials. Electrotonic potentials were evoked with very large (900 pA) current pulses. Left side, hyperpolarizing electrotonic potential from resting potential of −60 mV. Right side, depolarizing electrotonic potential from holding potential of −130 mV. Activation of A current when membrane depolarizes results in asymmetry of electrotonic potentials (arrows). (b) Current evoked by depolarizing voltage command from −94 to −56 mV. Left side, control. Right side, 4-aminopyridine (300 μM). The initial transient or A current was completely blocked by 4-aminopyridine. The effect of 4-aminopyridine reversed within 15 min of discontinuing the application. Tetraethylammonium (10 mM) and tetrodotoxin (300 nM) were present throughout.

Transient potassium current

AH cells displayed an outward current that was activated by membrane depolarization from potentials which were hyperpolarized with respect to rest (Figure 7). This current was studied in ten neurones. The amplitude of the transient current increased as the holding potential was made more negative and the threshold for activation was near −70 mV. The inactivation of the current was progressively removed by hyperpolarization according to the length of time at which the membrane was held at more negative potentials before eliciting the transient

outward current. At −140 mV the time constant for removal of inactivation was about 20 ms. The transient current decayed according to a single exponential function. At a holding potential of −60 mV the time constant was 22.5 ± 3.0 ms ($n = 4$). The transient outward current was completely blocked by 4-aminopyridine (300 μM) (see Figure 7b) but was not affected by tetrodotoxin (TTX, 300 nM), TEA (10 mM) or extracellular caesium (2 mM). The transient current was also unaffected by solutions containing no calcium/12 mM magnesium or cobalt (2 mM). The transient current caused an obvious delay in membrane repolarization following the termination of the hyperpolarizing current pulse (Figure 7a).

Oxotremorine (300 nM) had no effect on the transient outward current which flowed following a step depolarization from −100 mV to −60 mV. Conversely, 4-aminopyridine (300 μM), which greatly reduces the A current, did not alter the inward current evoked by iontophoretic application of ACh at −60 mV.

Discussion

Potassium currents in myenteric neurones

The present experiments distinguish four membrane potassium currents in myenteric neurones; one of these, the calcium-dependent potassium current, has already been described in detail (Hirst *et al.*, 1985; North & Tokimasa, 1987). The second current is the inward rectifier. This is similar in all respects to inwardly rectifying potassium channels in other tissues, including its dependence on extracellular potassium and voltage, and blockade by caesium and rubidium and relative insensitivity to TEA (Katz, 1949; Adrian, 1964; Hagiwara & Takahashi, 1974; Hagiwara *et al.*, 1976; 1977; Standen & Stanfield, 1980; Leech & Stanfield, 1981; Constanti & Galvan, 1983; Crepel & Penit-Soria, 1986; Williams *et al.*, 1988). The third current is the transient outward current. In gastropod neurones, a rapidly inactivating potassium current was described which was activated by depolarization from membrane potentials less than −80 mV. This current was called I_A (Connor & Stevens, 1971a) and functions to limit the rate of action potential firing during prolonged depolarization (Connor & Stevens, 1971b). The transient or A current has subsequently been described in many invertebrate and vertebrate neurones. The A current in myenteric neurones is similar to that in other neurones in its activation and inactivation curves (data not shown) and also in sensitivity to blockade by 4-aminopyridine. The fourth conductance is that present in S neurones at rest and in AH neurones in the presence of calcium-free or cobalt containing solutions; this can be described as a 'leak' conductance in the sense that it behaves according to the predictions of the constant field equation.

Muscarinic effects on potassium currents

Neither the inward rectifier nor the A current were affected by muscarine and nor was the depolarization caused by muscarine affected by blockers of these two potassium currents (caesium and 4-aminopyridine). Therefore, the effects of muscarine on potassium currents in myenteric neurones appears to be confined to the background or leak conductance and a calcium-dependent conductance. The reduction of the leak conductance is most clearly seen in S neurones. These cells have little or no tonically active calcium-dependent potassium current, and the after-hyperpolarization that results from calcium entry during the action potential is also much smaller than that seen in AH cells (Nishi & North, 1973; Morita *et al.*, 1982; North & Tokimasa, 1987). The muscarinic receptor involved in this effect has previously been shown to belong to the M_1 subtype (North *et al.*, 1985); acetylcholine released from presynaptic nerves acts as M_1-receptors to reduce this potassium conductance and depolarize myenteric neurones during the slow e.p.s.p. (North & Tokimasa, 1982; North *et al.*, 1985).

Our results show that muscarine has two effects in AH neurones. The first, which is seen in calcium-free solutions, is to reduce the background or leak potassium conductance in a way that is apparently similar to the effect on the S neurones. The second, is to reduce the calcium-dependent potassium conductance. Under resting conditions, this second action also contributes to the membrane depolarization because the AH neurones have a persistent calcium-dependent potassium current at the resting potential (North & Tokimasa, 1987). It has previously been shown that this is the same current as that activated by calcium-entry during the action potential, and which causes the after-hyperpolarization (Hirst *et al.*, 1985; North & Tokimasa, 1987). Therefore, we determined the muscarinic receptor subtype involved in the inhibition by muscarine of the after-hyperpolarization; the dissociation equilibrium constant for pirenzepine (about 10 nM) indicates that this is the M_1 type. It is concluded that muscarine reduces both the background and the calcium-dependent potassium conductance by acting at M_1-receptors.

Experiments of the kind described cannot exclude the possibility that the two potassium conductances separated in these conditions by their dependence on extracellular calcium are in fact the same, with the

background conductance 'maintained' by the free intracellular calcium concentration (normally about 95 nM in S cells and 115 nM in AH cells; see Tatsumi *et al.*, 1988). It is possible that our finding that, on average, the AH neurones are about ten times less sensitive to the depolarizing effect of oxotremorine than are the S cells (Figure 5) is related to this difference in intracellular calcium concentration.

Muscarinic inhibition of the calcium-dependent potassium current has been described in a number of neurones (hippocampus, Madison *et al.*, 1987; olfactory cortex, Constanti & Sim, 1987; sympathetic ganglion, Cassell & McLachlan, 1987). In those cases, as in the myenteric neurones (North & Tokimasa, 1983; present study), the depression of the calcium-dependent current seems not to result from an effect on calcium entry. Other attempts to distinguish muscarinic receptor subtypes have been somewhat less quantitative and have concluded that the M_2-receptor mediates inhibition of the afterhyperpolarization (olfactory cortex, Constanti & Sim, 1987; hippocampus, Muller & Misgeld, 1986).

In summary, the principal action of ACh on S cells is a reduction in leakage or background potassium conductance. On the AH neurones, ACh reduces both the calcium-dependent potassium conductance and the background potassium conductance: M_1 receptors are responsible for both these effects. A pharmacological consequence is that it may not be possible to develop muscarinic agonists or antagonists that would selectively act on rapidly firing neurones, at least on the basis of differential receptor selectivity.

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Modulation by glycyrrhetinic acid derivatives of TPA-induced mouse ear oedema

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1 The anti-inflammatory effects of glycyrrhetinic acid and its derivatives on TPA (12-O-tetradecanoylphorbol-13-acetate)-induced mouse ear oedema were studied. The mechanisms of TPA-induced ear oedema were first investigated with respect to the chemical mediators.

2 The formation of ear oedema reached a maximum 5 h after TPA application (2 μg per ear) and the prostaglandin E_2 (PGE_2) production of mouse ear increased with the oedema formation.

3 TPA-induced ear oedema was prevented by actinomycin D and cycloheximide (0.1 mg per ear, respectively) when applied during 60 min after TPA treatment.

4 Of glycyrrhetinic acid derivatives examined, dihemiphthalate derivatives (IIe, IIe', IIIa, IIIa', IVa, IVa') most strongly inhibited ear oedema on both topical (ID_{50}, 1.6 mg per ear for IIe, 2.0 mg per ear for IIIa and 1.6 mg per ear for IVa) and oral (ID_{50}, 88 mg kg^{-1} for IIe', 130 mg kg^{-1} for IIIa' and 92 mg kg^{-1} for IVa') administration.

5 Glycyrrhetinic acid (Ia) and its derivatives applied 30 min before TPA treatment were much more effective in inhibiting oedema than when applied 30 min after TPA. A dihemiphthalate of triterpenoid compound IVa completely inhibited oedema, even when applied 3 h before TPA treatment.

6 Glycyrrhetinic acid (Ia) and deoxoglycyrrhetol (IIa), the parent compounds, produced little inhibition by oral administration at less than 200 mg kg^{-1}

7 These results suggest that the dihemiphthalate derivatives of triterpenes derived from glycyrrhetinic acid by chemical modification are useful for the treatment of skin inflammation by both topical and oral application.

Introduction

Glycyrrhizin is a major saponin of liquorice root (*Glycyrrhiza* spp.), which has been reported to have anti-allergic (Kuroyanagi & Saito, 1966), ocular anti-inflammatory (Tanaka *et al.*, 1987) and interferon-inducing (Shinada *et al.*, 1986) activities as well as anti-viral effects (Pompei *et al.*, 1979) including those against varicella-herpes zoster virus (VZV) and human T-cell lymphotropic virus (HTLV-III) (AIDS virus) (Ito *et al.*, 1987). Its aglycone, glycyrrhetinic acid (Ia), also has pharmacological activities as an anti-inflammatory (Finney & Somers, 1958; Capasso *et al.*, 1982) and anti-tumour promoter agent (Nishino *et al.*, 1986), as well as inhibiting growth of mouse melanoma (Abe *et al.*, 1987) and inhibiting communication across intercellular gap junctions (Davidson *et al.*, 1986). Some glycyrrhetinic acid derivatives have been prepared in an attempt to enhance the therapeutic activity and suppress the unfavourable side-effect (pseudo-aldosteronism) of the parent compound (Takahashi *et al.*, 1980; Shibata *et al.*, 1987). Clinically, carbenoxolone sodium (glycyrrhetinic acid 3-O-hemisuccinate sodium salt) has been used to treat gastric ulcer (Doll *et al.*, 1962). Of the derivatives of glycyrrhetinic acid, three kinds of dihemiphthalate compounds (disodium salt of 18β-olean-12-ene-3β,30-diol di-O-hemphthalate (IIe), 18β-olean-9(11),12-diene-3β,30-diol di-O-hemiphthalate (IIIa), olean-11,13(18)-diene-3β,30-diol di-O-hemiphthalate (IVa)) have

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been found to inhibit experimental gastric ulcer (Yano & Watanabe, unpublished). Furthermore, we have reported that these dihemiphthalate compounds inhibit lipoxygenase and cyclo-oxygenase activities in a cell-free system from cloned mastocytoma cells (Inoue *et al.*, 1986) and show anti-nociceptive and anti-type IV allergic effects (Inoue *et al.*, 1987). Recently, these compounds have been found to inhibit arachidonic acid-induced mouse ear oedema which is mainly mediated by arachidonic acid metabolites such as leukotriene C_4 (LTC_4) and prostaglandin E_2 (PGE_2) (Inoue *et al.*, 1988).

Mouse ear oedema induced by croton oil and its irritant principle, TPA (12-O-tetradecanoylphorbol-13-acetate), has been used as a test for anti-inflammatory activity (Carlson *et al.*, 1985; Bird *et al.*, 1986; Tubaro *et al.*, 1986), being suppressed by non-steroidal and steroidal anti-inflammatory agents. In addition, lipoxygenase inhibitors and dual inhibitors of lipoxygenase/cyclo-oxygenase also inhibited TPA-induced ear oedema (Young *et al.*, 1983; Carlson *et al.*, 1985). However, the mechanism involved in the induction of this type of oedema has not been completely elucidated even though TPA-induced ear oedema was well-characterized by Young *et al.* (1983). In the present paper we measured the PGE_2 and LTC_4 content of TPA-treated mouse ears and investigated the contribution of new protein synthesis to the oedema response. In addition, we investigated the inhibitory effect of glycyrrhetinic acid derivatives on the oedema response.

Methods

Assay for TPA-induced ear oedema

Six week old male ddY mice (Shizuoka Laboratory Animal Center, Japan) were assigned to eight treatment groups, housed together and acclimatized under standard conditions for one week with free access to food and water. 12-O-Tetradecanoylphorbol-13-acetate (TPA, Sigma Chemical Co., U.S.A.) was dissolved in acetone at a concentration of $100\,\mu g\,ml^{-1}$. Each mouse was then treated with $20\,\mu l$ ($2\,\mu g$ per ear) of TPA on both surfaces of an ear. Test compounds were dissolved in acetone or ethanol for topical application, and were suspended with 1% polyoxyethylene sorbitan monooleate (Tween 80, Tokyo Kasei Chemical Industry, Japan) in saline (0.9% NaCl) for oral administration. Both treatments were given 30 min before and/or after TPA application. Control mice received the vehicle only. Ear oedema was measured 5 h after TPA application with dial calipers (Ozaki Factory, Japan) measuring to an accuracy of 0.01 mm. Statistical significance was assessed by Student's *t* test.

Determination of LTC_4 and PGE_2 in ear oedema

Animals treated with TPA were killed at designated intervals, and each ear was removed immediately and stored on dry-ice. It was homogenized with ice-cold 80% ethanol for LTC_4 and 95% ethanol for PGE_2 by a motor driven homogenizer (5,000 r.p.m. 30 s). The homogenate was centrifuged at $4,500\,g$ for 15 min at 4°C. LTC_4 in 80% ethanol extract or a further purified fraction by Sep-Pac C_{18} cartridge was measured by LTC_4-radio-immunoassay (New England Nuclear, Boston, MS, U.S.A.). PGE_2 in ethanol was absorbed by octadecylsilyl silica (ODS) and then washed with ethanol/0.05 N HCl and petroleum ether to remove protein and liquid. Finally, PGE_2 was eluted with ethyl acetate. The eluate was further purified by a silica minicolumn (BOND ELUT Si) with the following eluate solvent: acetonitrile-chloroform-acetic acid (20:80:0.5). PGE_2 was measured by radio-immunoassay (New England Nuclear, U.S.A.). The recovery of LTC_4 from the 80% ethanol-homogenate and that of PGE_2 from 95% ethanol were 79% and 97.5%, respectively.

Materials

Glycyrrhetinic acid and its derivatives were prepared according to Shibata *et al.* (1987). Other compounds were purchased as follows: aspirin from Nakarai Chemical Co., Japan; nordihydroguaiaretic acid (NDGA), indomethacin, cyproheptadine and dexamethasone from Sigma Chemical Co., U.S.A.; actinomycin D and cycloheximide from Sigma Chemical Co., U.S.A. AA 861 (2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone) was a generous gift from Takeda Chemical Industry Co., Japan.

Results

The ear oedema induced by a single topical application of $2\,\mu g$ TPA was examined at various intervals by measuring ear thickness. As found previously (Young *et al.*, 1983), the ear thickness had increased 3 h after TPA application and reached a maximum at 5 h (Figure 1). Oedema then decreased gradually but swelling still remained 24 h after TPA application and resulted in eventual necrosis. The level of endogenous PGE_2 began to increase 1 h after TPA

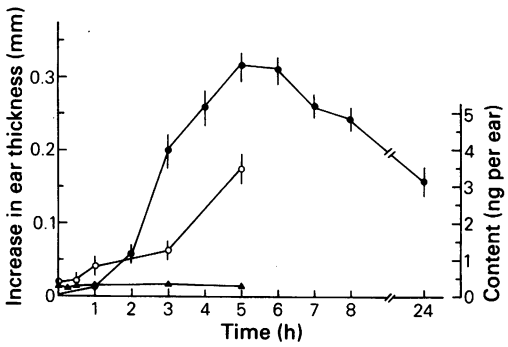


Figure 1 The time course of ear oedema formation and chemical mediator production induced by TPA. Mouse ears were treated by topical application of TPA (2 μg). The measurement of prostaglandin E_2 (PGE_2) and leukotriene C_4 (LTC_4) was as described in Methods. Each point represents the mean value from 8 animals; vertical lines show s.e.mean. Ear oedema is the mean ear thickness of test animals minus that of control animals. (●) Ear thickness; (○) PGE_2; (▲) LTC_4.

application and continued for a further 4 h in accordance with the oedema formation, whereas LTC_4 production did not increase 5 h after application (Figure 1), and LTB_4 in the ear treated with TPA was not detected by radio-immunoassay. Since TPA is known to induce DNA synthesis (Hennings & Boutwell, 1970) and activate ornithine decarboxylase (ODC) (O'Brien *et al.*, 1975), the role of RNA and newly synthesized protein in development of

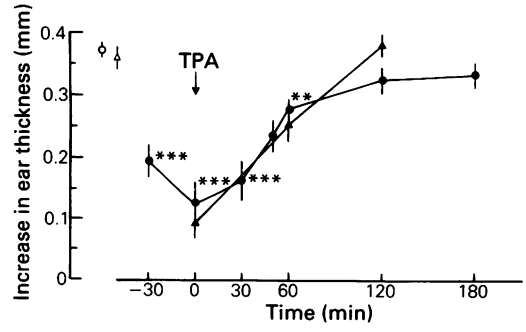


Figure 2 Inhibitory effects of cycloheximide (●) and actinomycin D (▲) on TPA-induced ear oedema. These compounds (0.1 mg per ear) were applied topically at various times before or after TPA treatment. Ear thickness was measured 5 h after TPA application. Each point represents the mean from 10 animals; vertical lines show s.e.mean. $^{**}P < 0.01$, $^{***}P < 0.001$ compared by paired Student's *t* test with the appropriate TPA control groups (○ and △).

oedema was investigated by use of inhibitors applied topically. Figure 2 shows that TPA-induced ear oedema was significantly inhibited by either actinomycin D or cycloheximide (each at a dose of 0.1 mg per ear) when applied during the first 60 min after TPA application. These compounds were particularly effective when applied immediately after TPA (64% inhibition, $P < 0.001$), suggesting that the new protein synthesis was induced immediately after TPA application.

The anti-inflammatory effects of glycyrrhetinic acid (Ia) and its derivatives (for structures see Figure 3) were examined by topical and oral application in the ear oedema model and the results are summarized in Table 1. The compounds were tested 30 min before or after TPA treatment at a dose of 1 mg per ear. Dexamethasone (0.1 mg per ear) was included as the positive control. With the exception of compounds IIb and IId, glycyrrhetinic acid (Ia) and its analogues were very effective, inhibiting oedema by 80% ($P < 0.001$) when applied 30 min before TPA treatment. Of these active compounds the three dihemiphthalate compounds, IIe, IIIa and IVa, inhibited oedema most effectively when applied to the ear before TPA: their effects decreased to 40% ($P < 0.001$) when applied after TPA. Similarly, NDGA was marginally more effective when applied before, rather than following, application of TPA. AA 861, aspirin and cyproheptadine were modest inhibitors, whereas dexamethasone was very effective when applied after TPA.

The dose-dependence of the antioedema effect was studied and IIe, IIIa and IVa, were found to have ED_{50} values of 1.6 mg per ear, 2.0 mg per ear, and 1.6 mg per ear, respectively (Figure 4). In contrast, NDGA, aspirin and cyproheptadine did not inhibit oedema in a dose-dependent fashion (data not shown). Compound IVa, a strong inhibitor of oedema, was then applied topically (1 mg per ear) to the ear at various times either before or after TPA application. Figure 5 shows that compound IVa inhibited ear oedema when applied before TPA treatment, but its activity decreased markedly when it was administered following TPA application.

The water soluble disodium salts of the hemiphthalates (Ic', IId', IIe', IIIa' and IVa') were given orally 30 min after TPA treatment. Compounds IIe', IIIa' and IVa' inhibited ear oedema dose-dependently with ED_{50} values of 88 mg kg^{-1}, 130 mg kg^{-1} and 92 mg kg^{-1}, respectively. Glycyrrhetinic acid 3-O-hemiphthalate (Ic') also showed a slight inhibition ($P < 0.05$) at a dose of 200 mg kg^{-1}, but had little effect at less than 100 mg kg^{-1}. In contrast, glycyrrhetinic acid (Ia) and deoxoglycyrrhetol (IIa) were inactive by the oral route even when applied 30 min before TPA treatment (data not shown).

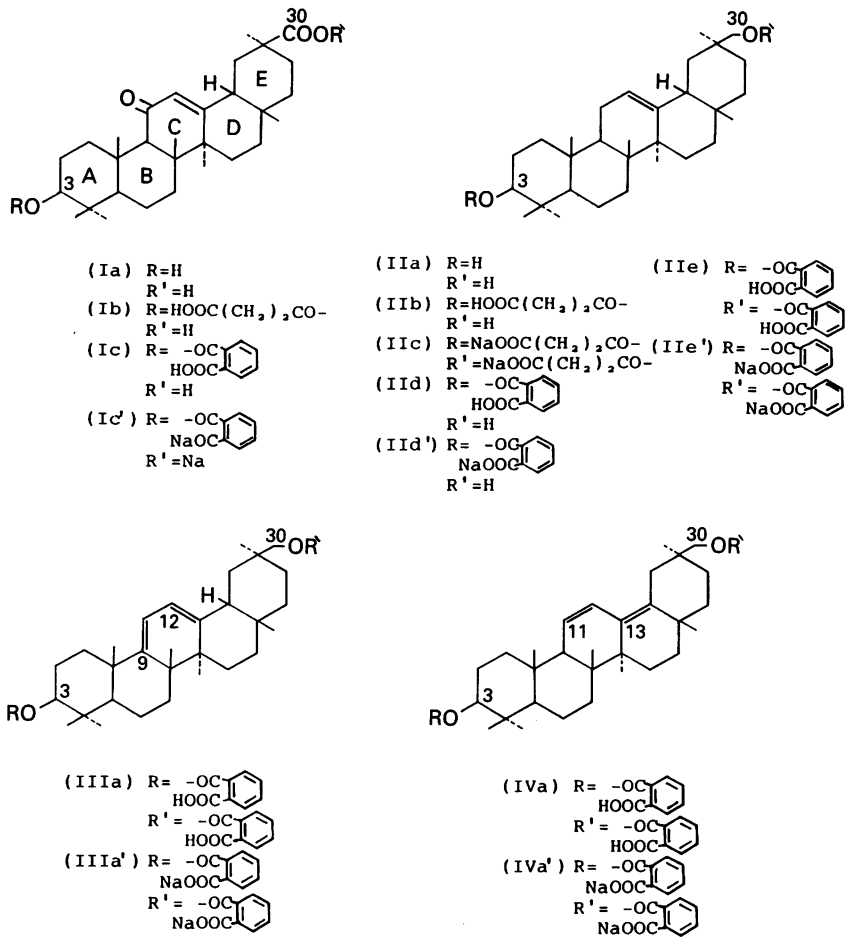


Figure 3 The structures of glycyrrhetinic acid derivatives.

Discussion

The application of TPA to mouse skin leads to oedema, cellular infiltration and skin tumour promotion (Diamond *et al.*, 1980; Blumberg *et al.*, 1981). Young *et al.* (1983) suggested the participation of arachidonate metabolites in the oedema formation caused by TPA, and TPA has been shown to induce PGE_2 production from epidermal cells (Bresnick *et al.*, 1979). We have now demonstrated that TPA induces PGE_2 production by the cells of the ear, and PGE_2 content increased with the same time course as oedema formation (Figure 1). In contrast, LTC_4 did not increase and LTB_4 was not detected. Since cyclo-oxygenase inhibitors such as indomethacin, aspirin and naproxen have been shown to suppress TPA-induced ear oedema (Young *et al.*, 1983; Carlson *et al.*, 1985; present results), PGE_2 is presumably a mediator of TPA-induced oedema. It is also probable that histamine and 5-hydroxytryptamine contribute to oedema since cyproheptadine was inhibitory. Since TPA did not stimulate increased levels of lipoxygenase products LTB_4 and LTC_4, the lipoxygenase inhibitors AA 861 and NDGA may have inhibited oedema formation by virtue of their weak cyclo-oxygenase-inhibitory activity or some other, unknown, property.

It is known that application of TPA can induce changes in the epidermal protein pool which are prevented by anti-inflammatory steroids and cycloheximide (Slaga & Nelson, 1984). We have now shown that the oedema induced by TPA is strongly

Table 1 Inhibition (%) of TPA-induced mouse ear oedema by glycyrrhetinic acid derivatives administered either orally or topically

	Topical inhibition (%)			*Oral inhibition* (%)	
Compound	*Dose* (mg per ear)	*Pre-treat*	*Post-treat*	*Dose* (mg per kg)	*Post-treat*
Ia	1	81***	24**	200	8
Ib	1		21*	200	5
Ic (c')	1	88***	23***	100	9
				200	40*
IIa				200	0
IIb	1	48**	25***	200	2
IIc	1			200	10
IId (d')	1	49***	20***	200	15
IIe (e')	1	93***	40***	25	10
				50	28*
				100	54**
				150	96***
IIIa (a')	1	91***	41***	25	1
				50	28**
				100	43**
				150	78***
IVa (a')	1	96***	48***	25	18
				50	29*
				100	71***
				150	93***
NDGA	1	38***	24**		
AA 861	1	19**	30**		
Aspirin	1	8	16**		
Indomethacin	1	32**	31**		
Cyproheptadine	1	0	35**		
Dexamethasone	0.1		83**		

Compounds were applied 30 min before and after TPA treatment.
Oral application was performed 30 min after TPA treatment.
Statistical significance from the control at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ ($n = 8$). For further details, see text.

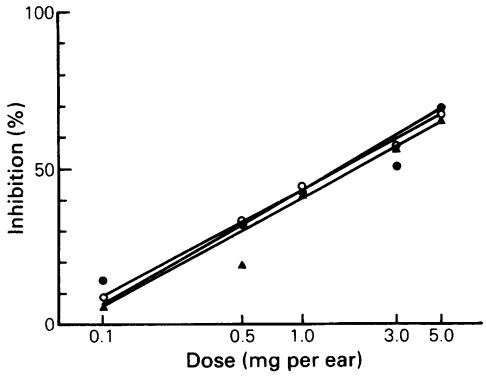


Figure 4 The dose-dependent inhibition of dihemiphthalate compounds on TPA-induced ear oedema. Various doses of the compounds were applied topically to the ear 30 min after TPA treatment. Each point represents mean percentage inhibition compared with control for 8 animals. (●) Compound IIe; (▲) compound IIIa; (○) compound IVa.

inhibited by dexamethasone, actinomycin D and cycloheximide. Therefore, it is likely that new protein synthesis induced by TPA contributes to the oedema response.

Among the glycyrrhetinic acid derivatives examined on TPA-induced ear oedema, compounds (IIe, IIe', IIIa, IIIa', IVa and IVa') with hemiphthalate groups at the 3- and 30-positions of ring A and E in the oleanane skeleton were found to inhibit ear oedema most strongly whether administered topically or orally. Glycyrrhetinic acid (Ia) was weakly effective when given topically but orally this compound and deoxoglycyrrhetol (IIa), the parent compounds of the dihemiphthalate derivatives, had little effect. Glycyrrhetinic acid hemiphthalate (Ic') and deoxoglycyrrhetol 3-O-monohemiphthalate (IId') also had little effect. These results suggest that the dihemiphthalate substitution on the oleanane skeleton of these compounds is essential for the potent inhibitory effect on TPA-induced ear oedema. This coincides with the results obtained from biochemical

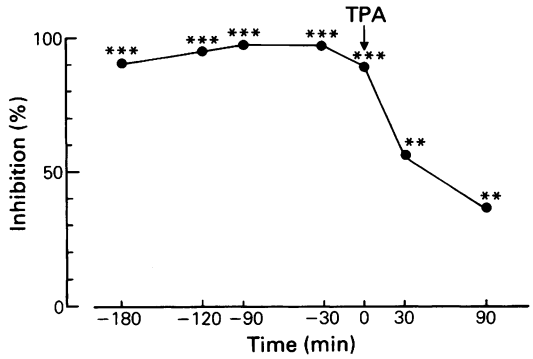


Figure 5 The time effect for topical application of compound IVa on TPA-induced ear oedema. The compound was applied at various times before and after TPA treatment. Ear thickness was determined 5 h after TPA treatment. Each point represents mean percentage inhibition compared with control for 8 animals. The statistically significant difference from control indicated by ** $P < 0.01$ and *** $P < 0.001$.

and pharmacological studies on the inhibitory effect of these compounds on lipoxygenase and cyclo-oxygenase activities (Inoue *et al.*, 1986), on the analgesic and the anti-type IV allergic effect (Inoue *et al.*, 1987) and on the inhibition of arachidonic acid-induced mouse ear oedema (Inoue *et al.*, 1988).

It is noticeable that the topical inhibitory effect of glycyrrhetinic acid and its derivatives was more potent when they were applied 30 min before TPA treatment than when applied 30 min after TPA. Particularly, compound IVa showed more than 90% inhibition when applied up to 3 h before TPA treatment. This characteristic suggests that modulation of oedema by the dihemiphthalate compounds could be due to interaction of the compounds with the intracellular receptor for TPA, protein kinase-C. These compounds were more effective in inhibiting 5-lipoxygenase than cyclo-oxygenase which was suppressed about 20% in cloned mastocytoma cells at 10^{-5} M (Inoue *et al.*, 1986). In addition, we have demonstrated that these dihemiphthalate compounds inhibit arachidonic acid-induced mouse ear oedema by suppressing the production of LTC_4 and PGE_2 (Inoue *et al.*, 1988). However, glycyrrhetinic acid analogues could not inhibit PGE_2 production in TPA-induced mouse ear oedema when given topically (2 mg per ear) or orally (100 mg per ear) 30 min after TPA treatment, whereas indomethacin (1 mg per ear) completely suppressed PGE_2 formation (data not shown). Thus the dihemiphthalate derivatives of glycyrrhetinic acid analogues appear to have anti-inflammatory effects by a mechanism(s) in addition to inhibition of arachidonate metabolism. It has been shown previously that the dihemiphthalate compounds inhibit vascular permeability and type IV allergy (Inoue *et al.*, 1987) and protect the gastric mucosa in an experimental model of gastric ulceration (Yano & Watanabe, unpublished), the mechanism of which is being studied. Thus, the dihemiphthalate compounds described have an interesting profile of action and may be of value for the treatment of inflammatory skin diseases.

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1,3,8- and 1,3,7-substituted xanthines: relative potency as adenosine receptor antagonists at the frog neuromuscular junction

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1 The ability of 1,3,8-substituted xanthines (1,3-dipropyl-8-(4-(2-aminoethyl)amino) carbonylmethyloxyphenyl)xanthine (XAC), 1,3-dipropyl-8-(4-carboxymethyloxyphenyl)xanthine (XCC), 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), 1,3-diethyl-8-phenylxanthine (DPX) and 8-phenyltheophylline (8-PT)), of 1,3,7-substituted xanthines (1-propargyl-3,7-dimethylxanthine (PGDMX) and caffeine), and of a 3-substituted xanthine (enprofylline) to antagonize the inhibitory effect of 2-chloroadenosine (CADO) on the amplitude of nerve-evoked twitches was investigated in innervated sartorius muscles of the frog.

2 All the 1,3,8-substituted xanthines, in concentrations virtually devoid of effect on neuromuscular transmission, shifted to the right, in a near parallel manner the log concentration-response curve for CADO. Linear Schild plots with slopes near to unity at concentration-ratios less than 14 were obtained for XAC, XCC, DPCPX, DPX and 8-PT.

3 The order of potency of the 1,3,8-substituted xanthines as antagonists of the effect of CADO was XAC (K_i = 23 nM) ≥ DPCPX (35 nM) > 8-PT (200 nM) ≥ DPX (295 nM) > XCC (1905 nM) ≥ PACPX (2291 nM). No correlation was found between the potency of these xanthines as antagonists of the adenosine receptor at the frog neuromuscular junction and their reported potency as antagonists of the A_1- or A_2-adenosine receptors.

4 The 1,3,7-substituted xanthines, PGDMX and caffeine, in concentrations virtually devoid of effect on neuromuscular transmission, also caused parallel shifts to the right of the log concentration-response curves for CADO, but were less potent than the 1,3,8-substituted xanthines. PGDMX was more than 20 times more potent than caffeine.

5 Enprofylline in concentrations up to 100 μM did not antagonize the inhibitory effect of CADO on neuromuscular transmission.

6 It is concluded that the antagonist profile of the adenosine receptor mediating inhibition of transmission at the frog neuromuscular junction is different from the antagonist profile of the A_1- and A_2-adenosine receptors.

Introduction

Two subtypes of xanthine-sensitive adenosine receptors have been postulated on biochemical grounds: A_1 (R_i) operating inhibition and A_2 (R_a) operating stimulation of adenylate cyclase (Van Calker *et al.*, 1979; Londos *et al.*, 1980). These two subtypes of adenosine receptor have different pharmacological profiles for agonists. At A_1-adenosine receptors the order of agonist potency is L-N^6-phenylisopropyladenosine (L-PIA), N^6-cyclohexyladenosine (CHA) > 2-chloroadenosine (CADO) > 5'-N-ethylcarboxamide adenosine (NECA). At A_2-adenosine receptors the agonist profile is reversed, i.e. NECA > CADO > L-PIA, CHA (e.g. Daly *et al.*, 1981).

The adenosine receptor mediating the adenosine-induced presynaptic inhibition of neuromuscular transmission in the innervated frog sartorius muscle has an agonist profile with L-PIA, CHA and NECA being of similar potency and more potent than CADO (Ribeiro & Sebastião, 1985). The observation that this agonist profile is similar to that described for the adenosine receptors mediating a decrease in

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neurotransmitter release in most of the peripheral and central synapses, but is different from the agonist profile of both A_1- and A_2-adenosine receptors, prompted the hypothesis that the adenosine receptor-mediating presynaptic inhibition of synaptic transmission by adenosine belongs to a third (A_3) subtype of xanthine-sensitive adenosine receptors (Ribeiro & Sebastião, 1986). Agonist potency can, however, be influenced by factors unrelated to receptor type (see e.g. Kenakin, 1987), which complicates receptor classification on the basis of different agonist profiles.

Xanthine derivatives with some antagonist selectivity for A_1- and A_2-adenosine receptors have recently been developed (e.g. Daly *et al.*, 1987). In the present work we have studied the relative potency of some of the substituted xanthines as antagonists of the adenosine receptor that mediates the presynaptic inhibition of neuromuscular transmission in the innervated sartorius muscle preparation of the frog. Several 1,3,8- and 1,3,7-substituted xanthines were used. CADO was used as the agonist because this adenosine analogue is a full agonist of the adenosine receptor in the frog innervated sartorius preparation and is easily washed out (Ribeiro & Sebastião, 1985).

A brief account of some of the results has already been published (Sebastião & Ribeiro, 1988a).

Methods

The experiments were carried out at room temperature (22–25°C) on innervated sartorius muscles of the frog (*Rana ridibunda*) (see e.g. Kharkevich, 1986). The preparations were set up in a 25 ml organ bath 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 25 ml min^{-1} during the first 2 min after changing the solutions and 5 ml min^{-1} until the next changeover of solutions. Rectangular pulses of 0.1 ms duration and supramaximal voltage were applied to the nerve 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 7.0) contained (mM): NaCl 117, KCl 2.5, $CaCl_2$ 1.8, $MgCl_2$ 1.2, NaH_2PO_4 1, Na_2HPO_4 1. The average nerve-evoked twitch amplitude in the control bathing solution for the experiments described in this paper was 22 ± 1.3 mN.

Concentration-response curves and Schild regressions

In each experiment the cumulative concentration-response curves to the adenosine receptor agonist, 2-chloroadenosine (CADO), were obtained first in the absence of the antagonist. CADO was then washed out and the preparation equilibrated with the antagonist for at least 40 min. A second cumulative concentration-response curve for CADO, now in the presence of the antagonist, was then performed. After a washing out period of 60–90 min, a third cumulative concentration-response curve for CADO again in the absence of the antagonist was finally obtained. The usual procedure was to average the responses in the same experiment to the same concentration of CADO in the absence of antagonist, to obtain the control responses to CADO. Whenever the control effect of CADO after washing out the antagonist was smaller than the effect of the same concentration of CADO before exposure to the antagonist, we considered that the action of the antagonist was not fully reversible; in these cases the post-control concentration-response curve for CADO was not included in the calculations. This was frequently observed in the experiments using the highest concentration (100 nM) of 1,3-dipropyl-8-(4-(2-aminoethyl)amino) carbonylmethyloxyphenyl)-xanthine (XAC), but not in the experiments using other xanthines.

The concentration of CADO producing 35% decrease in the amplitude of nerve-evoked twitches was calculated in each experiment by regression analysis of the linear part of the log concentration-response curves for CADO. The concentration of CADO causing 35% effect is near its EC_{50} value since the maximal effect of CADO (determined from the double reciprocal plot of the averaged concentration-response curves for CADO, $n = 51$) was 65% decrease in twitch amplitude. The ratios between equi-active concentrations (CR) of CADO (causing 35% effect) in the presence and in the absence of the 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 and K_i the equilibrium dissociation constant of the antagonist for the receptor. 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 (n) approximated to unity, the K_i value was taken as the negative antilog of the pA_2 value.

Drugs

Drugs used were: caffeine, 2-chloroadenosine (CADO) (Sigma); 1,3-diethyl-8-phenylxanthine (DPX), 1,3-dipropyl-8-(2-amino-4-chlorophenyl)-xanthine (PACPX), 1,3-dipropyl-8-cyclopentylxan-

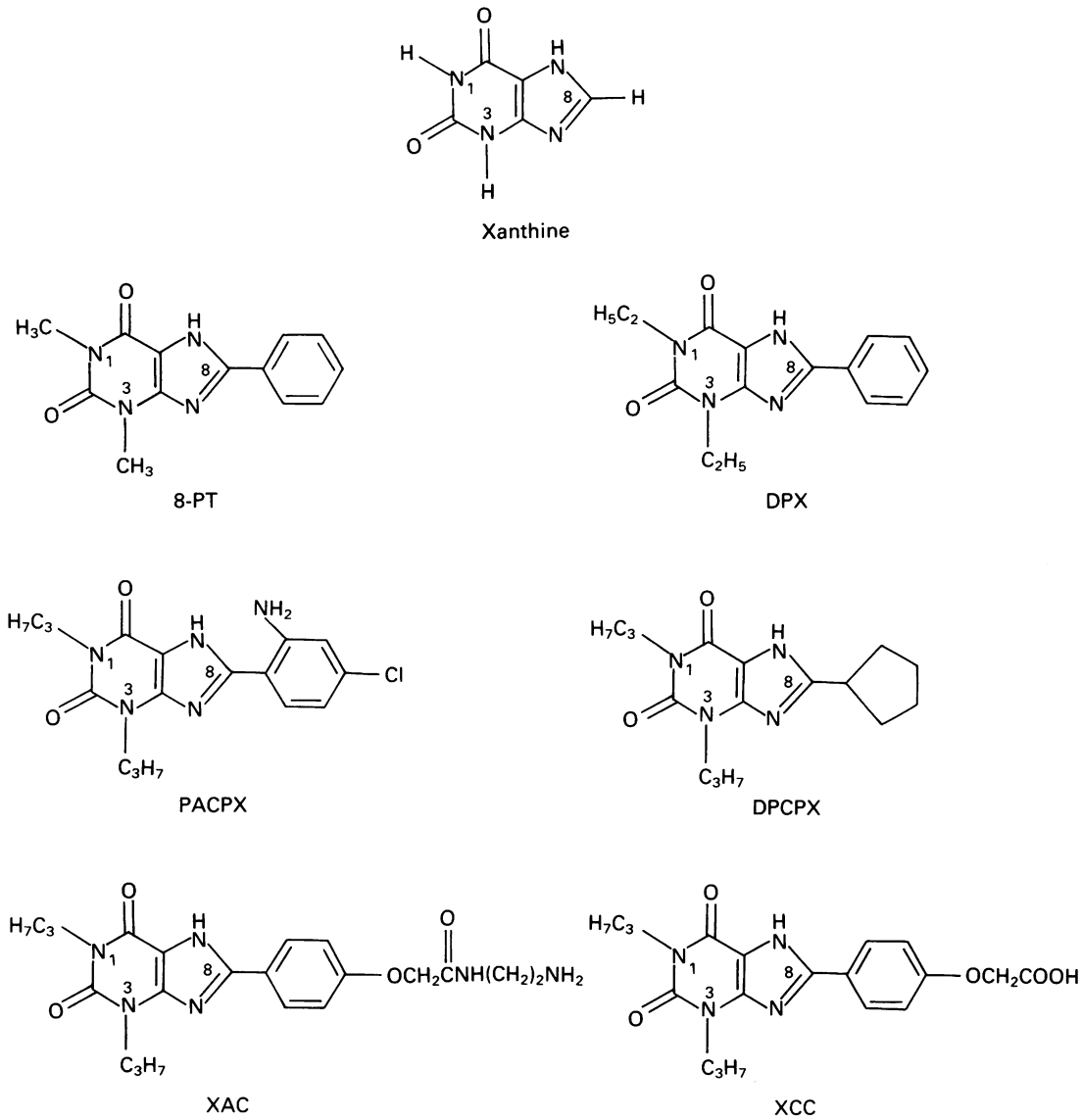


Figure 1 Structures of the 1,3,8-substituted xanthines. The structure of xanthine is illustrated at the top. 8-PT: 8-phenyltheophylline; DPX: 1,3-diethyl-8-phenylxanthine; PACPX: 1,3-dipropyl-8-(2-amino-4-chlorophenyl)-xanthine; DPCPX: 1,3-dipropyl-8-cyclopentylxanthine; XAC: 1,3-dipropyl-8-(4-((2-aminoethyl)amino)-carbonylmethyloxyphenyl)xanthine; XCC: 1,3-dipropyl-8-(4-carboxymethyloxyphenyl)xanthine.

thine (DPCPX), enprofylline, 8-phenyltheophyline (8-PT) (R.B.I.); 1,3-dipropyl-8-(4-((2-aminoethyl) amino) carbonylmethyloxyphenyl)xanthine (XAC), 1,3-dipropyl-8-(4-carboxymethyloxyphenyl)xanthine (XCC) and 1-propargyl-3,7-dimethylxanthine (PGDMX) were kindly supplied by Drs J.W. Daly and K.A. Jacobson (National Institute of Health, Bethesda, U.S.A.). 8-PT and DPX were made up into 10 mM and 50 mM stock solutions, respectively, in 80% methanol/20% M NaOH (v/v); PACPX and DPCPX were made up into 10 mM and 5 mM stock solutions, respectively, in 99% dimethylsulphoxide (DMSO)/1% M NaOH (v/v); XAC and XCC were made up into 80 μM and 2.5 mM stock solutions,

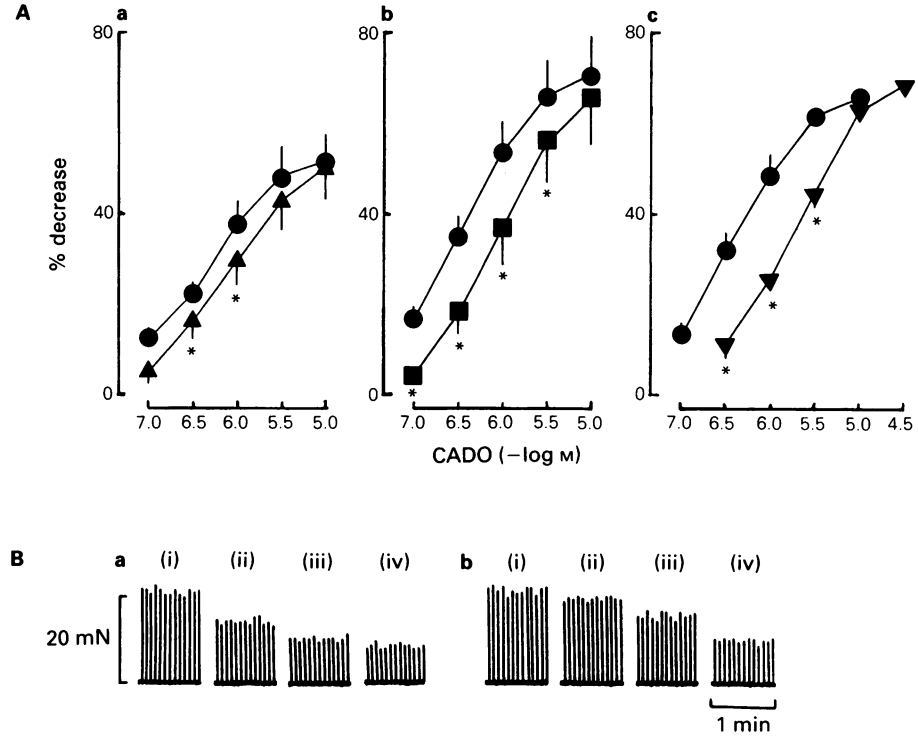


Figure 2 Action of the adenosine receptor antagonist 1,3-dipropyl-8-(4-((2-aminoethyl)amino)-carbonylmethyloxyphenyl)xanthine (XAC) on the inhibitory effect of 2-chloroadenosine (CADO) on twitch responses evoked by nerve stimulation of frog sartorius muscles. (A) Concentration-response curves for the inhibitory effect of CADO. (a) Antagonism by XAC (25 nM) ($n = 3$); (b) antagonism by XAC (50 nM) ($n = 4$); (c) antagonism by XAC (100 nM) ($n = 4$). Each panel represents pooled data from the same experiments. The ordinates are percentage decreases in twitch amplitude caused by CADO in the absence (●) and in the presence (▲, ■, ▼) of XAC. Zero% is the twitch amplitude in the control bathing solution and 100% represents a complete inhibition of the nerve-evoked twitches. The vertical bars represent s.e.mean and are shown when they exceed the symbols in size; * $P < 0.05$ (paired Student's t test as compared with the effect of CADO alone in the same experiments). When testing the effect of CADO in the absence of XAC, the solvent of XAC (DMSO) was present in the same concentration (0.03–0.125% v/v) as that present in the XAC solutions applied to the preparations. Average twitch amplitude in the control bathing solution: 20.5 ± 2.1 mN. (B) shows pen-recorder traces of nerve-evoked twitch responses obtained in one experiment using XAC (100 nM). (a) Effect of CADO in the absence of XAC; responses recorded in control bathing solution (i) before applying CADO, and after 10 min in (ii) 0.3 μM, (iii) 1 μM and (iv) 3 μM CADO. (b) Effect of CADO in the presence of XAC; responses recorded (i) 55 min after applying XAC (100 nM), and after 10 min in (ii) 0.3 μM, (iii) 1 μM and (iv) 3 μM CADO + XAC (100 nM).

respectively, 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 differences between the means was calculated by paired or unpaired Student's t test. P values of 0.05 or less were considered to represent significant differences.

Results

1,3,8-substituted xanthine derivatives

The structures of the 1,3,8-substituted xanthines used in the present work are shown in Figure 1. These

include xanthines with substitutions at the 1- and 3-positions by methyl, ethyl or propyl groups, the 8-position being substituted either by aryl or cycloalkyl groups.

Figure 2 shows the effect of the adenosine receptor agonist, 2-chloroadenosine (CADO, 0.1–30 μM) on nerve-evoked twitch responses of the frog sartorius, in the absence and in the presence of 1,3-dipropyl-8-(4 - ((2 - aminoethyl)amino) - carbonylmethyloxy - phenyl)xanthine (XAC, 25–100 nM). CADO decreased, in a concentration-dependent manner, the amplitude of the nerve-evoked twitches, its maximal effect being almost obtained with 3–10 μM of this adenosine analogue. The comparisons between the concentration-response curves for CADO in the absence and in the presence of antagonist were made using data from the same experiments because there is considerable variation between the quantitative effects of the same concentration of CADO in different experiments. XAC (25–100 nM) used alone was virtually devoid of effect on nerve-evoked twitches, but shifted to the right the concentration-response curve for CADO (0.1–30 μM), this shift being greater the higher the concentration of XAC. The maximal effect of CADO was not significantly modified by XAC (25–100 nM) (Figure 2). The pA_2 value for XAC calculated from the data shown in Figure 2 was 7.63 ($n = 11$) and the slope of the regression line of the Schild plot was 1.05, giving a dissociation constant (K_i) for XAC of 23 nM.

Concentration-dependent shifts to the right of the concentration-response curve for the inhibitory effect of CADO (0.1–30 μM) on nerve-evoked twitches were also observed with 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 50–250 nM), 8-phenyltheophyline (8-PT, 0.25–2.5 μM), 1,3-diethyl-8-phenylxanthine (DPX, 0.5–2.5 μM) and 1,3-dipropyl-8-(4-carboxymethyloxyphenyl)xanthine (XCC, 2.5–10 μM). The slope of the linear part of the log concentration-response curves for CADO as well as the maximal effect of CADO were not appreciably modified by these xanthines. In the concentrations used, the xanthines were devoid of effect on the nerve-evoked twitches, when applied to the preparations in the absence of CADO. In Figure 3 the Schild plots obtained for XAC, DPCPX, 8-PT, DPX and XCC are compared. As can be seen, linear Schild plots with slopes near unity at low concentration-ratios (less than 14) were obtained.

1,3-Dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX, 2.5 μM) shifted the log concentration-response curve for CADO (0.1–10 μM) to the right by a factor of 2.2 ± 0.3 ($n = 6$), without affecting in an appreciable manner the maximal effect of CADO. The averaged slopes of the linear part of the log concentration-response curves for CADO in the absence and in the presence of PACPX (2.5 μM) were

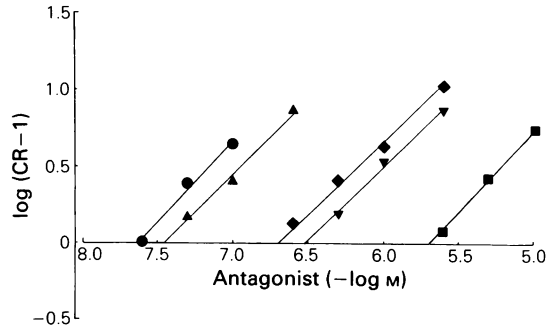


Figure 3 Schild plots for the antagonism by 1,3,-dipropyl-8-(4-((2-aminoethyl)amino) carbonylmethyloxyphenyl)xanthine (XAC, ●), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, ▲), 8-phenyltheophylline (8-PT, ◆), 1,3-diethyl-8-phenylxanthine (DPX, ▼)and 1,3-dipropyl-8-(4- carboxymethyloxyphenyl)xanthine (XCC, ■) of the inhibitory effect of 2-chloroadenosine (CADO) on twitch responses evoked by nerve stimulation of frog sartorius muscles. 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 the individual CR values; the correlation coefficients ranged from 0.876 to 0.760 ($P < 0.05$ in all cases). CR values were calculated at the level of 35% decrease in twitch amplitude caused by CADO, which is near its EC_{50} value since the maximal effect of CADO (determined from the double reciprocal plot of the averaged concentration-response curves for CADO, $n = 51$) was 65% decrease in twitch amplitude.

not statistically different (27.6 ± 4.0 in the absence and 28.6 ± 4.3 in the presence of PACPX, $P > 0.05$). When 0.25 μM and 0.5 μM PACPX were used, no measurable modifications of the inhibitory effect of CADO (0.1–10 μM) on nerve-evoked twitch amplitude were observed ($n = 3$), and 1 μM PACPX shifted the concentration-response curve for CADO (0.1–10 μM) to the right by a factor of 1.5 (one experiment). To avoid the use of concentration-ratios below 2 to calculate pA_2 values, and since concentrations of PACPX higher than 2.5 μM could not be used due to its solubility limit in the bathing solution, the pA_2 value for PACPX was estimated using the concentration-ratios obtained with 2.5 μM PACPX in an abbreviated Schild analysis where a slope of unity was assumed for log(CR − 1) on log[PACPX]. PACPX (0.25–2.5 μM) was virtually devoid of effect on nerve-evoked twitches when applied to the preparations in the absence of CADO.

A comparison between the different pA_2 values as well as the K_i values calculated for PACPX, XCC, DPX, 8-PT, DPCPX and XAC is shown in Table 1. It is evident that XAC and DPCPX were the most

Table 1 Potencies of the 1,3,8-substituted xanthine derivatives as antagonists of the inhibitory effect of 2-chloroadenosine on nerve-evoked twitch amplitude in the frog sartorius

	pA_2	*Max. CR*	*Slope*[a]	K_i(nM)	n
XAC	7.63 ± 0.08	5.39	1.05	23	11
DPCPX	7.46 ± 0.05	9.78	1.02	35	10
8-PT	6.70 ± 0.08*	13.17	0.94	200	13
DPX	6.53 ± 0.05	8.45	0.96	295	10
XCC	5.72 ± 0.10**	7.50	0.99	1905	7
PACPX[b]	5.64 ± 0.08	2.22	—	2291	6

Max. CR: averaged concentration-ratio obtained with the maximum concentration used. [a]Slope of the regression line from the Schild plot shown in Figure 3. [b]Due to solubility limits, pA_2 and K_i values were estimated using one concentration (2.5 μM) of PACPX in an abbreviated Schild analysis, where a slope of unity was assumed for log(CR − 1) on log[PACPX]. * $P < 0.05$ compared with DPCPX; ** $P < 0.05$ compared with DPX (unpaired Student's *t* test). For key to abbreviations used see legend to Figure 1.

potent antagonists, XCC and PACPX were the least potent, 8-PT and DPX having intermediate potency as antagonists of the inhibitory effect of CADO on nerve-evoked twitch responses in the frog sartorius.

1,3,7-substituted xanthine derivatives

The ability of 1-propargyl-3,7-dimethylxanthine (PGDMX, 20 μM) to antagonize the inhibitory effect of CADO (0.1–3 μM) on nerve-evoked twitches was investigated in two experiments. In this concentration PGDMX caused a small parallel shift to the right of the concentration-response curve for CADO (0.1–3 μM), with an average concentration-ratio obtained in the two experiments of 1.9 ± 0.04. Caffeine (1,3,7-trimethylxanthine) in concentrations from 50 μM to 100 μM was virtually devoid of antagonistic action against the inhibitory effect of CADO (0.1–10 μM) on the amplitude of nerve-evoked twitches ($n = 3$). Used in a higher concentration (250 μM) caffeine shifted to the right in a near parallel manner the concentration-response curve for CADO (0.1–10 μM) by a factor of 1.7 ± 0.1 ($n = 3$). Neither PGDMX (20 μM) nor caffeine (50–250 μM) affected the nerve-evoked twitch responses when applied to the preparations in the absence of CADO.

Enprofylline

Enprofylline (3-propylxanthine) (50–100 μM) did not antagonize in an appreciable manner the inhibitory effect of CADO (0.1–10 μM) on nerve-evoked twitches ($n = 3$).

Discussion

The present results show that several 1,3,8- and 1,3,7-substituted xanthine derivatives, antagonize the inhibitory effect of CADO on the amplitude of nerve-evoked twitch responses in the innervated sartorius muscle of the frog. The rank order of potency of the xanthines was XAC ≥ DPCPX > 8-PT ≥ DPX > XCC ≥ PACPX > PGDMX > caffeine. The 3-substituted xanthine, enprofylline, was virtually devoid of antagonistic action, which conforms with the results obtained by others (cf. Persson *et al.*, 1986; but see Ukena *et al.*, 1985).

In concentrations similar to those used in the present work, CADO is devoid of effect on the amplitude of twitches evoked by direct stimulation of curarized frog sartorius muscles (Ribeiro & Sebastião, 1985) and decreases the amplitude and quantal content of the endplate potentials (e.p.ps) recorded from frog muscle fibres (Silinsky, 1980; Ribeiro & Sebastião, 1987). Thus, the inhibitory effect of CADO on nerve-evoked twitches is due to inhibition of neuromuscular transmission by decreasing transmitter release.

The xanthines XAC, DPCPX, 8-PT, DPX and XCC, appear to be competitive antagonists of the adenosine receptor stimulated by CADO at the frog neuromuscular junction, as can be concluded from the finding that the slopes of the Schild plots for these xanthines were near unity. Also, they appear to antagonize an homogeneous population of adenosine receptors since the Schild plots were linear at low concentration-ratios (cf. Kenakin, 1987). Schild plots for PACPX, PGDMX and caffeine were not done, but the findings that the concentration-response curves for CADO were shifted to the right in a near parallel manner and that the maximal effect of CADO was virtually unaffected by these xanthines, indicate that they also may behave as competitive antagonists.

The presence of an 8-phenyl or 8-cycloalkyl group greatly increases the xanthine antagonistic potency at the adenosine receptor mediating inhibition of

transmission in the frog sartorius neuromuscular junction. This has also been shown to occur at A_1 (Bruns *et al.*, 1983) and A_2 (Bruns, 1981) adenosine receptors. Within the series of 8-substituted xanthines studied, 8-PT and DPX were of similar potency, indicating that the adenosine receptor at the frog sartorius neuromuscular junction does not discriminate between methyl or ethyl groups in the 1- and 3-positions of the xanthine molecule. XAC and DPCPX were about equipotent and more potent than 8-PT, whereas XCC and PACPX were less potent than 8-PT. It thus appears that the increase in the length of the 1,3-alkyl substituents from methyl to propyl groups can either increase or decrease the potency of the 1,3,8-substituted xanthines, the nature of the substituent in the 8-position being more important in determining the xanthine potency. This contrasts with that which was found at the A_1-adenosine receptor where 1,3-dipropyl-8-substituted xanthines are more potent than 1,3-dimethyl-8-substituted xanthines (see e.g. Daly, 1985).

The potencies of the 1,3,8-substituted xanthine derivatives as antagonists of the A_1-adenosine receptor have been determined by its ability to inhibit binding of [^{3}H]CHA or [^{3}H]-L-PIA to rat brain membranes (Ukena *et al.*, 1986a; Daly *et al.*, 1987; Lohse *et al.*, 1987) or by its ability to inhibit L-PIA-induced inhibition of adenylate cyclase activity in rat adipocytes (Ukena *et al.*, 1986b; Lohse *et al.*, 1987; Martinson *et al.*, 1987). The antagonistic profile found in brain membranes is DPCPX (K_i = 0.3–1.2 nM), XAC (1.2–3.5 nM), PACPX (2.5 nM) > XCC (58 nM), DPX (70 nM), 8-PT (76 nM), and in fat cells is DPCPX (K_i = 0.45–0.47 nM) > PACPX (3 nM), XAC (4.4–15 nM) > XCC (83 nM) > DPX (170 nM) > 8-PT (310 nM). The high potency of DPCPX observed in the present work could indicate that the adenosine receptor at the frog neuromuscular junction has an antagonist profile compatible with that of the A_1-adenosine receptor, and the finding that DPCPX and XAC are of similar potency suggests that the adenosine receptor at the frog neuromuscular junction is closer to the adenosine receptor in brain membranes than in fat cells. However, no correlation was found between the potencies of these xanthines as antagonists of the A_1-adenosine receptor in brain membranes or fat cells and as antagonists of the adenosine receptor at the frog sartorius neuromuscular junction (Figure 4a). This indicates that the antagonist profile of the adenosine receptor at the frog neuromuscular junction is different from the antagonist profile of the A_1-adenosine receptor. Even excluding PACPX, which in some preparations behaves as a non-competitive antagonist of adenosine receptors (Burnstock & Hoyle, 1985; Collis *et al.*, 1987; Wil-

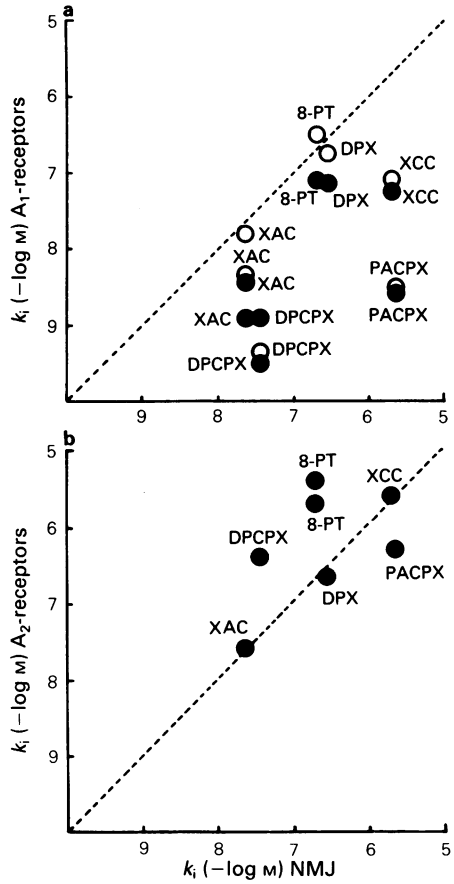


Figure 4 Scatter diagrams comparing the affinities of the 1,3,8-substituted xanthines for the adenosine receptor at the frog sartorius neuromuscular junction (NMJ) (expressed as the K_i values shown in Table 1) and for the A_1-adenosine receptor (a) in rat brain membranes (●) or rat fat cells (○), and for the A_2-adenosine receptor (b) in human platelets. The correlation coefficients in (a) were $r = 0.154$, $0.7 < P < 0.80$, for (●) and $r = -0.093$, $0.8 < P < 0.9$ for (○), and the correlation coefficient in (b) was 0.104, $0.8 < P < 0.9$. Excluding PACPX, the correlation coefficients were $r = 0.502$, $0.1 < P < 0.2$, for (●), and $r = 0.125$, $0.7 < P < 0.8$, for (○), and the correlation coefficient in (b) was $r = 0.374$, $0.3 < P < 0.4$. Data which are not from the present study are from Daly *et al.* (1987), Lohse *et al.* (1987), Martinson *et al.* (1987), Schwabe *et al.* (1985) and Ukena *et al.* (1986a,b,c); when different K_i values for the same xanthine have been reported they are all represented (e.g. XAC (●, ○), DPCPX (●) in (a) and 8-PT in (b)) and used in the calculation of the correlation coefficients. For key to abbreviations used see legend to Figure 1. The broken line indicates a gradient of unity. Note the absence of correlation between the affinities of the xanthines for the adenosine receptor at the neuromuscular junction and for the A_1- or the A_2-adenosine receptors.

liams *et al.*, 1987), no correlation was observed between the potencies of the 1,3,8-substituted xanthines at the A_1-adenosine receptor and at the adenosine receptor at the frog neuromuscular junction (see legend to Figure 4).

The ability of 1,3,8-substituted xanthine derivatives to inhibit NECA-induced stimulation of adenylate cyclase in human platelets has been studied in order to characterize the antagonist profile of the A_2-adenosine receptor (Schwabe *et al.*, 1985; Ukena *et al.*, 1986b; Lohse *et al.*, 1987). In these studies it was shown that XAC (K_i = 24–25 nM) > DPX (210 nM), DPCPX (390 nM), PACPX (470 nM) > 8-PT (1.9–4.1 μM), XCC (2.4 μM). The low potency of XCC observed in the present work, taken together with the finding that PGDMX was much more potent than caffeine (also a characteristic of the A_2-adenosine receptor (Ukena *et al.*, 1986c)), could indicate that the adenosine receptor at the frog neuromuscular junction has an antagonist profile compatible with that of the A_2-adenosine receptor. However, in disagreement with this idea is the absence of correlation between the potencies of the 1,3,8-substituted xanthines as antagonists of the A_2-adenosine receptor at human platelets and as antagonists of the adenosine receptor at the frog neuromuscular junction (Figure 4b). Also, no correlation was observed when PACPX was not included (see legend to Figure 4).

The adenosine receptor at the frog (Ribeiro & Sebastião, 1985) and rat (Sebastião & Ribeiro, 1988b) neuromuscular junctions has an agonist profile different from that of the A_1- and A_2-adenosine receptors and was recently named A_3 (Ribeiro & Sebastião, 1986). In the present work it was shown that the antagonist profile of the adenosine receptor at the frog sartorius neuromuscular junction is also different from the antagonist profile of the A_1- and A_2-adenosine receptors. Since differences might result from different experimental methods, i.e., binding vs cyclase vs functional studies, or from species differences (Ukena *et al.*, 1986a), i.e., rat vs human vs frog, characterization of the antagonist profile of the adenosine receptor mediating presynaptic inhibition of transmission in other preparations and species is needed to know whether the adenosine receptor antagonist profile found at the frog sartorius neuromuscular junction represents the antagonist profile of the A_3-adenosine receptor.

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Atenolol, but not mexiletine, protects against stimulus-induced ventricular tachycardia in a chronic canine model

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1 In a placebo-controlled study of the antiarrhythmic and electrophysiological properties of atenolol and mexiletine, programmed electrical stimulation (PES) was performed in three groups of six conscious greyhounds, 7–30 days after coronary artery ligation.

2 In the placebo group, repeated PES challenge resulted in the consistent induction of ventricular tachycardias (VT) in 4/6 dogs and ventricular fibrillation in 2/6. Atenolol prevented arrhythmia induction in 4/6 dogs, one continued to demonstrate a VT and one died ($P < 0.05$ compared with placebo). In the mexiletine group 5/6 dogs continued to demonstrate a VT and one died.

3 Electrocardiographic parameters were not affected by any treatment. There was no change in blood pressure in any group but when compared with placebo, heart rate fell ($P < 0.05$) after atenolol ($256\,\mu g\,kg^{-1}$) and increased ($P < 0.05$) after mexiletine ($16\,mg\,kg^{-1}$). Effective (ERP) and functional (FRP) refractory periods did not change after mexiletine, but ERP was prolonged ($P < 0.05$) after atenolol.

4 The results indicate that atenolol but not mexiletine is effective in preventing re-entrant arrhythmias in this conscious canine model. Antiarrhythmic efficacy may be related to a fall in heart rate and/or a prolongation of refractoriness.

Introduction

Cardiovascular disease is the single largest cause of death in the Western World with sudden cardiac death responsible for up to 25% of all deaths (Lown & Graboys, 1977). Experience with electrocardiographic monitoring has suggested that ventricular fibrillation is the most common terminal mechanism (Gradman *et al.*, 1977; Hinkle *et al.*, 1977; Nikolic *et al.*, 1982) and that chronic ventricular ectopy is a significant factor in predicting those at risk (The Coronary Drug Project Research Group, 1973; Bigger *et al.*, 1984). As a result, there is now considerable importance attached to the adequate control of chronic ventricular ectopy in survivors of acute myocardial infarction. Yet despite their demonstrated efficacy as antiarrhythmic drugs, many currently available agents have not been shown to reduce mortality in controlled trials (Myerburg *et al.*, 1979; May *et al.*, 1983; Furberg, 1983). However, there is good evidence that the use of β-adrenoceptor antagonists in the post-infarction period is associated with a reduction in both reinfarction and sudden death (Multicenter International Study, 1975; Norwegian Multicenter Study Group, 1981; Beta-Blocker Heart Attack Trial Research Group, 1982), although the mechanism responsible for a protective action remains unclear. In particular, it is not known whether the drugs are primarily antiarrhythmic or if mortality is reduced by alternative (anti-ischaemic) mechanisms (The MIAMI Trial Research Group, 1985; ISIS-1 Collaborative Group, 1986).

This department has recently developed a chronic canine model of myocardial infarction where re-entrant arrhythmias similar to those responsible for sudden death in man may be initiated by programmed electrical stimulation (Uprichard *et al.*,

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1988). The purpose of the present study was therefore to evaluate the antiarrhythmic and electrophysiological properties of atenolol, a cardioselective β-adrenoceptor antagonist, and mexiletine, a commonly used class I antiarrhythmic agent.

Methods

Coronary artery ligation

Adult greyhounds of either sex were anaesthetized by the intravenous administration of sodium methohexitone ($10\,mg\,kg^{-1}$) intubated and ventilated at a rate of 18 per min using a tidal volume of $13\,ml\,kg^{-1}$ (Palmer Ideal Pump). Anaesthesia was maintained with a 1.5% concentration of halothane in room air. Following thoracotomy the fourth or fifth left rib was dissected free and removed and the heart exposed. The anterior descending branch of the left coronary artery was dissected free below its second branch and ligated in a two-stage procedure (Harris, 1950). Two ligatures were placed around the artery and a 21 gauge needle. The first ligature was tied around the artery and the needle which was then removed. After 30 min the second ligature was tied tightly around the artery producing a complete occlusion. Myocardial pacing wires (Medtronic 6400 temporary myocardial pacing leads) were sutured into the heart: one in the centre of the area supplied by the occluded artery and a second in an area jointly supplied by an adjacent branch of the anterior descending artery. Interelectrode distance was maintained at 1.0–1.5 cm in all cases. Both pacing wires were brought out through skin distant from the wound, the chest was closed in layers and the dog allowed to recover. Routine antibiotic prophylaxis (250 mg streptomycin and 300,000 iu procaine penicillin) was given intramuscularly for three days.

Programmed electrical stimulation – protocol

Further observations were made on the conscious dogs, 7 to 30 days after coronary artery ligation. The 7 day lag period was to ensure that the experiments were not influenced by spontaneous postinfarction ectopy which in our experience may be unmasked by electrical stimulation up to the fifth-post operative day. The dogs were positioned on their left side and remained in this position throughout the duration of the experiment. The myocardial pacing wires were attached to a Digitimer 4279 modular pacing system; leads II and III of the electrocardiogram and blood pressure (recorded by means of a cannula in the femoral artery) were continuously displayed on a Device MX4 polygraph. Drugs were administered intravenously through a catheter in a foreleg vein. Using unipolar pulses of 4 ms duration at twice diastolic threshold, ventricular pacing (S_1) was introduced with a basic cycle length of 350 ms (170 beats min^{-1}). An extrastimulus (S_2) was introduced with a delay of 350 ms after the eighth basic pacing stimulus. The delay was subsequently reduced at 20 ms intervals until the extrastimulus failed to produce a ventricular response (R_2). S_2 was then set at the shortest delay (to the nearest 5 ms) which produced a ventricular response, and S_3 introduced with a delay of 350 ms after S_2. The procedure was repeated with S_3 and S_4 until an arrhythmia was produced or the protocol was exhausted.

The arrhythmias considered suitable for the continuation of the study were sustained ventricular tachycardia (S-VT, defined as a self-perpetuating arrhythmia of 5 min duration), and non-sustained ventricular tachycardia (NS-VT, defined as a reproducible arrhythmia of 4 or more ventricular ectopic beats at any given setting of S_2–S_3–S_4). Dogs that developed S-VT or NS-VT in response to PES were termed 'inducible'. Dogs that failed to produce an arrhythmia in response to PES were deemed 'non-inducible'.

Drug therapy

A total of 18 inducible dogs were entered into the study. Animals were randomly allocated to receive increasing intravenous doses of mexiletine, atenolol or placebo. Drugs were administered at 5 min intervals until the arrhythmia was abolished, side effects were apparent or the dog died (ventricular fibrillation (VF) in response to stimulation). Dogs with NS-VT were re-challenged every 5-min with the stimulus setting that had produced the arrhythmia. If stimulation failed to produce an arrhythmia after drug therapy the protocol was continued as described above. Only if this failed to produce the required ectopic response was the arrhythmia considered abolished and the dog deemed non-inducible. If a dog with S-VT reverted to sinus rhythm with drug therapy the protocol was continued as above. Any dog that was still in S-VT at the end of the experiment was paced back into sinus rhythm.

PR intervals and QRS duration were measured to the nearest 5 ms (paper speed $10\,cm^{-1}$) at the start and end of each experiment; QT intervals were measured and QT_c was calculated from the formula $QT_c = QT/\sqrt{RR}$ (Bazett, 1920). The effective refractory period (ERP) was taken as the shortest S_1–S_2 interval (to the nearest 5 ms) which produced a ventricular response. Functional refractory period (FRP was taken as the shortest R_1–R_2 interval measured during the pacing protocol. Blood pressures and heart rates were monitored throughout the experiments.

Drugs

The following drugs were used: mexiletine hydrochloride (Boehringer-Ingelheim Laboratories), atenolol hydrochloride (Stuart Laboratories).

All drugs were freshly prepared by dissolution in 0.9% saline. All doses expressed are in terms of the hydrochloride.

Statistics

Results of programmed electrical stimulation for each drug group were ranked for abolition of arrhythmias, no change, or death and compared with placebo by the non-parametric Mann-Whitney U test. Blood pressures and heart rates were compared by one-way analysis of variance with the method of contrasts at each dose for the 3 groups. Statistical analyses of the ECG parameters were performed using Student's t tests and significance measured at the $P < 0.05$ level. ERP and FRP were only available after treatment in those dogs that survived programmed electrical stimulation and were therefore compared with pretreatment values using unpaired t tests.

Results

Effects of programmed electrical stimulation

In the placebo group, five of the dogs had a reproducible NS-VT and one was in S-VT at the start of the experiment. Of the 5 with NS-VT, 3 consistently produced a NS-VT when stimulated after each of the 7 doses of placebo; the other 2 developed VF when stimulated 5 min after the first dose of placebo. The sixth dog remained in a S-VT throughout the duration of the experiment (40 min) (Figure 1). The effects of atenolol are illustrated in Figure 2. Of the 6 dogs studied, 5 had a reproducible NS-VT and one was in S-VT. Of the 5 with NS-VT, 3 became non-inducible after doses of $8\,\mu g\,kg^{-1}$, $64\,\mu g\,kg^{-1}$ and $128\,\mu g\,kg^{-1}$ respectively, one was still inducible after $256\,\mu g\,kg^{-1}$ and one died when stimulated after $1\,\mu g\,kg^{-1}$. The dog with S-VT became non-inducible after $256\,\mu g\,kg^{-1}$. No adverse effects were apparent at the doses used.

In the mexiletine group all 6 dogs had a reproducible NS-VT at the start of the experiment. Of these, 5 remained inducible after doses of up to $16\,mg\,kg^{-1}$ and one dog died when stimulated after $4\,mg\,kg^{-1}$ (Figure 2). All the dogs that received $16\,mg\,kg^{-1}$ exhibited generalised rigidity which prevented continuation of the experiment.

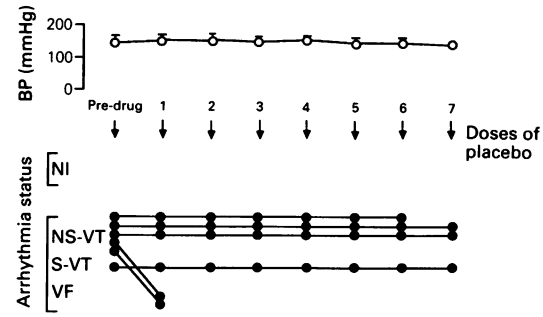


Figure 1 Effect of placebo upon the arrhythmias produced by programmed electrical stimulation (PES) in 6 conscious dogs 7–30 days after ligation of the left anterior descending coronary artery: BP, mean systolic blood pressure; NI, non-inducible; NS-VT, non-sustained ventricular tachycardia; S-VT, sustained ventricular tachycardia; VF, ventricular fibrillation.

A noticeable feature of 3 of the 4 dogs which developed VF during the course of the study was that fibrillation occurred only after several seconds of monomorphic ventricular tachycardia (Figure 3).

Statistical analysis revealed that atenolol was significantly ($P < 0.05$) better than placebo in abolishing arrhythmias produced by PES and protecting against VF. The difference between mexiletine and placebo was not significant.

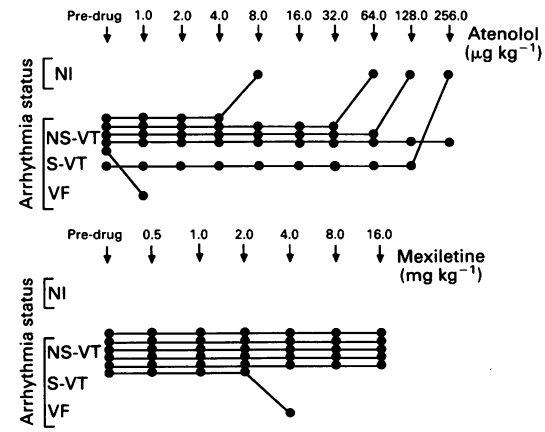


Figure 2 Effect of atenolol and mexiletine upon the arrhythmias produced by programmed electrical stimulation (PES) in 2 groups of 6 conscious dogs, 7–30 days after ligation of the left anterior descending coronary artery. All the dogs which received $16\,mg\,kg^{-1}$ of mexiletine exhibited generalized rigidity which prevented continuation of the experiment. Abbreviations as in Figure 1.

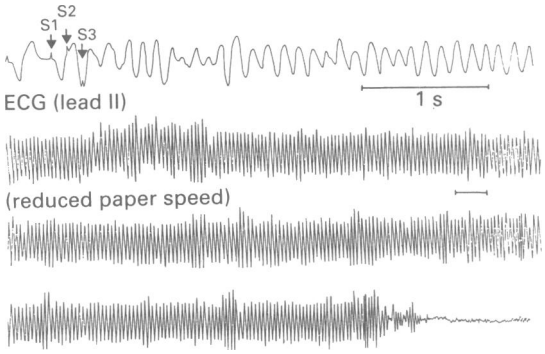


Figure 3 Example of ventricular fibrillation following several seconds of monomorphic ventricular tachycardia in response to programmed electrical stimulation.

Blood pressure and heart rate

In the placebo group, mean systolic pressure for the 6 dogs at the start of the experiments was 155 ± 6.9 mmHg (mean $\pm$ s.e.mean). The corresponding value for heart rate was $114.2 \pm 12.0\,min^{-1}$. Neither value altered significantly during the experiment.

Mean initial systolic pressure for the atenolol group was 162 ± 9.0 mmHg, after $256\,\mu g\,kg^{-1}$ of drug this had fallen to 147.5 ± 27.4 mmHg but the drop was not significant. Heart rate, however, fell from an initial $110.4 \pm 7.3\,min^{-1}$ to $90.4 \pm 6.5\,min^{-1}$ ($P < 0.05$ compared with placebo).

Mean initial systolic pressure for the mexiletine group was 165.5 ± 10.7 mmHg and did not vary significantly during the course of experiments. Mean initial heart rate in this group was $119.0 \pm 12.5\,min^{-1}$; this did not vary significantly with doses of up to $8\,mg\,kg^{-1}$ of mexiletine but after $16\,mg\,kg^{-1}$ increased to $176.5 \pm 11.5\,min^{-1}$ ($P < 0.05$ compared with placebo).

Electrophysiological measurements

Mean PR for the 6 dogs before placebo was 0.11 ± 0.005 s; after placebo this had increased to 0.115 ± 0.005 s (not statistically significant). Similarly there was no change in mean QRS (0.08–0,85 s), mean QT_c (0.30–0.31 s), mean ERP (0.115–0.11 s) or mean FRP (0.165–0.17 s) before and after placebo respectively. The pretreatment figures for the placebo group were not significantly different from the corresponding values for either drug group. There was no significant change in any parameter as the result of treatment with mexiletine, but atenolol resulted in a significant ($P < 0.05$) prolongation of the ERP (Table 1). FRP was also noted to lengthen (0.175–0.185 s) but in this case the change was not significant (Table 1).

Table 1 Effects of atenolol upon QT_c, QRS, PR, effective refractory period (ERP) and functional refractory period (FRP) in a group of 6 dogs

Parameter	*Before drug*	*After drug*
QT_c	0.31 ± 0.015	0.25 ± 0.005
QRS	0.08 ± 0.005	0.075 ± 0.005
PR	0.115 ± 0.005	0.12 ± 0.01
ERP	0.115 ± 0.005	$0.13^* \pm 0.005$
FRP	0.175 ± 0.005	0.185 ± 0.01

All results in seconds (mean $\pm$ s.e.mean).
* $P < 0.05$ (compared with placebo).

Discussion

Using a conscious canine model we have demonstrated that atenolol but not mexiletine is effective in preventing ventricular arrhythmias which share the same reentrant mechanisms as those that are responsible for sudden death in man (El-Sherif *et al.*, 1977; Josephson *et al.*, 1978). It has been argued that the induction of sustained ventricular tachycardia by programming techniques is more clinically relevant than the non-sustained arrhythmias used by this and other laboratories, but since the survival of the animals is a major consideration in all studies with programmed stimulation, it is our policy not to pursue a reproducible non-sustained VT with more aggressive pacing methods in an attempt to achieve a sustained arrhythmia. Our experience has been that increasing the number of extrastimuli and reducing coupling intervals may result in a higher incidence of sustained VT, but at the expense of greater pretreatment mortality. In this respect our results are similar to the experiences of Lucchesi and co-workers (Lynch *et al.*, 1987). Furthermore, results from the same laboratory have shown a highly predictive relationship between the vulnerability to initiation of either sustained or non-sustained VT (by programmed stimulation) to the development of sudden death in response to a subsequent ischaemic insult at a distant site (Wilber *et al.*, 1985). When ventricular fibrillation did occur, in the present study, it was frequently preceded by a variable period of sustained ventricular tachycardia; in this respect the model closely resembles the clinical situation, where studies using ambulatory monitoring have identified sustained tachyarrhythmias as the most common terminal mechanisms in sudden death

(Panidis & Morganroth, 1983; Kempf & Josephson, 1984; Milner *et al.*, 1985).

Experience from our laboratory has indicated that inducibility of arrhythmias by programmed stimulating techniques is determined largely by infarct size (Harron *et al.*, 1988). This is similar to other investigators who have also demonstrated that sham-operated animals are non-inducible at baseline testing (Michelson *et al.*, 1981; Patterson *et al.*, 1982a; Wilber *et al.*, 1985). In our experience, there is a time-dependent reduction in inducibility of arrhythmias after infarction (Harron *et al.*, 1988), but there is no reason to believe that the arrhythmia, once established, is any different with regard to either mechanisms of initiation or response to drug therapy.

To date, numerous antiarrhythmic agents representing the various classes of antiarrhythmic drug action have been evaluated in chronic coronary artery occluded or occluded/reperfused preparations, either in the conscious or anaesthetized state. The effects of β-adrenoceptor antagonists have varied widely with the agent under test: results with propranolol (Echt *et al.*, 1983), timolol (Gang *et al.*, 1982) and sotalol (Patterson *et al.*, 1984) have each demonstrated significant antiarrhythmic activity, while metoprolol (Cobbe *et al.*, 1983) and nadolol (Patterson & Lucchesi, 1982) have not. In general, class 1 agents have been only marginally effective in suppressing these arrhythmias; in fact, several studies have shown significant proarrhythmic effects (Patterson *et al.*, 1982b; Zimmerman *et al.*, 1985; DiCarlo *et al.*, 1985).

Unlike propranolol and certain other β-adrenoceptor antagonists, atenolol is devoid of the membrane-stabilizing properties that have been thought responsible for antiarrhythmic effects in the past (Somani & Lum, 1965). It is probable therefore that the antiarrhythmic action of atenolol is due to direct antagonism of circulating and (neurogenic) catecholamines. Catecholamine concentrations are abnormally high in the acute infarction period (Richardson, 1963) and may be inherently arrhythmogenic by nature of the associated increase in myocardial oxygen demand and effects on rate and automaticity. Various metabolic factors have also been held responsible for arrhythmogenesis in the acute infarction period and many of these are consequent upon increased catecholamine activity (Opie *et al.*, 1979). Hypokalaemia is a particular hazard which may be induced by adrenaline and inhibited by certain β-adrenoceptor antagonists (Struthers *et al.*, 1981).

The importance of an intact autonomic nervous system in the genesis of cardiac arrhythmias has been recognized for some time (Lown & Verrier, 1976; Corr & Gillis, 1978). In a similar chronic canine model, Schwartz & Stone (1980) have demonstrated that sympathetic stimulation can facilitate the induction of ventricular fibrillation, and it is known that neurogenic activity can modify the effects of antiarrhythmic drugs (Bigger *et al.*, 1982). The relevance of a conscious model (such as that used in the present study) for evaluating antiarrhythmic agents must also be of particular importance when dealing with drugs with inherent sympatholytic activity.

We have noted two characteristics that distinguish atenolol from both mexiletine and the placebo group. Prolongation of the effective refractory period (and functional refractory period, a derivative of the ERP which also varies inversely with conductivity) has been noted previously (Gang *et al.*, 1982) and is most likely a reflection of antagonism of the high sympathetic tone pertaining in the conscious dogs than any direct electrophysiological effect. Both sympathetic nerve stimulation (Kralios *et al.*, 1975) and exogenous catecholamine administration (Hoffman & Singer, 1967) have been shown to shorten refractory periods but β-adrenoceptor antagonism has been shown to have no effect on refractoriness in anaesthetized dogs (Hoffman & Singer, 1967). Furthermore, any direct action of the drug would seem unlikely without associated changes in action potential duration, as reflected by the QT interval on the surface ECG. Variations in action potential duration resultant upon catecholamine tone, however, will not necessarily be reflected in changes in the corrected QT interval, as the calculation employed accounts for heart rate variations. Nevertheless, by delaying depolarization of myocardial fibres in possible re-entrant pathways, any drug-induced antagonism of catecholamines-dependent reductions in refractoriness must be considered protective.

The significant fall in heart rate observed in the study may be similarly antiarrhythmic; El-Sherif (1978), in a study of reentrant ventricular arrhythmias in the late myocardial infarction period in the dog, demonstrated that the propensity of sympathetic stimulation to induce arrhythmias was primarily due to its effect on heart rate. A previous study with propranolol (Hope *et al.*, 1974) had shown a protective action of propranolol which was antagonized when the heart rate was returned to control values by atrial pacing.

We demonstrated no change in any electrophysiological parameter when mexiletine was compared with placebo. This is in agreement with previous studies which have shown no effect on action potential duration (Singh & Vaughan Williams, 1972), conduction times (Okuma *et al.*, 1976) or refractory periods (Campbell, 1982). A recent study from this laboratory found a similar lack of significant electrophysiological effect with the related class Ib agent

tocainide (Uprichard *et al.*, 1988). In the present study, 16 mg kg^{-1} of mexiletine produced a significant positive chronotropic effect. Similar effects have previously been ascribed to a vagal inhibitory action (Touboul *et al.*, 1978) but this would seem unlikely without associated delays in conductivity, in particular atrioventricular times (as reflected in PR intervals). Since the increase in heart rate was only apparent after 16 mg kg^{-1}, a dose that produced invariable features of agitation and rigidity, it would seem more likely that this finding was a reflection of drug toxicity.

We are indebted to Mr R Wilson for his invaluable technical assistance. Our thanks also to Boehringer Ingelheim and Stuart Laboratories for their respective supplies of mexiletine and atenolol. During the course of the study, A.C.G.U. was the holder of a DHSS (NI) research fellowship and D.W.G.H. the holder of a BHF grant 84/14. The study described was carried out, as far as possible, in accordance with the Lambeth conventions.

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Decreased arterial responsiveness to multiple cyclic AMP-generating receptor agonists in spontaneously hypertensive rats

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1 Arterial relaxant responses via β-adrenoceptors are decreased in spontaneously hypertensive rats (SHR) when compared with normotensive Wistar-Kyoto rats (WKY). Recent studies from this laboratory proposed that a reduced function of stimulatory guanosine 5'-triphosphate (GTP)-binding protein (Gs) is responsible for the decreased β-adrenoceptor responsiveness in the SHR femoral artery. Since the Gs is common to all tissues, as opposed to receptors, which are tissue specific, the reduced function of Gs should lead to resistance to multiple receptors that act by activating adenylate cyclase (AC). To test this hypothesis, relaxant responses via β-adrenoceptors, A_2-adenosine, H_2-histamine and D_1-dopamine receptors were compared between arterial strips from 13 week-old WKY and age-matched SHR.

2 The relaxant responses to noradrenaline (NA) via β-adrenoceptors in femoral, mesenteric, renal and carotid arteries were significantly decreased in the SHR, when compared with the respective arteries from WKY.

3 However, under the same conditions arterial relaxant responses to forskolin, an activator of AC, were not significantly different between the WKY and SHR.

4 The relaxant responses due to activation of A_2-adenosine. H_2-histamine and D_1-dopamine receptors were significantly decreased in the SHR arteries.

5 Nitroprusside and nifedipine, agents which are independent of the Gs · AC system, produced similar arterial relaxations in the WKY and SHR.

6 These results support the hypothesis that a reduced function of Gs in the SHR is responsible for the decreased arterial responsiveness to a variety of receptor agonists whose mechanism of action involves AC activation.

Introduction

β-Adrenoceptor-mediated relaxation has been demonstrated to be decreased in a variety of hypertensive animals including spontaneously hypertensive rats (SHR; Amer. 1973; Triner *et al.*, 1975; Cohen & Berkowitz, 1976; Asano *et al.*, 1982). In strips of the SHR femoral artery, the decreased β-adrenoceptor responsiveness is reflected in an enhanced arterial contraction through the activation of α-adrenoceptors (Asano *et al.*, 1982). This decreased β-adrenoceptor responsiveness may contribute to the elevation of total peripheral resistance which occurs in essential hypertension. β-Adrenoceptor-mediated relaxation has been proposed to involve increased cellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) through the activation of adenylate cyclase (AC) and subsequent activation of cyclic AMP-dependent protein kinase in a variety of smooth muscles including vascular smooth muscle (for review, see Anderson & Wilsson, 1977; Kukovetz *et al.*, 1981). According to current models (Gilman, 1987), the activation by β-adrenoceptor agonists of AC-catalyzed cyclic AMP formation involves the interaction of at least three membrane-bound components: β-adrenoceptor, stimulatory guanosine 5'-triphosphate (GTP)-binding protein (Gs) and catalytic component (C) of AC. The interaction of agonists with β-adrenoceptors results in the binding of GTP to Gs, which then activates C, resulting in conversion of adenosine 5'-triphosphate (ATP) to cyclic AMP. The activation of AC can be modified by agents which interact directly with Gs, e.g., cholera toxin (Northup

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et al., 1980) or with C, e.g., forskolin (Seamon & Daly, 1981). Numerous receptors act to stimulate AC, cumulatively leading to an increase in the cellular concentration of cyclic AMP. Stimulation of A_2-adenosine, H_2-histamine and D_1-dopamine receptors usually results in an increase in cyclic AMP concentrations and relaxation of arterial smooth muscle (Goldberg et al., 1978; Bolton, 1979; Daly, 1985; Hilditch & Drew, 1985; Stiles, 1986). However, arterial relaxant responses via these cyclic AMP-generating receptors were not determined in SHR.

In previous studies, we have examined the possible mechanism responsible for the decreased β-adrenoceptor responsiveness in the femoral artery of SHR using several agents which interact with different processes of the β-adrenoceptor · AC system, and have obtained evidence suggesting that a reduced function of Gs is responsible for the decreased responsiveness in the SHR (Asano et al., 1988a,b). Since the Gs and AC are common to the cyclic AMP-generating receptors, the reduced function of Gs could lead to resistance to the stimulation of multiple receptors that link to AC. Moreover, the reduced β-adrenoceptor responsiveness would occur in all the blood vessels from SHR. Therefore, the aim of the present study was two fold: (1) to determine whether the reduced β-adrenoceptor responses can be observed in other arteries than the femoral artery, and (2) to compare relaxant responses due to activation of A_2-adenosine, H_2-histamine and D_1-dopamine receptors in the arteries from normotensive Wistar-Kyoto rats (WKY) and SHR.

Methods

Preparation of arterial strips for recordings of mechanical activity

Male SHR, 13 weeks of age, and age-matched male WKY were used (Asano et al., 1988a). The rats were stunned and exsanguinated. The femoral artery (0.7–0.9 mm outside diameter), distal portion of the superior mesenteric artery (0.7–0.9 mm o.d.), renal artery (0.4–0.6 mm o.d.) and carotid artery (0.9–1.1 mm o.d.) were quickly dissected and placed in the Krebs-bicarbonate solution of the following composition (in mM): NaCl 115.0, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and dextrose 10.0. Helical strips (0.8 mm in width and 7 mm in length) of these arteries were prepared according to the method described previously (Asano et al., 1988a). The size of the strips of renal arteries was somewhat smaller (0.6 mm × 5 mm). To avoid the possible effects of the endothelium-derived relaxing factor, the endothelium of the strip was removed (Asano et al., 1988a).

Arterial strips were mounted vertically between hooks in water-jacketed (37 ± 0.5°C) muscle baths containing 20 ml of the Krebs-bicarbonate solution. Muscle bath solutions were maintained at 37 ± 0.5°C and continuously bubbled with a mixture of 95% O_2 and 5% CO_2. The hook anchoring the upper end of the strip was connected to the lever of a force-displacement transducer (TB-612T, Nihon Kohden Kogyo Co., Tokyo, Japan) for isometric tension recording. The strips were stretched passively to optimal length by imposing the optimal resting tension (femoral, 0.6 g; mesenteric, 0.5 g; renal, 0.3 g; carotid, 0.4 g). The length-passive tension studies failed to demonstrate differences in the resting tension between the strips from WKY and SHR.

After the 90 min equilibration period, the submaximally effective concentration of K^+ (30 mM) was administered two or three times at 40 min intervals until the responses were reproducible. At the final response, 60 mM K^+ was cumulatively added to obtain the maximum contraction of the strip.

Relaxation of arterial strips

To compare agonists that relax blood vessels, arterial strips were contracted with K^+ to an equivalent magnitude of tension (85% of the maximum contraction developed by 60 mM K^+) before challenge with the agonists (Asano et al., 1988a). These contractions induced by ED_{85} K^+ were well sustained for at least 3 h. Cumulative dose-response curves for the arterial relaxant responses to agonists were determined by producing a stepwise increase in the concentration of the agonist as soon as a stable response to the preceding dose had been obtained. At the end of experiments, 10^{-4} M papaverine was added to obtain the maximum relaxation of the strip. In some experiments, strips were contracted to 70% of the maximum contraction developed by 60 mM K^+ before challenge with the agonists.

All the experiments were conducted in phenoxybenzamine (Pbz)-treated strips to eliminate possible α-adrenoceptor responses. Strips were treated with 2×10^{-6} M Pbz during the first 60 min of the 90 min equilibration period. Using these Pbz-treated strips, relaxant responses to NA via β-adrenoceptors could be consistently demonstrated (Asano et al., 1982; 1988a).

Statistical analysis

When assessing the ED_{50} value, responses to agonists were calculated as % of the maximum response obtained with each agonist. The ED_{50} value was obtained visually from a plot of % response vs log

concentration of the agonist and expressed as a negative log (pD_2 value).

Unless specified, results shown in the text and figures are expressed as the mean value ± s.e. (n = number of preparations). Statistical analysis of the data was done by Student's t test for unpaired data, or by completely randomized design, one-way analysis of variance followed by Newman-Keuls test for a significant F ratio ($P < 0.05$), depending on which test was statistically appropriate. Two groups of data were considered to be significantly different when $P < 0.05$.

Drugs and chemicals

The following drugs were used: (−)-noradrenaline bitartrate (NA; Sigma Chemical Co., St. Louis, MO), adenosine (Sigma), 5'-N-ethylcarboxamide adenosine (NECA; Sigma), histamine dihydrochloride (Wako Pure Chemical Industries, Osaka, Japan), dopamine hydrochloride (Sigma), forskolin (Nippon Kayaku Co., Tokyo, Japan), phenoxybenzamine hydrochloride (Pbz; Nakarai Chemicals, Kyoto, Japan), timolol maleate (Banyu Pharmaceutical Co., Tokyo, Japan), cimetidine (Fujisawa Pharmaceutical Industries, Osaka, Japan), pyrilamine maleate (Sigma), SCH 23390 maleate ((R)-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol; Schering Corp., Kenilworth, NJ), sodium nitroprusside dihydrate (Wako), nifedipine (Bayer Yakuhin Ltd., Osaka, Japan) and papaverine hydrochloride (Wako).

Stock solutions of Pbz (10^{-3} M) and forskolin (10^{-3} M) were prepared using 50% ethanol with further dilution in distilled water before use. Nifedipine was dissolved in 99.5% ethanol to make a stock solution of 10^{-3} M with further dilution in distilled water before use. NA, histamine and dopamine were prepared daily in Krebs-bicarbonate solution and kept on ice during the course of the experiment. Aqueous stock solutions were prepared for other drugs. Concentrations of drugs are expressed as final molar concentrations in the muscle bath.

Results

Relaxation of arterial strips induced by NA and forskolin

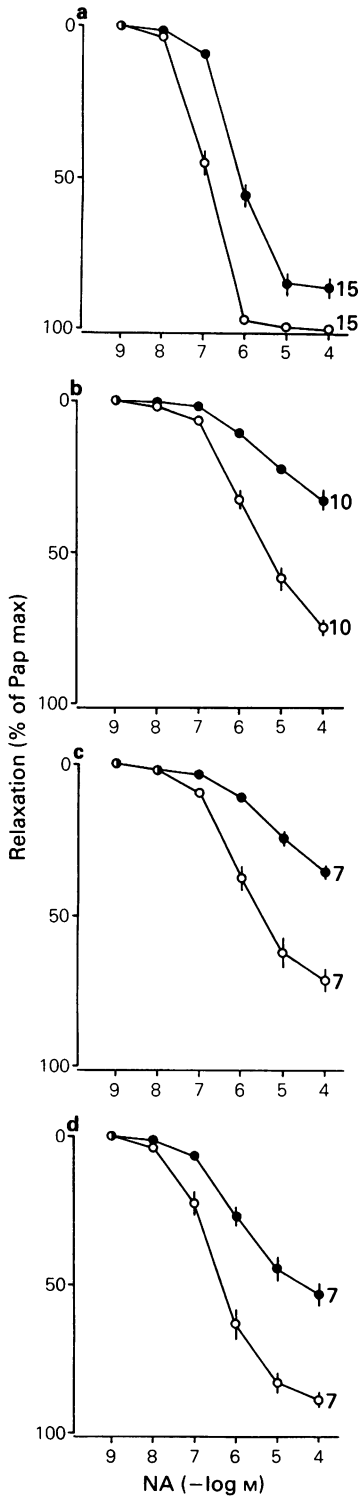
Relaxant responses to NA were first compared between the Pbz-treated, K^+-contracted strips of femoral arteries from WKY and SHR. The addition of 17.5 ± 0.6 mM K^+ ($n = 15$) to a strip of the WKY femoral artery caused a sustained contraction which was approximately 85% of the maximum contraction developed by 60 mM K^+ (Figure 1a). On the other hand, for an equivalent contraction to be observed in a SHR strip, 12.7 ± 0.8 mM K^+ ($n = 15$) was required (Figure 1a). The addition of NA (10^{-9}–10^{-4} M) produced a dose-dependent relaxation in Pbz-treated, ED_{85} K^+-contracted strips of femoral arteries from both strains (Figure 1a). These relaxations were dose-dependently antagonized by 5×10^{-8} and 5×10^{-7} M timolol, confirming that the relaxation induced by NA is due to the activation of β-adrenoceptors. The relaxant responses of femoral arteries to NA were significantly weaker in the SHR than in the WKY (Figure 1a). When the relaxant responses to NA were determined in Pbz-treated, ED_{85} K^+-contracted strips of mesenteric, renal and carotid arteries, there was a decreased responsiveness to NA in the three arteries from SHR when compared with the respective arteries from WKY (Figure 1b,c,d). The relaxant responses to NA in the mesenteric, renal and carotid arteries were significantly weaker than the responses in the femoral arteries. The pD_2 value for NA-induced relaxation in the WKY femoral artery (6.87 ± 0.06, $n = 15$) was significantly different from the pD_2 value in other WKY arteries. The maximum relaxant response to NA in the WKY femoral artery (99.2 ± 0.2%, $n = 15$) was significantly greater than the maximum response in other WKY arteries. Similar results were also obtained in the SHR arteries (Figure 1).

Similar experiments were performed on the relaxant responses to forskolin in the femoral, mesenteric, renal and carotid arteries. The addition of forskolin (3×10^{-9}–1×10^{-6} M) produced a dose-dependent relaxation in the Pbz-treated, ED_{85} K^+-contracted strips of these arteries (Figure 2). Under the same experimental conditions as those used to obtain NA-induced relaxation, the relaxant responses to forskolin were not significantly different between the WKY and SHR (Figure 2). Moreover, the relaxant responses to forskolin were not significantly different between the four arteries (Figure 2).

Relaxation of arterial strips due to activation of A_2-adenosine, H_2-histamine and D_1-dopamine receptors

Relaxant responses to either adenosine or NECA were compared between the Pbz-treated, ED_{70} K^+-contracted strips of femoral arteries from WKY and SHR (Figure 3a,b). For an equivalent 75% contraction to be observed in the femoral arteries, 15.2 ± 0.5 ($n = 6$) and 10.8 ± 0.7 ($n = 6$) mM K^+ were required for WKY and SHR, respectively. Both agonists produced a dose-dependent relaxation in these strips. However, the relaxant responses to the two agonists were significantly weaker in the SHR than in the WKY (Figure 3a,b).

Relaxant responses to histamine were determined in the presence of 1×10^{-6} M pyrilamine and



5×10^{-7} M timolol in Pbz-treated, ED_{70} K^+-contracted strips of femoral arteries (Figure 3c). Histamine produced a dose-dependent relaxation in these strips. The maximum relaxation was obtained when 1×10^{-4} M histamine was added, and a higher concentration of this agonist (3×10^{-4} M) produced a significant contraction. These relaxations were diminished by 1×10^{-4} M cimetidine, an H_2-histamine receptor antagonist, suggesting that histamine produces the relaxation through the activation of H_2-histamine receptors. The extent of relaxation induced by histamine was significantly weaker in the SHR than in the WKY (Figure 3c).

Relaxant responses to dopamine were determined in Pbz-treated, ED_{70} K^+-contracted strips of renal arteries, because renal arteries have been demonstrated to possess vasodilator D_1-dopamine receptors (Goldberg *et al.*, 1978; 1984; Alkahdi *et al.*, 1986). The relaxant responses to dopamine were significantly weaker in the SHR than in the WKY (Figure 3d). These relaxations were diminished by 1×10^{-7} M SCH 23390, a selective D_1-dopamine receptor antagonist (Iorio *et al.*, 1983; Goldberg *et al.*, 1984; Hilditch & Drew, 1985), suggesting that dopamine produces the relaxation through activation of D_1-dopamine receptors.

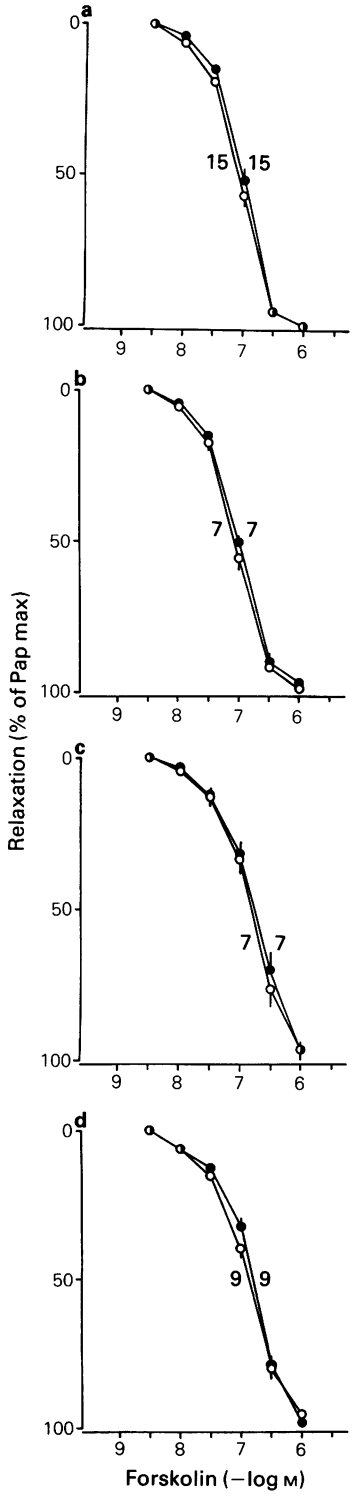
Relaxation of arterial strips induced by nitroprusside and nifedipine

The addition of either nitroprusside or nifedipine also produced a dose-dependent relaxation in the Pbz-treated, ED_{85} K^+-contracted strips of femoral arteries from WKY and SHR. Dose-response curves for the relaxation induced by either nitroprusside or nifedipine were not significantly different between the two strains (Figure 4).

Discussion

The present study evaluated the relaxant responses due to activation of a cyclic AMP-generating system in strips of several peripheral arteries isolated from

Figure 1 Dose-response curves for the relaxant responses to noradrenaline (NA) determined after precontraction with ED_{85} K^+ in phenoxybenzamine-treated strips of femoral (a), mesenteric (b), renal (c) and carotid (d) arteries isolated from 13 week-old WKY (○) and age-matched SHR (●). ED_{85} K^+ = 17.5 and 12.7 mM in (a), 18.2 and 20.3 mM in (b), 22.2 and 21.6 mM in (c), and 19.2 and 16.0 mM in (d) for WKY and SHR, respectively. Relaxation induced by 10^{-4} M papaverine (Pap) was taken as 100%. Vertical lines represent s.e. Numbers beside the dose-response curves indicate the number of preparations used.



WKY and SHR. β-Adrenoceptor-mediated relaxations were decreased in SHR femoral, mesenteric, renal and carotid arteries when compared with the respective WKY arteries. The relaxant responses due to activation of A_2-adenosine, H_2-histamine and D_1-dopamine receptors were also decreased in SHR arteries. However, forskolin-induced relaxations did not differ between these arteries from WKY and SHR.

A consistent loss in responsiveness of arterial smooth muscle to β-adrenoceptor agonists has been demonstrated in SHR (Amer, 1973; Triner *et al.*, 1975; Klenerova *et al.*, 1975; Cohen & Berkowitz 1976; Sands *et al.*, 1976). However, the data were limited to the aorta. Decreased β-adrenoceptor responses were observed in all the arterial strips used in the present study. Thus, the β-adrenoceptor · AC system appears to be systemically impaired in SHR. Judging from the NA-induced relaxations (Figure 1), β-adrenoceptor activities were quite different between these arteries. On the other hand, the relaxant responses of these arteries to forskolin, an activator of AC, were not decreased in the SHR, suggesting that components of the β-adrenoceptor · AC system distal to and including AC are not responsible for the decreased responsiveness to multiple cyclic AMP-generating vasodilators. This suggestion is also supported by observations from our previous study (Asano *et al.*, 1988a) that the relaxant responses of femoral arteries to dibutyryl cyclic AMP were the same in WKY and SHR. Thus, the abnormality in SHR arteries is assumed to occur at the level of the β-adrenoceptor or Gs. Judging from the forskolin-induced relaxations (Figure 2), the activity of AC appears to be similar in the four arteries used.

Adenosine receptors are linked to the AC system in various tissues (for review, see Daly, 1985; Stiles, 1986). The studies of Burnstock and his colleagues demonstrated that the effects of adenosine and adenine nucleotides were mediated by two distinct purine receptors termed P_1 and P_2 (Burnstock, 1979). By using adenosine and its derivatives, Londos & Wolff (1977) obtained evidence supporting the existence of two distinct receptors that

Figure 2 Dose-response curves for the relaxant responses to forskolin determined after precontraction with ED_{85} K^+ in phenoxybenzamine-treated strips of femoral (a), mesenteric (b), renal (c) and carotid (d) arteries isolated from 13 week-old WKY (○) and age-matched SHR (●). ED_{85} K^+ = 18.1 and 12.9 mM in (a), 17.9 and 20.0 mM in (b), 22.0 and 21.3 mM in (c), and 20.1 and 15.4 mM in (d) for WKY and SHR, respectively. Relaxation induced by 10^{-4} M papaverine (Pap) was taken as 100%. Vertical lines represent s.e. Numbers beside the dose-response curves indicate the number of preparations used.

altered the activity of AC. Depending upon the type of cells, cellular effects of adenosine are mediated by two separate subtypes of P_1-receptor, termed A_1 (or Ri) and A_2 (or Ra) (Van Calker *et al.*, 1979; Londos *et al.*, 1980). By interacting with one of these two receptor subtypes, adenosine can initiate a transmembrane signal to either inhibit (A_1) or stimulate (A_2) the AC activity. Adenosine receptor subtypes can be defined by a unique agonist potency series of adenosine derivatives such that the A_2-adenosine receptors have a potency series of NECA > adenosine > N^6-phenylisopropyladenosine (PIA) while the reverse is true for the A_1-receptors (Brown & Collis, 1982; Mustafa & Askar, 1985; Stiles, 1986). NECA is 10 to 100 times more potent than adenosine at A_2-adenosine receptors (Brown & Collis, 1982; Edvinsson & Fredholm, 1983; Mustafa & Askar, 1985). In the present study of femoral arteries, NECA was approximately 30 times more potent than adenosine in producing the arterial relaxation, suggesting that the two agonists produce relaxation through activation of A_2-adenosine receptors.

Both H_1- and H_2-histamine receptors are located on smooth muscle of resistance vessels. In various smooth muscles, relaxations induced by histamine through the activation of H_2-histamine receptors are associated with a rise in cyclic AMP. In some smooth muscles, however, the H_2-receptor responses were mediated by the release of catecholamines from

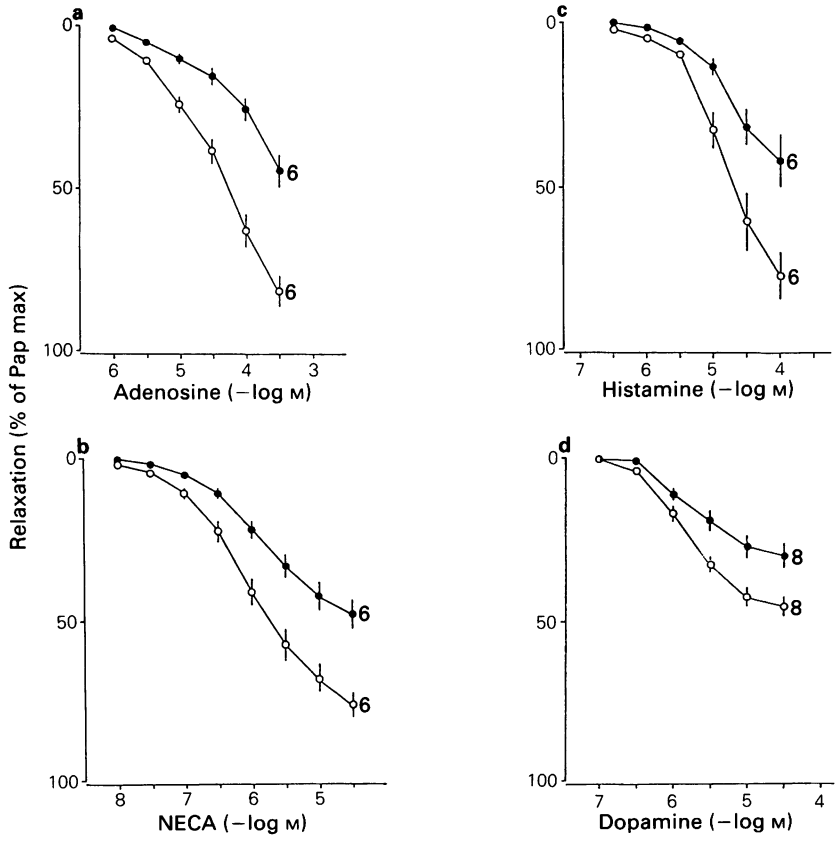


Figure 3 Dose-response curves for the relaxant responses to adenosine (a), 5'-N-ethylcarboxamide adenosine (NECA, b), histamine (c) and dopamine (d) in phenoxybenzamine-treated, ED_{70} K^+-contracted strips of femoral (a, b, c) and renal (d) arteries isolated from 13 week-old WKY (○) and age-matched SHR (●). The relaxant responses to histamine were determined in the presence of 1×10^{-6} M pyrilamine and 5×10^{-7} M timolol after precontraction with ED_{70} K^+. ED_{70} K^+ = 15.2 and 10.8 mM in (a), 14.6 and 10.2 mM in (b), 14.2 and 10.0 mM in (c) and 20.1 and 17.5 mM in (d) for WKY and SHR, respectively. Relaxation induced by 10^{-4} M papaverine (Pap) was taken as 100%. Vertical lines represent s.e. Numbers beside the dose-response curves indicate the number of preparations used.

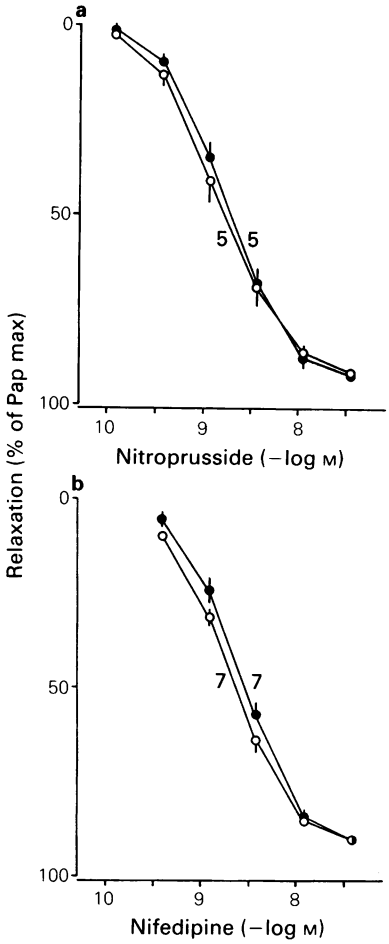


Figure 4 Dose-response curves for the relaxant responses to nitroprusside (a) and nifedipine (b) in phenoxybenzamine-treated, ED_{85} K^+-contracted strips of femoral arteries isolated from 13 week-old WKY (○) and age-matched SHR (●). ED_{85} K^+ = 18.0 and 12.7 mM in (a), and 18.2 and 13.0 mM in (b) for WKY and SHR, respectively. Relaxation induced by 10^{-4} M papaverine (Pap) was taken as 100%. Vertical lines represent s.e. Numbers beside the dose-response curves indicate the number of preparations used.

sympathetic nerve endings (for review, see Bolton, 1979). Moreover, histamine has also been demonstrated to produce arterial relaxation through the activation of H_1-histamine receptors in the endothelium (Toda, 1986). Therefore, to exclude these possibilities, the relaxant responses to histamine in the present study were conducted in Pbz-treated, endothelium-removed strips of femoral arteries in the presence of timolol and pyrilamine. Under these conditions, relaxant responses through the activation of smooth muscle H_2-histamine receptors could be consistently demonstrated.

Peripheral dopamine receptors are subdivided into D_1 and D_2 subtypes, primarily on the basis of agonist and antagonist profiles of action (Goldberg *et al.*, 1978; 1984; Hilditch & Drew, 1985). The D_1-dopamine receptors produce smooth muscle relaxation including vasodilatation in the renal, mesenteric, coronary and cerebral vascular beds, and these relaxations are selectively antagonized by SCH 23390. The activation of D_1-receptors in vascular blood vessels leads to an increase in the AC activity (Alkadhi *et al.*, 1986). On the other hand, D_2-receptors inhibit the release of NA from sympathetic nerve endings, and these effects are selectively antagonized by domperidone. In the present study of renal arteries, the dopamine-induced relaxations were antagonized by SCH 23390, suggesting that dopamine was acting on D_1-receptors.

The relaxant responses to the stimulation of cyclic AMP-generating receptors, including β-adrenoceptors, A_2-adenosine, H_2-histamine and D_1-dopamine receptors, were decreased in SHR arteries. On the other hand, relaxant responses to nitroprusside and nifedipine, agents which are independent of the Gs · AC system, were not decreased in SHR, suggesting that the decreased responsiveness of SHR arteries is specific for the receptor agonists whose mechanism of action involves AC activation.

Recent studies from this laboratory (Asano *et al.*, 1988b) have provided evidence suggesting that a reduced function of Gs is involved in the decreased β-adrenoceptor responses in the SHR femoral artery. In these studies (Asano *et al.*, 1988b) the inhibitory effects of cholera toxin, an activator of Gs (Northup *et al.*, 1980), on the contractile responses to α-adrenoceptor stimulation with NA were compared with the contractile responses to NA determined in the absence of β-adrenoceptor antagonists. When the contractile responses of femoral arteries to NA were determined in the absence of timolol, we observed that the responses consisted of α-adrenoceptor-mediated contractions and β-adrenoceptor-mediated relaxations. The interaction between α- and β-adrenoceptors was quite different between WKY and SHR. On the other hand, the ability of cholera toxin to antagonize the α-adrenoceptor-mediated contractions was weaker in SHR than in WKY (Asano *et al.*, 1988b). The dose-response curve for NA determined in the absence of timolol was in good agreement with the dose-response curve for α-adrenoceptor-mediated contractions determined after the pretreatment with cholera toxin in either WKY or SHR. These results strongly suggest that the difference at the level of Gs between WKY and SHR reflected the difference at the level of β-adrenoceptors. If both Gs and β-adrenoceptors are

assumed to be impaired, such agreement of the dose-response curves cannot be expected, because the activation by NA of β-adrenoceptors contains two components and the activation by cholera toxin of Gs contains one component. For these reasons, we proposed that a reduced function of Gs is responsible for the decreased β-adrenoceptor responsitveness in the SHR femoral artery (Asano *et al.*, 1988b). Since the Gs and AC are common to multiple cyclic AMP-generating receptors, the reduced function of Gs could lead to resistance to the activation of A_2-adenosine, H_2-histamine and D_1-dopamine receptors. The results of the present study support this hypothesis.

Although radioligand bindings for each receptor were not actually measured, it is concluded that a reduced function of Gs is responsible for the decreased arterial responsiveness to multiple cyclic AMP-generating receptor agonists in SHR. The present pharmacological approach to the Gs·AC system in SHR arteries needs to be strengthened by the biochemical analysis of Gs and receptor binding studies in the same tissues, and this is the subject of our current investigations.

Generous gifts of forskolin (Nippon Kayaku), SCH 23390 (Schering Corp.) cimetidine (Fujisawa Pharmaceutical), nifedipine (Bayer Yakuhin) and timolol (Banyu Pharmaceutical) are greatly acknowledged. This work was supported in part by a Grant-in-Aid for Scientific Research (No. 62570401) from the Ministry of Education, Science and Culture, Japan, and grants from The Research Foundation for Oriental Medicine and from The Tokai Science Academy.

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Reduction of food intake by central administration of cholecystokinin octapeptide in the rat is dependent upon inhibition of brain peptidases

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1 The effects of intracerebroventricular (i.c.v.) injections of cholecystokinin-octapeptide (CCK-8) and caerulein, an amphibian decapeptide structurally related to CCK-8, are inconsistent in the rat. We have therefore investigated the possibility that enzymatic degradation could be responsible for the lack of activity of CCK-8 seen in some studies on food intake.

2 Injections of CCK-8 at doses of 2.5 nmol and 25 nmol into the lateral cerebral ventricle of rats did not reduce the intake of a highly palatable diet whereas injections of the same doses of caerulein reduced food intake potently and dose-dependently.

3 Co-administration of CCK-8 with a combination of the peptidase inhibitors bestatin (70 nmol), captopril (100 nmol) and thiorphan (120 nmol) resulted in an inhibition of feeding similar to that seen after the injection of caerulein alone. The peptidase inhibitors alone did not affect food intake.

4 When caerulein was injected i.c.v. in combination with bestatin, captopril and thiorphan the effect of caerulein was potentiated, suggesting that enzymatic breakdown of caerulein does occur.

5 It is concluded that the effect of centrally administered CCK-8 on food intake is dependent on the activity of cleaving enzymes in the brain. It is emphasized that the action of brain peptidases is a major factor which has to be considered when investigating the role of peptides in the central nervous system.

Introduction

The gut peptide cholecystokinin (CCK) has been shown to be present in the brain in considerable amounts (Rehfeld, 1978; Larsson & Rehfeld, 1979; Beinfeld & Palkovits, 1981; 1982) and to be synthesized *in vivo* in rat cortex (Goltermann *et al.*, 1980). Of the several different fragments of CCK, the octapeptide (CCK-8) is believed to be the predominant form in the brain (Rehfeld, 1978; Larsson & Rehfeld, 1979). CCK-8 has been found in cell bodies, neurones and nerve terminals (Pinget *et al.*, 1978; Innis *et al.*, 1979; Vanderhaeghen *et al.*, 1980), and specific CCK-receptors have been demonstrated in discrete areas of the brain (Zarbin *et al.*, 1983; Van Dijk *et al.*, 1984; Day *et al.*, 1986) including a number of regions that are considered to be involved in the regulation of feeding behaviour. Schick *et al.* (1987) have shown that neuronal CCK-like immunoreactivity is released postprandially from primate hypothalamus.

In the rat results of studies in which CCK-8 or caerulein, a decapeptide structurally related to CCK-8, were administered intracerebroventricularly (i.c.v.) show some inconsistencies. There are studies in which CCK-8 administered by i.c.v. injection had no demonstrable effect on food intake (Della-Fera & Baile, 1979; Grinker *et al.*, 1980; Woods *et al.*, 1981; Smith *et al.*, 1984), whereas other workers have shown that central administration of CCK-8 does result in a reduction in food intake although the doses required were higher than those which were active peripherally (Nemeroff *et al.*, 1978; Lorenz & Goldman, 1982; Makovec *et al.*, 1986; Schick *et al.*, 1986).

The aim of the present study was to investigate whether the co-injection of peptidase inhibitors

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enhanced the ability of CCK-8 to reduce food intake following central administration.

Methods

Surgery

Male Hooded Lister rats (250–300 g initial weight, Olac, Bicester, U.K.) were anaesthetized with diazepam (Dialar, Lagap Pharmaceuticals Ltd., U.K.; 2.5 mg kg^{-1}, i.p.) and Hypnorm (fentanyl citrate 0.315 mg ml^{-1} and fluanisone 10 mg ml^{-1}, Janssen Pharmaceuticals Ltd., U.K.; 0.3 ml, i.m.) and placed in a stereotaxic frame with the incisor bar fixed at −3.3 mm. A 22 gauge stainless steel guide cannula (Plastic Products Comp., Roanoke, VA, U.S.A.) was aimed at a lateral ventricle with the tip of the cannula 8.2 mm anterior, 1.4 mm lateral and 6.6 mm dorsal to the midpoint of the interaural line according to the atlas of Paxinos & Watson (1986). The cannula was anchored to the skull with three stainless steel screws (10 BA) and acrylic cement (Howmedica Int. Ltd., U.K.) and sealed with an obturator pin. After surgery the animals were injected i.m. prophylactically with 0.2 ml Streptopen (Glaxovet Ltd., U.K.) and were given an i.p. injection of 5 ml 0.9% (w/v) NaCl and 5% (w/v) dextrose. A recovery period of at least seven days following surgery was allowed before experiments were begun.

Experimental procedure

For the first experiment the animals were randomly assigned to three experimental groups. Experimental groups for each of the subsequent experiments were formed in a way that provided equal means and variances of food intake on the day before the experiment. Immediately before injections the normal rat chow (CRM, Labsure, U.K.) and drinking water were removed from the cages. Intracerebroventricular injections were made in a volume of 20 μl over a period of 1 min through a 28 gauge stainless steel cannula (Plastic Products Comp., U.S.A.) which protruded 1 mm beyond the tip of the guide cannula. The volume for intraperitoneal injections was 1 ml kg^{-1}. Immediately after i.c.v. injections or 5 min after i.p. injections the animals were given a highly palatable diet similar to that described by Cooper *et al.* (1985) consisting of 100 ml sweetened condensed milk (Nestlé, U.K.), 300 ml tap water and 400 g powdered rat chow (CRM, Labsure, U.K.) in perspex Petri dishes. Intake of this palatable diet was then determined after a period of 30 min at which time the palatable diet was removed and the normal food and water returned to the cages. A training period of 5 days was used to accustom the animals to this procedure before the experiments were started. This feeding paradigm was used for all experiments and was carried out also on the days between the experiments for 5 days per week. All the experiments were performed between 13 h 00 min and 15 h 00 min. Animals were kept for a period of 5 weeks following cannula implantation, after which the correct placement of the cannula was verified by i.c.v. injection of 20 μl 2.5% (w/v) pontamine sky blue (BDH Chemicals Ltd., U.K.) in sodium acetate buffer (pH = 5.6).

Substances

Stock solutions of 1.25 mM cholecystokinin octapeptide (CCK-8; Cambridge Research Biochemicals, U.K.) were made up in 150 mM NaCl plus 5 mM $NaHCO_3$ or in 50 mM $NaHCO_3$. Caerulein (Bachem, U.S.A.) was dissolved at the same concentration in 50 mM $NaHCO_3$. When these peptides were used in combination with captopril (Squibb Pharma, U.S.A.), bestatin (Sigma Chem. Comp., U.S.A.) and thiorphan (Peninsula Labs. Europe, U.K.), the enzyme inhibitors were added sequentially to 50 mM $NaHCO_3$ or to the stock solution of each of the peptides to achieve final concentrations of 6 mM, 3.5 mM and 5 mM, respectively. For the intraperitoneal injections the stock solution of CCK-8 was diluted with 150 mM NaCl to concentrations of 10 μM and 1 μM.

Statistical analysis

All values presented represent means ± s.e.mean. Two-way analysis of variance followed by the Student-Newman-Keuls test was used to analyse the results of the i.c.v. injections of caerulein in the absence and presence of the peptidase inhibitors. All the other experiments were analysed separately by one-way analysis of variance followed by Dunnett's *t* test (two-tailed), because these experiments were carried out in different colonies of animals at different stages of their palatable diet training and in the case of CCK-8 with different vehicle solutions.

Results

Cholecystokinin octapeptide

CCK-8 was injected into a lateral cerebral ventricle at doses of 2.5 nmol and 25 nmol (Figure 1a). Although one-way analysis of variance showed a sig-

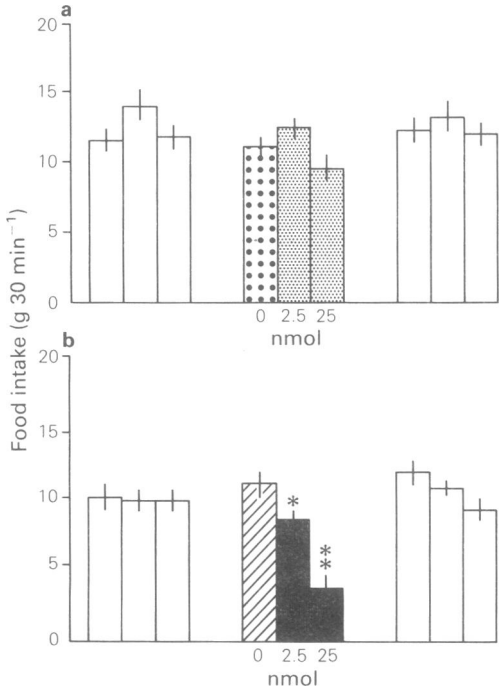


Figure 1 Effect of i.c.v. injections of cholecystokinin-octapeptide (CCK-8) on food intake: (a) on the day of the experiment rats received i.c.v. injections of CCK-8 at doses of 2.5 nmol and 25 nmol (stippled columns), or of the vehicle (150 mM NaCl plus 5 mM $NaHCO_3$, dotted column). The injection volume was 20 μl. Immediately after the injections the animals were presented with a palatable diet. No significant differences in 30 min food intake (g) were found between the treatment groups and the vehicle control group. (b) Rats received i.c.v. injections of CCK-8 at doses of 2.5 nmol and 25 nmol (solid columns), or of the vehicle (50 nM $NaHCO_3$ + 6 mM captopril + 3.5 mM bestatin + 5 mM thiorphan, hatched column). In the presence of the peptidase inhibitors CCK-8 reduced food intake in a dose-dependent manner: * $P < 0.05$, ** $P < 0.01$; significantly different from vehicle control group (Dunnett's t test).

Open columns show food intake on the day before (left) and on the day after the experiment (right) when no injections were given. In the group which had received 25 nmol CCK-8, food intake on the day after the experiment was slightly, but significantly ($P < 0.05$), lower than that of the group of animals which had served as the control group. Columns represent mean values, $n = 9$–11; vertical lines show s.e.mean.

nificant difference in food intake between the three groups ($\hat{F}_{(2;27)} = 1.3998$, $P < 0.05$), no significant differences could be found comparing the treatment groups with the vehicle control group.

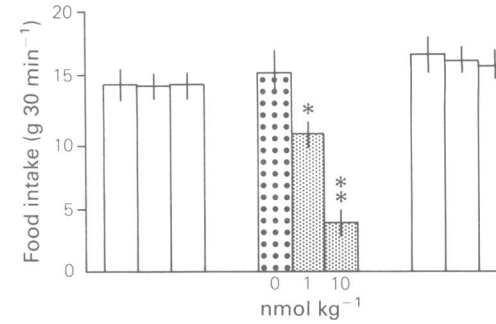


Figure 2 Effect of i.p. injections of cholecystokinin-octapeptide (CCK-8) on food intake: Rats were assigned to three groups according to their food intake on the day before the experiment so that the groups showed equal mean values and variances of 30 min food intake (open columns, left). On the day of the experiment the animals were injected i.p. with CCK-8 at doses of 1 nmol kg^{-1} and 10 nmol kg^{-1} (stippled columns), or with the vehicle (150 mM NaCl, dotted column) 5 min before presentation of food. CCK-8 reduced 30 min food intake (g) in a dose-dependent manner. Significance of difference to vehicle control group: * $P < 0.05$, ** $P < 0.01$ (Dunnett's t test). The open columns on the right show food intake on the day after the experiment. Columns represent mean values, $n = 10$; vertical lines show s.e.mean.

To verify the integrity of the CCK-8 solution, the same stock solution of the peptide was diluted and used for intraperitoneal injections in the same colony of animals (Figure 2). The inhibition of feeding ($\hat{F}_{(2;27)} = 22.372$, $P < 0.001$) was significant at dose levels of 1 nmol kg^{-1} and 10 nmol kg^{-1} ($P < 0.05$ and $P < 0.01$, respectively).

When CCK-8 was injected i.c.v. in combination with captopril, bestatin and thiorphan (Figure 1b), a significant reduction of food intake ($\hat{F}_{(2;27)} = 26.651$, $P < 0.001$) was observed at dose levels of 2.5 nmol ($P < 0.05$) and 25 nmol ($P < 0.01$). On the day after the experiment food intake of the three groups did not return to equal values ($\hat{F}_{(2;27)} = 3.8975$, $P < 0.05$), i.e. the group of animals that had received the high dose of CCK-8 on the day before still showed a slightly, but significantly, lower food intake than the rats from the control group ($P < 0.05$).

Caerulein

Significant reductions in food intake ($\hat{F}_{(2;24)} = 47.675$, $P < 0.001$) were observed following intracerebroventricular injections of caerulein (Figure 3a) at doses of 2.5 nmol and 25 nmol ($P < 0.01$,

Dunnett's t test). The high dose of caerulein almost abolished feeding during the test period of 30 min after the injection.

When caerulein (2.5 nmol) was injected i.c.v. in combination with captopril, bestatin and thiorphan (Figure 3b) the reduction in food intake was greater than that observed when the same dose of caerulein was given alone ($P < 0.01$, Student-Newman-Keuls test). Two-way analysis of variance indicates a statistically significant interaction between the caerulein and the peptidase inhibitors ($\hat{F}_{(1;20)} = 4.5571$; $P < 0.05$).

Discussion

Although several investigators (Della-Fera & Baile, 1979; Grinker *et al.*, 1980; Woods *et al.*, 1981; Smith *et al.*, 1984) have failed to demonstrate an action of centrally administered CCK-8 on feeding behaviour in the rat, these results can possibly be explained by the findings of other workers that higher doses of CCK-8 are necessary to inhibit feeding following i.c.v. injection than with intraperitoneal administration (Nemeroff *et al.*, 1978; Lorenz & Goldman, 1982; Makovec *et al.*, 1986; Schick *et al.*, 1986). Results of these studies are not easily comparable because of the diversity of experimental procedures used, particularly regarding the length of periods of food deprivation and palatability of the test meals. Furthermore, variations between different strains of animals cannot be excluded.

In the present study large doses of CCK-8 (25 nmol) had no effect on food intake after i.c.v. administration (Figure 1a), whereas caerulein reduced food intake potently and dose-dependently (Figure 3a). It is noteworthy that caerulein in a dose of 2.5 nmol (approximately 2.9 μg) decreased feeding significantly ($P < 0.01$), whereas Smith *et al.* (1984) did not see an alteration in food intake even at a dose of 4 μg. To verify the integrity of the stock solution of CCK-8, the same solution which was used for the i.c.v. injections was diluted with 150 mM NaCl and injected intraperitoneally (Figure 2). The potency of CCK-8 injected by this route was similar to that reported for intraperitoneal injections by Makovec *et al.* (1986).

Drugs injected into the cerebral ventricles have to reach the sites of their action in the brain by diffusion and may be susceptible to enzymatic degradation. CCK-8 has been shown to be broken down *in vitro* by crude synaptosomal fractions of rat brain with degrading activity present both in the cytosol and the synaptic membranes (Deschodt-Lanckman *et al.*, 1981). The inactivation of CCK-8 is likely to be the result of the action of several different peptidases. In synaptic membranes at least four cleaving

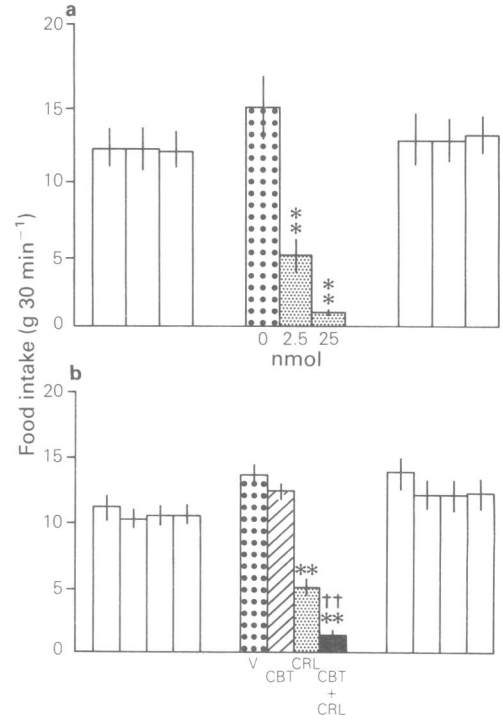


Figure 3 Reduction in food intake induced by i.c.v. injections of caerulein: (a) Immediately before presentation of the palatable diet rats were injected i.c.v. with caerulein at doses of 2.5 nmol and 25 nmol (stippled columns), or of the vehicle (50 mM $NaHCO_3$, dotted column). The injection volume was 20 μl; 30 min food intake (g) was reduced significantly and dose-dependently by caerulein. (b) Effect of peptidase inhibitors on the reduction in 30 min food intake induced by i.c.v. injections of caerulein: rats were injected i.c.v. with 2.5 nmol caerulein (CRL) either alone or together with 100 nmol captopril + 70 nmol bestatin + 120 nmol thiorphan (CBT). Control groups received i.c.v. injections of the enzyme inhibitors alone or of the vehicle (50 mM $NaHCO_3$, V, dotted column). The reduction of food intake induced by caerulein (stippled column) was potentiated by co-injection of the peptidase inhibitors (solid column). The inhibitors injected alone (hatched column) did not affect food intake. ** $P < 0.01$, significantly different from the respective control group; †† $P < 0.01$, significantly different from caerulein alone. Open columns show food intake on the day before and on the day after the experiments. Columns represent mean values, $n = 6$–10; vertical lines show s.e.mean.

enzymes have been identified: two aminopeptidases with tryptophan- and tyrosin-releasing activities which are also involved in the cleavage of enkephalins (Deschodt-Lanckman & Bui, 1981), a thiol protease which is capable of removing the N-terminal Asp in CCK-8, thus producing CCK-7 (Deschodt-Lanckman *et al.*, 1983), and a metallo-

endopeptidase which may be identical to 'enkephalinase A' (Deschodt-Lanckman & Strosberg, 1983) which can cleave CCK-8 at different bonds (Deschodt-Lanckman *et al.*, 1984; Matsas *et al.*, 1984).

Bestatin is a specific inhibitor for various aminopeptidases such as aminopeptidase B and leucine aminopeptidase (Suda *et al.*, 1976) or aminotripeptidase (Hayashi & Oshima, 1980). Captopril is well known as an inhibitor of angiotensin-converting enzyme (Rubin *et al.*, 1978), a peptidyldipeptidehydrolase which is also present in small amounts in rat brain (Cushman & Cheung, 1971) and in the cerebrospinal fluid of cats (Whalley & Wahl, 1988). Thiorphan has been designed as a specific inhibitor for enkephalinase by Llorens *et al.* (1980). A combination of these three enzyme inhibitors was used by McKnight *et al.* (1983) to increase the potency of opioid peptides *in vitro*. Similarly, this mixture of enzyme inhibitors has been shown to enhance the antinociceptive effect of i.c.v. injections of CCK-8 in the mouse (Hill *et al.*, 1987). We have therefore investigated the possibility that this same combination of bestatin, captopril and thiorphan might increase the potency of i.c.v. injected CCK-8 in our feeding study.

Indeed, co-injection of these peptidase inhibitors together with CCK-8 revealed clear, dose-dependent reduction of food-intake (Figure 1b). However, the action of CCK-8 was still not as potent as that of caerulein alone (Figure 3a), possibly due to incomplete inhibition of the enzymatic degradation of CCK-8 by the enzyme inhibitors used in this study. The peptidase inhibitors also potentiated the effect of i.c.v. injections of caerulein (Figure 3b), indicating that enzymatic breakdown of caerulein does occur. The slight differences in food intake on the day after the experiment in which CCK-8 in combination with bestatin, captopril and thiorphan was used (Figure 1b) do not seem to be of significance since no such differences were observed on the day after the i.c.v. injections of caerulein in the absence and presence of these enzyme inhibitors (Figure 3b). Caerulein has previously been reported to reduce food intake more potently than CCK-8 after peripheral administration in the rabbit (Houpt *et al.*, 1978), in the mouse (Zetler & Mörsdorf, 1984) and in man (Stacher *et al.*, 1982a,b). Similar differences in the potency of caerulein and CCK-8 following peripheral administration have also been demonstrated for analgesia and ptosis (Zetler, 1980) and sedative and anticonvulsive effects (Zetler, 1982) in mice. Jurna & Zetler (1981) also reported a difference in antinociceptive potency of centrally administered caerulein and CCK-8. Both peptides apparently bind to the same receptors with similar affinity (Innis & Snyder, 1980; Van Dijk *et al.*, 1984; Fujimoto *et al.*, 1985) but caerulein has been shown to be more resistant to enzymatic degradation than CCK-8 (Deschodt-Lanckman *et al.*, 1981). In the present study we have seen that after central administration, caerulein is more potent than CCK-8 in reducing food intake both in the absence and in the presence of enzyme inhibitors (compare Figures 1 and 3). There is no information available to show to what extent the mixture of enzyme inhibitors inhibits the degradation of CCK-related peptides *in vivo*. Given the multitude of enzymes capable of cleaving CCK it seems possible that the inhibitors may not block enzymatic breakdown completely.

Although the receptors mediating the satiety effect of peripherally injected CCK-related peptides are assumed to be located on vagal neurones (Zarbin *et al.*, 1981) rather than on non-neuronal cells as e.g. in the gall bladder or the pancreas (Yau *et al.*, 1973; Williams *et al.*, 1981), this effect seems to be mediated by the 'peripheral' receptor subtype since it could be inhibited effectively by CR 1409 (Makovec *et al.*, 1986) and by L-364,718 (Hewson *et al.*, 1988), whereas centrally administered CCK-8 could not be blocked by CR 1409 (Makovec *et al.*, 1986). Barthó *et al.* (1987) have pointed out that the sensitivity of CCK-receptors found outside the CNS is independent of the type of cells on which they are located. Several investigators (Innis & Snyder, 1980; Van Dijk *et al.*, 1984; Fujimoto *et al.*, 1985) have shown that the affinity of both CCK-8 and caerulein for the peripheral receptor is, if anything, only slightly higher than that for the central receptor. The finding that CCK-8 is active at much lower doses when injected i.p. may therefore indicate that enzymatic degradation possibly plays a greater role in the termination of the activity of CCK-8 in the central nervous system than it does in the periphery. An attempt to enhance the effects of peripheral administration of CCK-8 or caerulein by co-injection of bestatin, captopril and thiorphan has not been made since a great amount of these peptidase inhibitors would be necessary to perform this experiment.

Our present results show that the effect of centrally administered CCK-8 is dependent on the activity of cleaving enzymes in the brain. Different or apparently contradictory results of studies on the central role of CCK-8 may therefore reflect varying levels of activity of cleaving enzymes in the animals used by the investigators. Caerulein may be preferable to CCK-8 when administered centrally because of its greater resistance to enzymatic degradation. It is emphasized that the action of brain peptidases is a major factor which has to be considered when investigating the role of peptides in the central nervous system.

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Effect of endothelium removal on stimulatory and inhibitory modulation of rat aortic prostacyclin synthesis

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1 Removal of the endothelium (DE) enhanced the *in vitro* release of prostacyclin (PGI_2) by rat aortae in response to adrenaline (Ad), noradrenaline (NA), thromboxane A_2 analogue U46619, phorbol dibutyrate (PDBU) and sodium fluoride (NaF) when assessed at 3 h post DE. At 6 h post DE, there were no differences between the dose-response curves obtained from aortic rings with or without endothelium.

2 At 3 h post DE the antagonism of Ad- and NA-stimulated PGI_2 synthesis by yohimbine and prazosin, and NA-stimulated PGI_2 synthesis by nifedipine was markedly reduced in aortae without eudothelium when compared with controls. These effects were reversed by protracted incubation of aortic tissue post DE (6 h and 9 h).

3 Acetylcholine, carbachol, substance P and nitroprusside were without effect on *de novo* or NA-stimulated PGI_2 synthesis, whether or not the endothelium was present and irrespective of incubation time, post-DE.

4 These results indicate that: (a) PGI_2 synthesis linked to excitatory receptors (α-adrenoceptors, thromboxane A_2) and associated systems (G proteins, protein kinase C) in the smooth muscle component of the rat aorta is not influenced by endothelium-derived relaxing factor (EDRF); (b) the changes of response to stimulators and inhibitors of PGI_2 synthesis may be due to an increased reactivity of the vessels caused by the trauma of DE; and (c) vasodilators (parasympathomimetics, substance P and nitroprusside) that do not act directly on excitatory receptors do not influence PGI_2 synthesis.

Introduction

Removal of the endothelium (DE) of isolated blood vessels results in the abolition of relaxation elicited by vasodilators (Furchgott, 1983) and in enhanced responses to contractile agonists (e.g. Egleme *et al.*, 1984). These observations have led to the concept that vascular endothelium produces a substance (endothelium-derived relaxing factor; EDRF), the release of which is obligatory in mediating the action of vasodilators (Furchgott, 1983).

It has been demonstrated that vasoconstrictors (α-adrenoceptor agonists, thromboxane A_2 analogue U46619, fluoride, phorbol ester: Godfraind *et al.*, 1982; Coleman *et al.*, 1980; Martin *et al.*, 1986; Danthaluri & Deth, 1984) also stimulate the synthesis of the endogenous prostanoid, prostacyclin (PGI_2), by isolated vascular tissue (Stewart *et al.*, 1984; Golub *et al.*, 1985; Jeremy *et al.*, 1985a,b; 1986a; Jeremy & Dandona, 1987; 1988). Since PGI_2 is both a vasodilator (at low concentrations; Moncada & Vane, 1979) and a vasoconstrictor (at high concentrations; Van Dam *et al.*, 1986), and there is a direct relationship between vascular contractility and PGI_2 synthesis, the pharmacological properties of blood vessels, *in vitro*, following removal of the endothelium may be due, in part, to changes in endogenous PGI_2 synthesis. To explore this possibility, the effect of DE on a range of known stimulators of rat aortic PGI_2 synthesis (adrenaline, noradrenaline, thromboxane A_2 analogue U46619, phorbol dibutyrate [PDBU] and sodium fluoride [NaF]; Jeremy *et al.*, 1985a,b; Jeremy & Dandona, 1987; 1988) and known vasodilators (yohimbine, prazosin and the calcium channel blocker, nifedipine; Jeremy *et al.*, 1985a; 1986a) on rat aortic PGI_2 synthesis was investigated. Since acetylcholine (ACh) and substance P are established stimulators

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of EDRF (Furchgott & Zawadski, 1980; Bolton & Clapp, 1986), their action on rat aortic PGI_2 synthesis following DE was also investigated.

It has recently been proposed that EDRF is, in fact, nitric oxide (NO; Palmer *et al.*, 1987). Since the vasodilator action of nitroprusside is believed to be due to the release of NO from this agent (Freelisch & Noack, 1987), nitroprusside was also investigated. In preliminary investigations, marked variations in response were observed at different times following DE. Responses to PGI_2 stimulators and inhibitors were therefore studied at 3 h, 6 h and 9 h post DE.

Methods

Preparation of tissue

Male Sprague Dawley rats (300 g) were decapitated, their thoracic aortae excised (from aortic arch to sub diaphragm) and adventitia and fatty tissue removed. All aortae were opened longitudinally with scissors. Removal of the endothelium was achieved by immobilising the aortae (endothelium upward) on a cork board with surgical staples (at the aortic arch and abdominal portions of the vessel). The vessel was then rubbed twice, longitudinally in both directions, with a paper tissue wrapped around the index finger. Scanning electron microscopy revealed that this treatment completely removed the endothelium. Furthermore, in ten consecutive experiments aortic preparations without endothelium were consistently reduced by 5–8 per cent tissue mass. All aortae were then cut laterally into 2 mm segments with a scalpel blade on a teflon block. The segments were pooled in Dulbecco's Minimum Essential Medium (MEM; pregassed with 95% O_2:5% CO_2) and incubated for 3 h, 6 h and 9 h in MEM at 37°C in a shaking water bath, before incubation of the tissue with drugs. Medium was changed every 30 min. In separate experiments, aortic tissue was placed in formalin following incubation for 3 h, 6 h and 9 h post DE for histological appraisal of the tissue, following staining with haemotoxylin/eosin.

Effect of endothelium removal on stimulators and inhibitors of aortic prostacyclin release

Following DE, three aortic segments were placed in 1 ml MEM, in sextuplicate for each drug concentration. Drugs which stimulate PGI_2 formation (adrenaline, noradrenaline, U46619, PDBU, NaF; Jeremy *et al.*, 1985a,b; Jeremy & Dandona, 1987; 1988) were added over a concentration range of 1×10^{-7} to 1×10^{-4} mol l^{-1}, plus an untreated group (zero basal), and incubated at 37°C for 30 min in a shaking water bath. The effect of acetylcholine, substance P and nitroprusside on *de novo* PGI_2 synthesis was also investigated as for the above stimulatory agents. Aliquots of supernatant were then taken for estimation of 6-oxo-prostaglandin $F_{1\alpha}$ (the stable, spontaneous hydrolysate of PGI_2) by specific radioimmunoassay (Jeremy *et al.*, 1985a). For studies with inhibitors of stimulated PGI_2 synthesis, the following interactions were studied: adrenaline vs yohimbine, noradrenaline vs prazosin, noradrenaline vs nifedipine, noradrenaline vs substance P, noradrenaline vs acetylcholine, noradrenaline vs carbachol and noradrenaline vs nitroprusside. Three aortic segments, in sextuplicate for each drug dose, were placed in 1 ml MEM containing agonists (α-adrenoceptor antagonists: 1×10^{-7} to 1×10^{-5} mol l^{-1}; nifedipine, substance P, nitroprusside, acetylcholine, carbachol: 3×10^{-6} to 3×10^{-4} mol l^{-1}) and incubated for 15 min in a shaking water bath. The media was then aspirated and 1 ml media containing the same concentrations of inhibitors replaced. Stimulatory agents were then added immediately (final concentrations: adrenaline, noradrenaline and U46619, 3×10^{-6} mol l^{-1}; PDBU, 3×10^{-7} mol l^{-1}) and the tissues further incubated at 37°C for 30 min and an aliquot of supernatant taken for estimation of 6-oxo-$PGF_{1\alpha}$ as above.

Treatment of results

Data on concentration-response curves of stimulatory agents are expressed graphically as pg 6-oxo-$PGF_{1\alpha}$ mg^{-1} tissue min^{-1}. In these graphs, basal output has been subtracted from stimulated release, since DE elicits greater 'spontaneous' release of PGI_2 from aortic tissue (see Results: Spontaneous release of PGI_2); thus in these graphs only specific agonist-stimulated PGI_2 release is depicted. Data on inhibitors of stimulated PGI_2 synthesis are expressed as percentage inhibition of stimulated PGI_2 release, arbitrarily set at 0% (stimulated minus basal PGI_2 output); 100% inhibition is equivalent to basal output.

Statistical analyses

Values are expressed as mean $\pm$ s.d. of mean. A one-way analysis of variance was performed on the data and the significance determined by Student's t test. P values less than 0.01 were taken to be significant. Significant differences between responses at each drug concentration are expressed graphically with an asterisk.

Materials and drugs

Adrenaline bitartrate, noradrenaline bitartrate, sodium fluoride, phorbol dibutyrate, nifedipine, yohimbine HCl, substance P, acetylcholine, car-

bamyl choline chloride (carbachol), sodium nitroprusside and Dulbecco's minimum essential medium were purchased from Sigma Chemical Company (Poole, Dorset). Thromboxane A_2 analogue, U46619 (11,9 epoxymethano-PGH_2), was purchased from the Cayman Chemical Company (Ann Arbor, Michigan, U.S.A.). Prazosin was supplied by Pfizer Ltd (Sandwich, Kent).

Results

Spontaneous release of prostacyclin

Removal of the endothelium caused a marked increase in the spontaneous release of PGI_2 when compared to intact tissue (Figure 1). It has previously been demonstrated that preparative procedures (viz. cutting) of rat aorta cause an initial surge in PGI_2 release from the rat aorta *in vitro*, which subsides following incubation of tissue (Jeremy *et al.*, 1984; Boeynaems & Galand, 1983). Since in the first hour excessive 6-oxo-$PGF_{1\alpha}$ concentrations may mask changes caused by stimulatory agents, subsequent experiments were carried out at 3 h following tissue preparation. Using Student's *t* test, spontaneous PGI_2 release from DE tissue became non-significant at 3.5 h post DE. Furthermore, since at 3 h there was a greater basal output of PGI_2 from rubbed aortae compared with intact aortae, basal output was carefully assessed and subtracted from agonist-stimulated values (see Treatment of results, above).

Effect of endothelium removal on stimulated prostacyclin release

At 3 h post DE the concentration-PGI_2 response curves of the stimulatory agents were shifted markedly to the left (adrenaline, Figure 2; noradrenaline, Figure 3; U46619, Figure 4; PDBU, Figure 5; fluoride, Figure 6). At 6 h post DE there were no differences between the concentration-PGI_2 response curves of these stimulatory agents obtained from aortae without endothelium or intact aortae (Figures 2, 3, 4, 5 and 6). Substance P, acetylcholine and nitroprusside were without significant effect on *de novo* rat aortic PGI_2 synthesis, irrespective of incubation time or presence of the endothelium (Table 1).

At 3 h post-DE the inhibitory action of yohimbine on adrenaline-stimulated PGI_2 synthesis, prazosin on noradrenaline-stimulated PGI_2 synthesis and nifedipine on noradrenaline-stimulated PGI_2 synthesis were markedly diminished (Figure 7, 8 and 9), whilst those of control aortae were similar to previously-described data (Jeremy *et al.*, 1985a; 1986a). At 6 h post-DE, the inhibitory action of yohimbine (Figure 7), prazosin (Figure 8) and nifedipine (Figure 9) was partially restored, although still significantly diminished, whilst at 9 h post-DE it

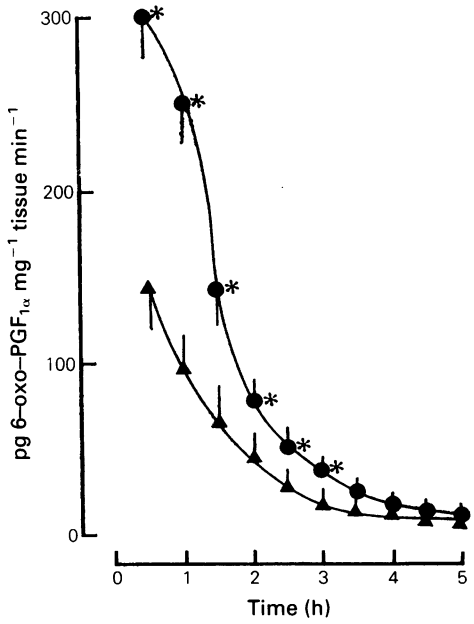


Figure 1 Spontaneous release of prostacyclin (PGI_2) following removal of the endothelium from the rat aorta. Intact aorta (▲); stripped aorta (●). Each point represents mean with s.d. shown by vertical lines, $n = 6$. *$P < 0.01$.

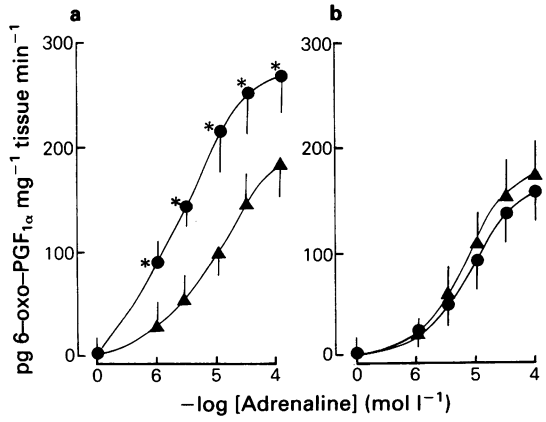


Figure 2 Effect of endothelium removal (DE) on adrenaline-stimulated rat aortic prostacyclin (PGI_2) release at (a) 3 h and (b) 6 h post-DE. Intact aorta (▲); stripped aorta (●). Each point represents mean with s.d. shown by vertical lines, $n = 6$. *$P < 0.01$.

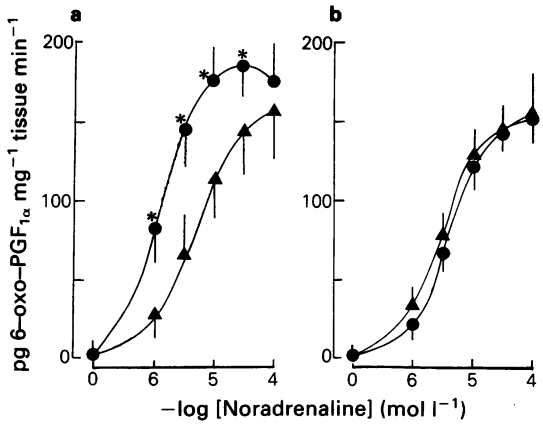


Figure 3 Effect of endothelium removal (DE) on noradrenaline-stimulated rat aortic prostacyclin (PGI_2) release at (a) 3 h and (b) 6 h post-DE. Intact aorta (▲); stripped aorta (●). Each point represents mean with s.d. shown by vertical lines, $n = 6$. $^*P < 0.01$.

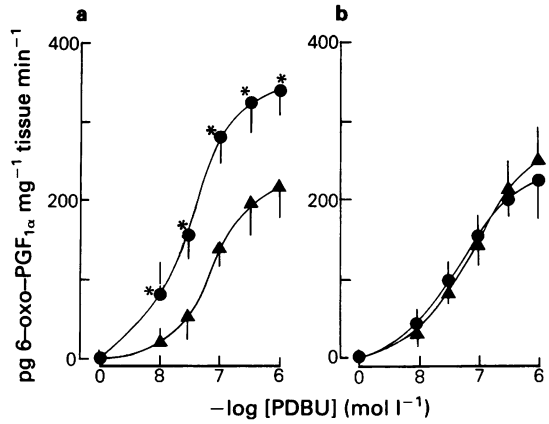


Figure 5 Effect of endothelium removal (DE) on phorbol dibutyrate (PDBU)-stimulated rat aortic prostacyclin (PGI_2) release at (a) 3 h and (b) 6 h post-DE. Intact aorta (▲); stripped aorta (●). Each point represents mean with s.d. shown by vertical lines, $n = 6$. $^*P < 0.01$.

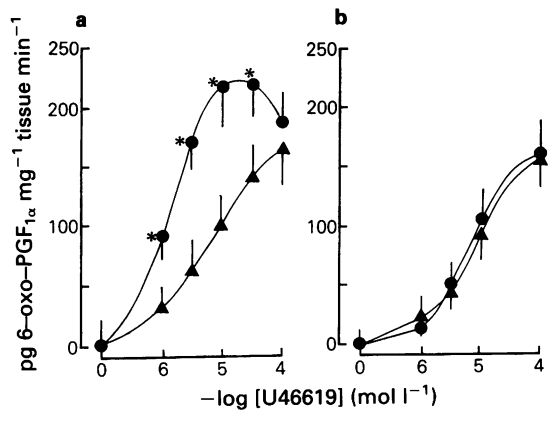


Figure 4 Effect of endothelium removal (DE) on thromboxane A_2 analogue U46619-stimulated rat aortic prostacyclin (PGI_2) release at (a) 3 h and (b) 6 h post-DE. Intact aorta (▲); stripped aorta (●). Each point represents mean with s.d. shown by vertical lines, $n = 6$. $^*P < 0.01$.

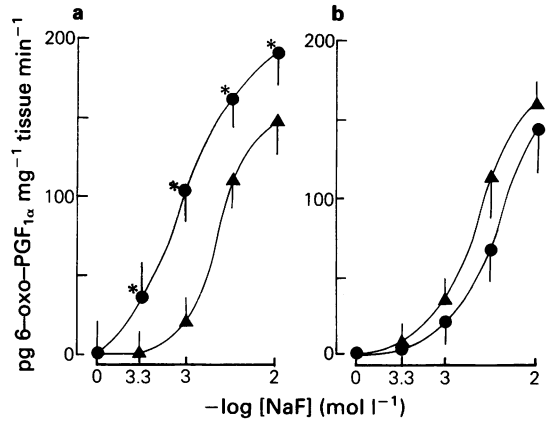


Figure 6 Effect of endothelium removal (DE) on sodium fluoride, (NaF, 5×10^{-3} mol l^{-1})-stimulated rat aortic prostacyclin (PGI_2) release at (a) 3 h and (b) 6 h post-DE. Intact aorta (▲); stripped aorta (●). Each point represents mean with s.d. shown by vertical lines, $n = 6$. $^*P < 0.01$.

was completely restored in aortae without endothelium (Figures 7, 8 and 9).

Substance P, acetylcholine, carbachol and nitroprusside (all up to 3×10^{-4} mol l^{-1}) were without significant effect on noradrenaline-stimulated PGI_2 synthesis in aortae with or without endothelium at 3 h, 6 h or 9 h post-DE (Table 2).

Scanning electron microscopy revealed that at 3 h, 6 h and 9 h post-DE the endothelium was absent from rubbed aortae. There were no detectable changes in the microscopic morphology of the smooth muscle cells following 3 h, 6 h and 9 h incubation of the tissue. This confirmed a previous study which demonstrated complete integrity of rat aortic

Table 1 Effect of the vasodilators nitroprusside (NP), acetylcholine (ACh) and substance P (SP) on *de novo* rat aortic 6-oxo-prostaglandin $F_{1\alpha}$ release (pg-6-oxo-$PGF_{1\alpha}$ mg^{-1} tissue min^{-1}) at 3 h, 6 h and 9 h after removal of endothelium

Drug	*Time*	*Concentration of vasodilators* ($nmol\,l^{-1}$)							
		0		3×10^{-6}		3×10^{-5}		3×10^{-4}	
		C	DE	C	DE	C	DE	C	DE
NP	(3 h)	15 ± 2	25 ± 3	16 ± 1	22 ± 4	16 ± 2	24 ± 3	15 ± 3	24 ± 2
NP	(6 h)	10 ± 1	12 ± 2	11 ± 1	10 ± 1	9 ± 1	10 ± 1	8 ± 0.8	8 ± 0.8
NP	(9 h)	8 ± 1	8 ± 1	9 ± 1	10 ± 1	8 ± 1	6 ± 0.5	8 ± 1	10 ± 1
ACh	(3 h)	15 ± 2	25 ± 3	15 ± 2	26 ± 2	16 ± 2	28 ± 1	15 ± 2	24 ± 3
ACh	(6 h)	10 ± 1	9 ± 2	10 ± 1	8 ± 2	10 ± 1	12 ± 1	6 ± 1	8 ± 1
ACh	(9 h)	7 ± 1	8 ± 1	10 ± 1	8 ± 1	6 ± 2	8 ± 1	4 ± 0.5	4 ± 0.3
SP	(3 h)	16 ± 2	26 ± 4	15 ± 1	25 ± 2	15 ± 2	27 ± 3	16 ± 1	28 ± 2
SP	(6 h)	8 ± 1	5 ± 0.5	8 ± 2	6 ± 1	10 ± 1	8 ± 1	8 ± 1	10 ± 1
SP	(9 h)	7 ± 1	6 ± 1	6 ± 1	6 ± 1	7 ± 1	6 ± 1	8 ± 1	6 ± 1

Value are mean ± s.d.; $n = 6$
C: intact aortae; DE: aortae with eudothelium removed.

tissue following prolonged incubation in MEM, when assessed by transmission electron microscopy (Jeremy & Dandona, 1986a).

Discussion

The present study clearly demonstrates that the endothelium contributes negligible quantities of PGI_2 to the total synthesis of this prostanoid by the rat isolated aorta, at least in response to the stimulatory agents investigated. The main source of PGI_2 in this vessel is therefore of medial and/or intimal origin. This conclusion is compatible with earlier findings which showed that the endothelium produced only 5% of the total PGI_2 in rabbit aortic rings (Moncada *et al.*, 1977). It has also been demonstrated that vasoconstrictors (α-adrenoceptor agonists and the thromboxane A_2 analogue U46619; Godfraind *et al.*, 1982; Coleman *et al.*, 1980) stimulate PGI_2 release in blood vessels through the mobilisation of a common activator, calcium (Stewart *et al.*, 1984; Golub *et al.*, 1985; Jeremy *et al.*, 1985a,b; 1986a). Similarly, in the rat aorta, phorbol ester (a protein kinase C activator; Nishizuka, 1984) and fluoride (a G protein activator; Bigay *et al.*, 1985)

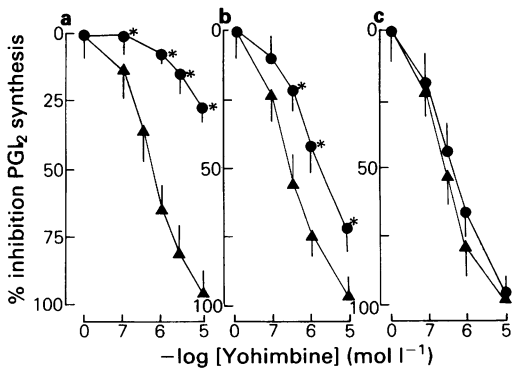


Figure 7 Effect of endothelium removal on yohimbine antagonism of adrenaline (3×10^{-6} $mol\,l^{-1}$)-stimulated prostacyclin (PGI_2) synthesis at (a) 3 h, (b) 6 h and (c) 9 h following removal of the endothelium. Intact aorta (▲); endothelium removed (●). Each point represents mean with s.d. shown by vertical lines; $n = 6$. *$P < 0.01$.

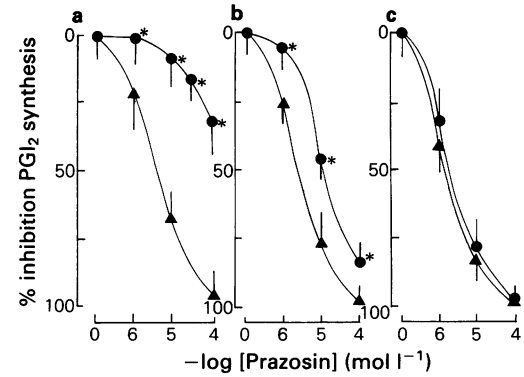


Figure 8 Effect of endothelium removal on prazosin antagonism of noradrenaline (3×10^{-6} $mol\,l^{-1}$)-stimulated prostacyclin (PGI_2) synthesis at (a) 3 h, (b) 6 h and (c) 9 h following removal of the endothelium. Intact aorta (▲); endothelium removed (●). Each point represents mean with s.d. shown by vertical lines; $n = 6$. *$P < 0.01$.

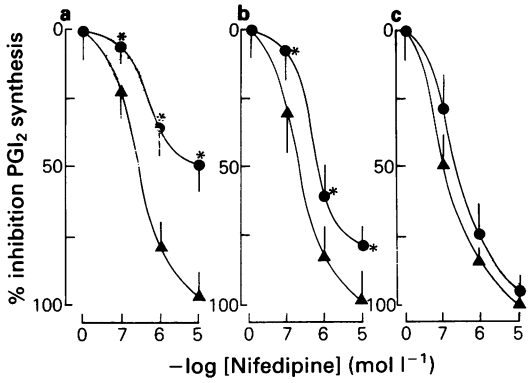


Figure 9 Effect of endothelium removal on nifedipine antagonism of noradrenaline-stimulated prostacyclin (PGI_2) synthesis at (a) 3 h, (b) 6 h and (c) 9 h following removal of the endothelium. Intact aorta (▲); endothelium removed (●). Each point represents mean with s.d. shown by vertical lines; $n = 6$. $^*P < 0.01$.

elicit both contraction and PGI_2 synthesis (Danthaluri & Deth, 1984, Martin *et al.*, 1986; Jeremy & Dandona, 1987; 1988). It was therefore proposed that G proteins and protein kinase C mediate both contraction and PGI_2 synthesis through mobilisation of activator calcium (Jeremy & Dandona, 1987; 1988). In the present study, we have shown that even after DE those agents that elicit contraction of the rat aorta also cause the release of PGI_2. The present study thus consolidates the concept that there is a direct relationship between vascular contraction and PGI_2 synthesis and that this phenomenon appears to occur at the smooth muscle rather than at the endothelial component of vessels.

At 3 h post-DE we observed enhanced stimulation of PGI_2 synthesis in response to adrenaline, noradrenaline, U46619, phorbol ester and fluoride in aortae without endothelium when compared with intact tissue. Similarly, at 3 h post-DE there was a marked reduction in the inhibition of noradrenaline-stimulated PGI_2 synthesis by yohimbine and prazosin and by calcium channel blockade in aortae without endothelium. Previous studies have shown that removal of the endothelium results in diminished responsiveness to vasodilators (Furchgott, 1983) and increased response to vasoconstrictors (Egleme *et al.*, 1984), which has led to the concept that vascular reactivity is controlled by EDRF (Furchgott, 1983). The data at 3 h post-DE would therefore seem to indicate that stimulation of rat aortic PGI_2 synthesis and inhibition of PGI_2 synthesis by specific α-adrenoceptor antagonism and calcium channel blockade are dependent on the secretion of EDRF. However, protracted incubation (6 h and 9 h) of the aorta without endothelium normalised these changes of response to stimulators and inhibitors of PGI_2 synthesis, despite the continued absence of the endothelium. These findings therefore suggest that removal of the endothelium *per se*, rather than the secretion of EDRF, determines these changes. Since DE constitutes a severe trauma to the vessel, it is possible that this mechanical injury sensitizes the vessel, thereby enhancing and diminishing responsiveness to stimulators and inhibitors of PGI_2 synthesis respectively. We are aware of no systematic

Table 2 Effect of the vasodilators nitroprusside (NP), acetylcholine (ACh), carbachol (CCh) and substance P (SP) on percentage inhibition (mean, $n = 6$) of noradrenaline (3×10^{-6} mol l^{-1})-stimulated prostacyclin synthesis (0%) at 3 h, 6 h and 9 h after endothelium removal

Drug	*Time*	*Concentration of vasodilator* (mol l^{-1})					
		3×10^{-6}		3×10^{-5}		3×10^{-4}	
		C	DE	C	DE	C	DE
NP	(3 h)	0	0	2	1	0	3
NP	(6 h)	0	2	1	3	0	0
NP	(9 h)	0	0	0	0	0	0
ACh	(3 h)	0	3	4	3	0	0
ACh	(6 h)	3	0	0	4	0	0
ACh	(9 h)	0	0	0	3	0	2
CCh	(3 h)	0	3	2	5	2	1
CCh	(6 h)	0	3	2	1	0	5
CCh	(9 h)	0	3	2	0	4	0
SP	(3 h)	0	0	2	1	2	0
SP	(6 h)	0	0	1	2	0	0
SP	(9 h)	0	0	3	0	0	0

C: intact aortae; DE: aortae without endothelium.

study on mechanical injury and vascular responsiveness *in vitro*, but it is known that trauma causes prolonged spasm in human arteries (Kinmonth *et al.*, 1962). Since there is a direct relationship between contractility and PGI_2 release, in that they share common activator systems (viz. Ca^{2+} channels, protein kinase C), the effect of more prolonged preincubation on contractile/relaxation responses of isolated vessels in organ bath systems warrants investigation.

It has been proposed that the vasodilator action of parasympathomimetics and substance P is due to the release of EDRF (Furchgott & Zawadski, 1980; Bolton & Clapp, 1986), and that nitroprusside elicits relaxation through the release of nitric oxide (Freelisch & Noack, 1987), which itself is currently believed to be EDRF (Palmer *et al.*, 1987). Thus the lack of effect of parasympathomimetics, nitroprusside and substance P on *de novo* or stimulated PGI_2 synthesis, whether or not the endothelium was present, indicates that PGI_2 synthesis in the rat aorta is not influenced by direct action of these vasodilators or by the stimulated release of EDRF by these agents. In the present system, modulation of PGI_2 synthesis appears to be confined to excitatory receptors (receptors that elicit contraction and PGI_2 release, α-adrenoceptors, thromboxane A_2) and directly linked systems (calcium channels, protein kinase C, G proteins; Jeremy & Dandona, 1987; 1988; Jeremy *et al.*, 1985a; 1986a) and not by vasodilators which elicit relaxation through separate receptors (viz. cholinomimetics, substance P, EDRF). This view is consistent with a conclusion reached on receptor-linked prostanoid synthesis in the rat urinary bladder (Jeremy *et al.*, 1986b). In this study it was observed that muscarinic stimulation of rat urinary bladder prostanoid synthesis was inhibited by muscarinic antagonists (atropine, ipratropium, gallamine) and calcium channel blockers but not by α-adrenoceptor agonists, although these agents cause relaxation in the urinary bladder of this species (Jeremy *et al.*, 1986b).

In conclusion, modulation of receptor-linked PGI_2 synthesis by the rat aorta is transiently altered by the removal of the endothelium. This effect is not due to the release of EDRF, but possibly to a trauma-induced increase in vascular reactivity. Since PGI_2 is a vasodilator, at least in the concentrations generated by the aorta in the present system (Moncada & Vane, 1979), the differential changes of endogenous vascular PGI_2 in response to DE may influence contractile responses of vessels in organ bath systems. Notwithstanding the lack of effect of acetylcholine, carbachol and nitroprusside on PGI_2 synthesis by the rat aorta, the apparent sensitization of vessels resulting from DE may influence vascular reactivity and therefore responses to vasoconstrictors and vasodilators *in vitro*.

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29 March–1 April 1989

International Symposium on Serotonin from Cell Biology to Pharmacology and Therapeutics, Florence, Italy (Secretariat, Fondazione Giovanni Lorenzini, Via Monte Napoleone 23, 20121 Milan, Italy).

2–5 April 1989

Seventh National Meeting of the Brain Research Association, Sheffield, U.K. (Dr. N. Payne, Dept. of Anatomy, Sheffield University, Sheffield, U.K.)

3–4 April 1989

British Neuroendocrine Group Meeting, Bristol, U.K. (Dr. C.D. Ingram, Department of Anatomy, The Medical School, University Walk, Bristol BS8 1TD).

5–7 April 1989

British Pharmacological Society Spring Meeting, Bristol, U.K. (Closed meeting for members and guests.)

6–7 April 1989

International Symposium on Psychiatric Neuropathology and Neurochemistry, London, U.K. (Dr. R.W. Kerwin, Department of Neuropathology, Institute of Psychiatry, De Crespigny Park, London SE5).

7 April 1989

British Pharmacological Society. Symposium on the NMDA Receptor, Bristol, U.K. (Dr. G.L. Collingridge, Department of Pharmacology, The Medical School, University of Bristol, Bristol, BS8 1TD, U.K.)

21–22 April 1989

Satellite Symposium of Int. Soc. for Neurochemistry Congress, Adenosine Receptors, Agarne, Portugal (Professor J.A. Ribeiro, Laboratory of Pharmacology, Gulbenkian Institute of Science, 2781 Oeiras, Portugal).

30 April–5 May 1989

Fifth Int. Symposium on the Adrenal Chromaffin Cell as a Neuroendocrine model; from Basic to Clinical Aspects, Jerusalem, Israel (Dr. K. Rosenheck, Dept. of Membrane Research, Weizmann Institute of Science, Rehovot 76100, Israel).

22–26 May 1989

Second Meeting of the International Neurotoxicology Association, Barcelona, Spain. (Dr. E. Rodriquez Farre, Dept. of Pharmacology & Toxicology, CSIC, Jorge Girona Salgado, 18–26, E-08034, Spain).

25–27 May 1989

4th International Symposium on Calcium Antagonists, Florence, Italy. (Secretariat, Fondazione Giovanni Lorenzini, Via Monte Napoleone 23, 20121 Milan, Italy).

1–2 June 1989

International Workshop on Sensory Neuropeptides, Florence, Italy. (Miss A. Giannini, Istituto Farmacobiologico Malesci, S.p.A., Dept. Pharmacology, Via Porpora 22, 50144 Florence, Italy).

1–3 June 1989

International Symposium on Kidney, Proteins & Drugs, Montecatani Terme, Italy. (Dr. Claudio Bianchi, Centro Nefrologico, Clinica Medica Generale 2, University of Pisa, 56100 Pisa, Italy).

28 June–2 July 1989

Ethnicity & Cardiovascular Diseases Conference, Nairobi, Kenya. (Ms. C. Cate, Int. Society on Hypertension in Blacks, 69 Butler Street, S. E., Atlanta, GA 30303, USA).

12–14 July 1989

British Pharmacological Society Summer Meeting. Leeds, U.K. (Closed meeting for members and guests)

16–21 July 1989

Vth International Congress of Toxicology, Brighton, U.K. (IUTOX '89, Congress House, 65 West Drive, Sutton, Surrey SH2 7NB, U.K.)

1–3 September 1989

Second International FIP Symposium on Disposition & Delivery of Peptide Drugs, Leiden, The Netherlands. (Dr. J. Verhoef, Centre for Bio-Pharmaceutical Sciences, PO Box 9502, 2300 RA Leiden, The Netherlands).

17–20 September 1989

1st International $GABA_B$ Symposium, Cambridge, U.K. (Professor N.G. Bowery. Department of Pharmacology, School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX.

13–15 September 1989

British Pharmacological Society Autumn Meeting. Manchester, U.K. (Closed meeting for members and guests)

3–5 January 1990

British Pharmacological Society Winter Meeting. London, U.K. (Closed meeting for members and guests)

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1–6 July 1990

IUPHAR 11th International Congress of Pharmacology, Amsterdam, Netherlands (Contact to be advised.)

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'Antigen-induced contraction of guinea-pig isolated trachea: studies with novel inhibitors and antagonists of arachidonic acid metabolites' T.R. Jones, L. Charette & D. Denis

On pages 314 and 315 of this paper, concentrations of AA-861, 651, 896, BW-755C, ETYA and theophylline should be in μM rather than mM.